Effects of bradykinin on Calcium signalling and contractility of the ureter

Thesis submitted in accordance with the

requirements of the University of Liverpool for the

degree of Doctor in Philosophy

by

Aisha Alfituri

June 2017

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Declaration

I declare that the studies and reviews presented in this thesis are the result of my own work, unless otherwise acknowledged

The content of this thesis has not and is not being currently submitted for candidature for any other degree

Aisha Alfituri

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Conference presentations and university local events

1) Alfituri A., and Burdyga T., Effects of bradykinin on calcium signalling and force of ureteric smooth muscle. Joint Meeting of the American Physiological Society and the Physiological Society. Convention Centre Dublin, Ireland, 29 - 31 July 2016. (Poster presentation).

2) Alfituri A., and Burdyga T., The importance of Rho-associated kinase -induced Ca^{2+} sensitization as a component of Bradykinin –induced pharmacomechanical coupling in rat ureteric smooth muscle. Pharmacology 2016. The Queen Elizabeth II Conference Centre, London, 13−15 December 2016. (Poster presentation).

3) Alfituri A., and Burdyga T., Effects of bradykinin on calcium signalling and force of ureteric smooth muscle. University of Liverpool Poster day, Liverpool, UK, 13 May 2016. (Poster presentation).

4) Alfituri A., and Burdyga T., The importance of Rho-associated kinase -induced Ca^{2+} sensitization as a component of Bradykinin –induced pharmacomechanical coupling in rat ureteric smooth muscle. University of Liverpool ITM research day, Liverpool, UK, 17-July 2016. (Poster presentation).

5) Alfituri A., and Burdyga T., Expression, distribution and functional importance of Bradykinin 2 subtype receptor in the rat ureteric smooth muscle. Pharmacology 2017. The Queen Elizabeth II Conference Centre, London, 11−13 December 2016. (Accepted as oral presentation).

List of abbreviations

Ach	Acetyl Choline
AC	Adenylate Cyclase
ADP	Adenosine-5'-diphosphat
ATP	Adenosine-5'-triphosphat
4-AP	4- aminopyridine
ACE	Angiotensin- converting enzyme
ANS	Autonomic nervous system
Ba^{2+}	Barium
BK	Bradykinin
B_1 and B_2	Bradykinin receptors
BK_{Ca}	Large conductance calcium-activated potassium channel
CGRP	Calcitonin Gene Related Peptide
Ca^{2+}	Calcium ions
CICR	Calcium induced calcium release
I_{CRAC}	Calcium release-activated Calcium Current
CaM	Calmodulin
CCh	Carbachol
CSU-10	Confocal scanning unit
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic Guanosine monophosphate
CCD camera	Charge coupled device
CICR	Calcium induced calcium release
Cl^-	Chloride ions
COX	Cyclooxygenase

CPI-17	C-kinase potentiated Protein phosphatase-1 Inhibitor
DAPI	4', 6 Diamidino-2-phenylindole, Dihydrochloride
DAG	Diacyl glycerol
DM	Dichroic mirror
DMSO	Dimethyl sulphoxide
ELC	Essential Light Chain
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EDHF	Endothelial Derived Hyperpolarizing Factor
Fps	Frame per second
GPCR	G-Protein Coupled receptor
GEF	Guanine nucleotide exchange factors
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K^+	Potassium ion
KCl	Potassium Chloride
HK	High molecular weight Kininogens
H_1, and H_2	Histamine receptor
5-HT	5-Hydroxytryptamine
IC_{50}	The half maximum inhibitory concentration
ICC	Interstitial cells of Cajal
IP3	Inositol triphosphate
IP3R	Inositol triphosphate receptor
IICR	Inositol triphosphate Induced –calcium release
KD	Kallidin
K	Rate constant
K+	Potassium ions

kDa	Kilo daltons
Kir	Inward-rectifier potassium channels
Kv	Voltage-operated potassium channel
LK	Low molecular weight Kininogens
M	Moles
mg	Milligrams
Mg^{2+}	Magnesium ions
MgSO4	Magnesium sulphate
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
mM	Millimolar
mRNA	Messenger ribonucleic acid
mV	Millivolts
M_2, M_3 and M_5	Muscarinic receptors
MYPT1	Myosin phosphatase target subunit 1
N	Newton
Na^+	Sodium ions
NEP	Neutral Endopeptidase
NaCl	Sodium chloride
NANC	Non-adrenergic non-cholinergic Innervation
NEP	Neutral endopeptidase
NO	Nitric Oxide
N.A.	Numerical aperture
PF	Paraformaldehyde

PLC	Phospholipase C
PLA$_2$	Phospholipase A$_2$
PIP$_2$	Phosphatidylinositol 2,5-biphosphate
PBS	Phosphate buffer saline
PG	Prostaglandins
PKA, G	Protein kinase A, G
PKC	Protein kinase C
PP$_{IC}$	Protein Phosphatase 1 Catalytic subunit
P$_2$X, P$_2$Y	Purinergic receptors
ROCCs	Receptor operated calcium channels
RLC	Regulatory light chain
ROCK 1 and 2	Rho-associated, coiled-protein kinase 1 and 2
RyR	Ryanodine receptors
SR	Sarcoplasmic reticulum
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATP$_{ase}$
Ser	Serine
SEM	Scanning Electron Microscope
SMs	Smooth muscle
SMC	Smooth muscle Cell
STOCs	Spontaneous transient outward currents
s.e.m.	Standard error of the mean
SOCCs	Store Operated Calcium Channels
SOCE	Store Operated Calcium Entry
STIM	Stromal Interaction molecule
TEA	Tetra-Ethyl-ammonium

Thr   Threonine

VIP   Vasoactive intestinal Peptide

VOCCs   Voltage Operated Calcium Channels

Abstract

The understanding of the mechanism, by which bradykinin (BK) effects ureteric contractility, is limited. The primary aim of this study was to determine the expression and the distribution of B_2 receptors along the length of the ureter and study the effects of BK on Ca^{2+} signalling and contractile activity of the rat ureteric smooth muscle. Both the Ca^{2+}-dependent and Ca^{2+}-independent pathways, underlying the stimulant action of BK on the ureteric smooth muscle, have been investigated. Immunohistochemistry was used to identify the presence and the distribution of B_2 receptors along the ureter. Confocal imaging combined with force measurements under different physiological conditions was used to study the effects of BK on Ca^{2+} signalling and force of the ureteric smooth muscle *in situ*. The effect of the selective blocker of Rho-associated kinase, Y-27632, on force/ Ca^{2+} relationship was investigated.

It was found that the distribution of the B_2 receptors is non-homologous along the length of the rat ureter, with the expression being greater in the upper ureter and lowest in the middle and the lower ureter. It was found that BK caused a complex contractile response, consisting of an initial phasic component, associated with the Ca^{2+} release from the store, which appeared as asynchronous Ca^{2+} waves, followed by a sustained component, associated with Ca^{2+} entry via store/receptor operated Ca^{2+} channels that was superimposed by multiple phasic contractions, associated with propagating intercellular Ca^{2+} waves. The L-type Ca^{2+} channel blocker, Nifedipine, selectively blocked the intercellular Ca^{2+} wave and the repeated phasic contraction, but had no effect on the initial phasic contraction, associated with the Ca^{2+} release from the sarcoplasmic reticulum. Nifedipine, also had no effect on the sustained slow component, associated with the Ca^{2+} entry via store/receptor operated Ca^{2+} channels. Y-27632 had no effects on the Ca^{2+} signalling, associated with the Ca^{2+} release from the store or the Ca^{2+} entry via store/receptor operated Ca^{2+} channels, but had significant inhibitory effects on the initial phasic and sustained tonic component of BK-induced contractile response.

There was a significant reduction in the amplitude and an increase in the rate of relaxation of the initial phasic component, suggesting an increase of the activity of myosin light chain phosphatase, responsible for dephosphorylation of MLC_{20} and relaxation of force in the smooth muscles. Collectively, the data obtained indicate that both Ca^{2+}-dependent and Ca^{2+}-independent mechanisms are involved in BK-induced stimulant action on the rat ureteric smooth muscle.

Chapter 1

General introduction

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Chapter 1

General introduction

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1.1 The ureter:

The ureters are fibromuscular tubes and their main role is to transport the urine from the kidney to the urinary bladder. The mechanical peristaltic activity of the ureters is myogenic and is controlled by propagating plateau type action potentials, triggered by pacemaker potential, generated by pacemaker cells, located in the pelvic region. Any disorders in the ureters causing an obstruction to the passage of the urine, would have a detrimental effect on the kidney function.

1.1.1 Anatomy and structure of the ureter:

The ureters are narrow, thick-walled ducts that transport the urine from the kidneys to the urinary bladder. There are two ureters in the mammalian bodies, with the upper half situated in the abdomen and the lower one in the pelvis. Their length in human is about 25–30 centimetres (Hannappel, 1981, Notley, 1978). The ureters extend from the renal pelvis to the urinary bladder and are wider at the renal pelvis, and then they gradually become narrower as they enter the urinary bladder (Figure 1.1.1-1).

Furthermore, the renal pelvis is different in different species. In human and pigs, it possesses a number of major calyces (multicalyceal), which is a combination of numerous minor calyces. While, in the rats, mice, dogs, guinea-pigs, sheep, cats and rabbits, the renal pelvis are unicalyceal, that it possesses one minor calyces (Richard et al., 2002).

The wall of the human and rats ureters is composed of three layers: the adventitia, the muscular layer, and the mucous layer (Drake et al., 2015, Hicks, 1965) (Figure 1.1.1-2).

In human, It is four to five millimetres thick (Hannappel, 1981).

The adventitia is the outer layer and comprises a fibroelastic connective tissue that joins with the connective tissue on the back of the peritoneum. The adventia plays a protective role and contains the blood vessels, lymphatic channels and the nerve plexus that run along the ureter in human (Hannappel, 1981, Notley, 1978), and rats (Hicks, 1965).

The muscular layer is responsible for ureteral peristalsis and is made up of interlacing smooth muscle (SM) bundles. The bundles consist of outer longitudinal fibres, which turn into a circular layer on its spiral turn. However, close to the lamina propria, the bundle converted again to longitudinal fibres (Hannappel, 1981). In the rat, the compact circular layer of the SMs is about 25μ thick (Hicks, 1965).

The inner layer, the mucous membrane, constitutes the transitional epithelium (the urothelium) and the lamina propria in human (Hannappel, 1981), and rats (Hicks, 1965). The mucous membrane is arranged in longitudinal folds to permit substantial dilation of the channel that accompanies the increase in the urine volume, thus helping to prevent rupturing of the ureters in human (Hannappel, 1981), and rats (Hicks, 1965). The mucous membrane has no true glands. The transitional epithelium resembles that of the urinary bladder. It consists of several layers of cells, with the innermost, the one in contact with the urine, is flattened. The lamina propria is merged with the muscle layer and consists chiefly of collagen, fibrils and fibroblast. It also contains small blood vessels and unmyelinated axon bundles (Lang et al., 1998b).

The scanning electron microscope (SEM) allows more detailed examination of the muscular architecture and the orientation of the ureteric smooth muscles (SMs) in different parts of the ureter. The SMs of the upper ureter of the guinea-pig, when examined by the SEM, showed a generally circular oriented fibres. While in the middle part, the fibres had an irregular pattern and in the lower ureter, the muscle bundles were longitudinal (Tachibana et al., 1985).

The architecture of the ureteric muscle bundles, revealed by the SEM, was confirmed by live confocal imaging of Fluo-4-loaded ureters (Burdyga et al., 2003). The SMs of the guinea pig ureter appeared as a network of interlacing muscle bundles of varying thicknesses and orientation, with no distinct separation into longitudinal or circular layers (Figure 1.1.1-3) (Burdyga, unpublished data).
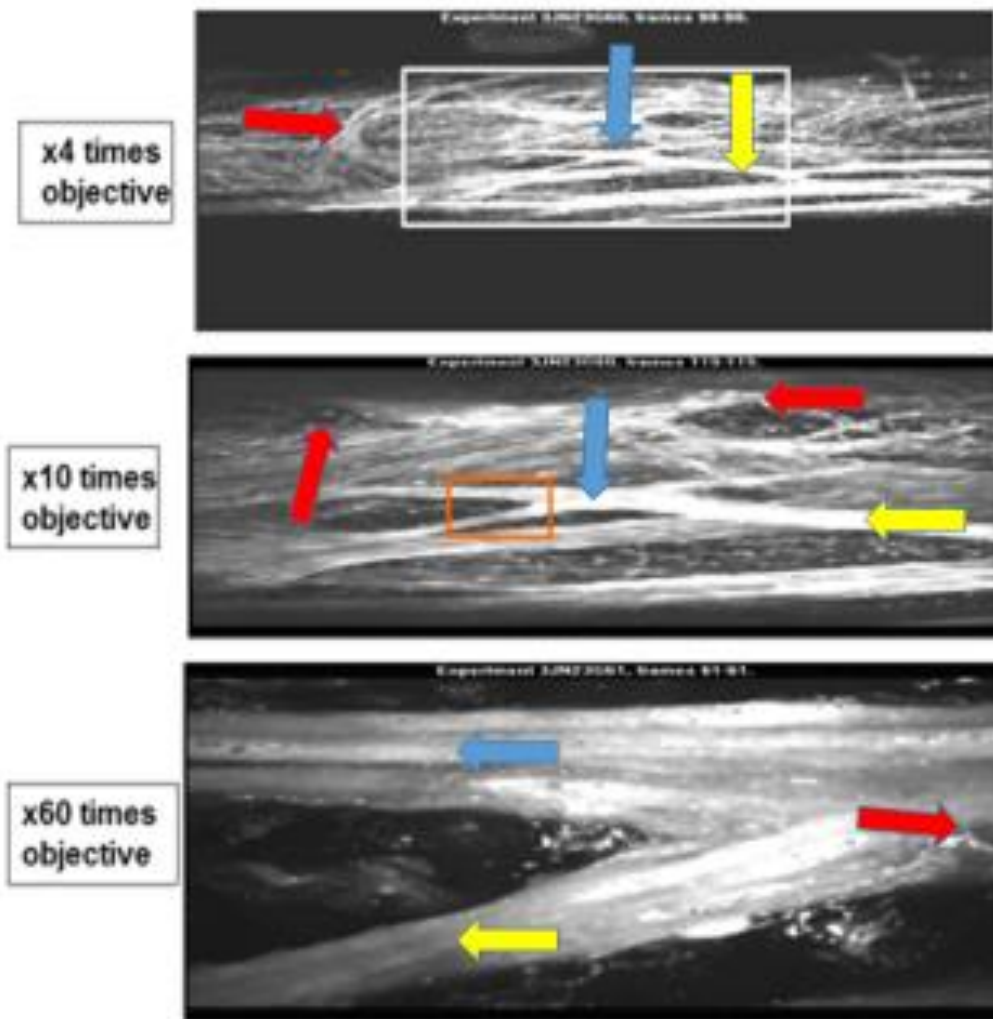
Figure 1.1.1-3. *X-Y grey scale images of Fluo-4 loaded guinea pig ureter reveals ureteral SM bundles morphology. The images, taken at different magnification, show SM bundles with different orientations and thicknesses. The blue arrows indicate longitudinal, red arrows indicate the circular and the yellow arrows indicate the oblique orientation (Burdyga, unpublished data).*

1.1.2 Ureters' blood supply:

The blood supply of the upper part of the ureter is received by the feed arteries, which branch off from the renal arteries that arise off the side of the abdominal aorta (Figure 1.1.2-1), or in few occasions, they may arise directly from the aorta, or the common iliac, or the internal iliac arteries. Whereas, in their distal end, the ureters get their blood supply from the gonadal, vesical, uterine, or vaginal arteries, and it is usually on their lateral side (Wood and Greenwell, 2010). At the ureteral wall, the blood vessels divide into long descending and ascending branches that run in the connective tissue and anastomose with each other to form the ureteral plexus (Abel, 1994). Penetrating branches from the ureteral plexus arise and spread from the muscle layer to the submucosal layer. Venous and lymphatic vessels follow blood supply (Wood and Greenwell, 2010).

The live architecture and morphology of the rat ureter was studied in detail by Borysova et al., (2013) using live confocal imaging (Figure 1.1.2-2). The ureter arteries start from the feed arteries that enter the ureter through the adventia and run into it. Then, the feed arteries divide into interconnected arcade arterioles and penetrating transverse arterioles, which later terminate as precapillary arterioles in the capillaries. From the capillaries, the collecting venules arise and flow to the transverse and the arcade venules and continue as ureteric venules (Borysova et al., 2013).
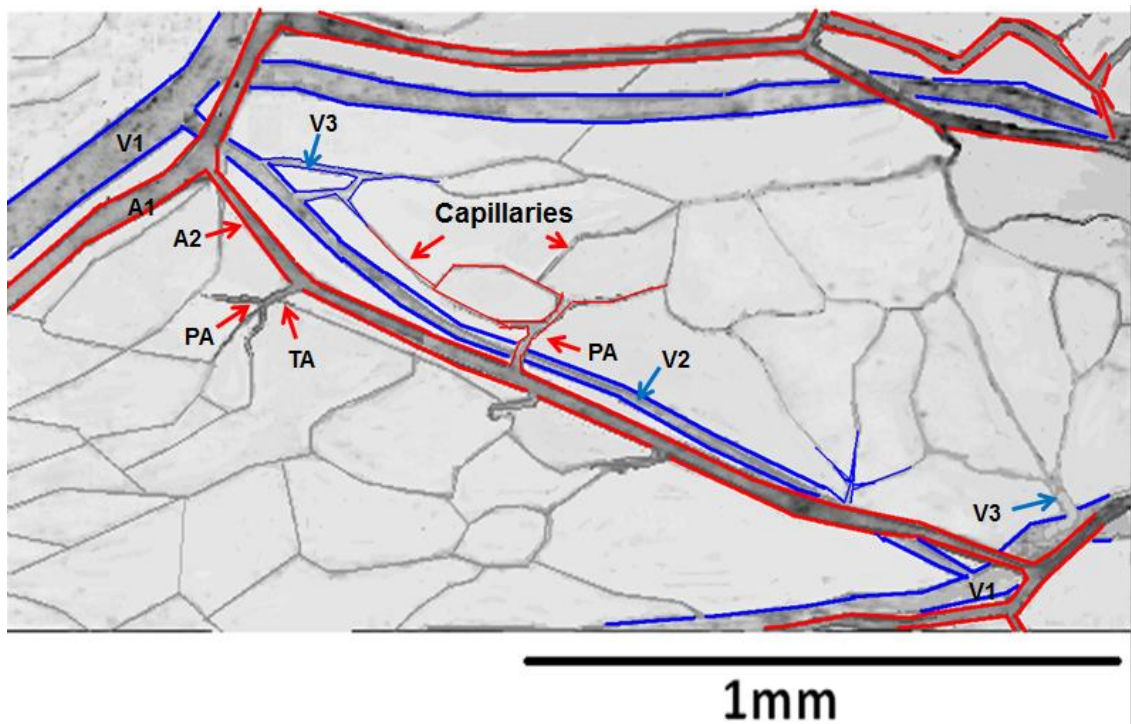
Figure 1.1.2-2. *Architecture of the rat ureteric microvascular networks in situ (Borysova et al., 2013). A_1 and A_2- Arcade arteriole; TA- Transverse arteriole; PA- Precapillary arteriole; V_1- Arcade venules; V_2- Transverse venules; V_3- Collecting venules*

1.1.3   Ureters' innervation:

The ureter has a rich nerve supply disseminated alongside its entire length. The nerves mainly arise from the celiac and mesenteric ganglia. However, in some circumstances, they may arise from the pelvic plexuses (Mitchell, 1935). Both parasympathetic and sympathetic nerves supply the ureter and have a modulatory effect.

1.1.3.1 The cholinergic (parasympathetic innervation):

The parasympathetic innervation of the upper two third of the ureter is via the vagus nerve, while in the lower third, it is by the sacral (S_2-S_3) segments of the spinal cord (Hannappel, 1981). Acetylcholine (ACh) is the neurotransmitter of the autonomic nervous system (ANS) released by the cholinergic nerves. Muscarinic-2 (M_2), Muscarinic-3 (M_3), and Muscarinic-5 (M_5) cholinergic receptors are identified in the ureter (Sakamoto et al., 2006). Activation of the muscarinic receptors, mainly M_3 leads to activation of Phospholipase C (PLC) enzyme, which subsequently causes the formation of Inositol 1, 4, 5-Trisphosphate (IP_3) and Diacylglycerol (DAG) (Berridge, 1984). The IP_3 binds to IP_3 receptors in the sarcoplasmic reticulum (SR), and thus promoting Ca^{2+} release from the store (Streb et al., 1983). On the other hand, DAG activates Protein kinase C (PKC) enzyme leading to an increase in Ca^{2+} influx across the cell membrane (Nishizuka, 1984). Conversely, activation of G_i proteins by M_2 receptors inhibits adenylate cyclase (AC) enzyme activity, thus inhibiting cAMP formation, and hence causing relaxation (Cooper, 1983).

1.1.3.2 The adrenergic (sympathetic innervation):

The sympathetic innervation is via thoracic-6 to lumbar-2 (T_6-L_2) (Hannappel, 1981). Noradrenaline (NA) is the main neurotransmitter released from the adrenergic nerves. Both α and β adrenoceptors exist in the ureter. However, α adrenoceptors effects predominate over β adrenoceptors effects in the ureter (Canda et al., 2007). $α_1$ adrenoceptors are found in the ureter of both animal and human (Morita et al., 1994, Scofield et al., 1995). Activation of $α_1$

adrenoceptors causes ureters' SMs contraction through the activation of a PLC-IP$_3$-DAG pathway (Canda et al., 2007). Whereas β adrenoceptors activation causes relaxation, through the cAMP-Protein kinase A activation (Canda et al., 2007). Both β$_2$ and β$_3$ adrenoceptors are expressed in human's ureter myocytes (Park et al., 2000).

1.1.3.2 Non adrenergic non-cholinergic innervation (NANC):

The ureter is also modulated by NANC nerves, which act by release neurotransmitters of the autonomic nervous system (ANS) other than acetylcholine and norepinephrine.

- **The purinergic system**: They play a vital role in both sensory and motor functions in the ureter, and ATP is the excitatory neurotransmitter that causes ureteric contraction (Andersson and Arner, 2004, Canda et al., 2007). Both rested and distended guinea pig ureter have shown to produce ATP, which stimulates the afferent nerve through purinergic receptors (Rong and Burnstock, 2004). The purinergic receptors (P$_2$X and P$_2$Y) sense pain in human and pigs and this travel along afferent nerve terminals to the pain centres in the brain, causing SMs contraction (Burnstock, 2006, Andersson and Arner, 2004, Canda et al., 2007). The P$_2$X receptors are ligand-gated cation channels promoting Ca^{2+} influx into the cell and the P$_2$Y purinergic receptors are G–protein coupled receptors inducing SMs contraction via PLC-IP$_3$ signalling pathway (Burnstock and Williams, 2000).

- **Nitric oxide (NO):** Arginine-derivative of NO is the major relaxant neurotransmitter of the lower urinary tract SMs in human (Andersson and Arner, 2004) and pigs (Iselin et al., 1998), as well as the rats (Mastrangelo et al., 2003). Additionally, the L-arginine/NO/cGMP pathway was the suggested mechanism to have a role in the regulation of uretervescical junction valve (Iselin et al., 1997).

- **Histamine:** Both Histamine-1 (H$_1$) and Histamine-2 (H$_2$) receptors are present in the ureter, with the predominance of H$_1$ (Canda et al., 2007). Although H$_1$ receptors cause

ureteric SMs contraction and H_2 receptors cause SMs relaxation in human (Dodel et al., 1996, Canda et al., 2007), canine (Lennon et al., 1993), sheep (Benedito et al., 1991) and buffalo (Canda et al., 2007), the H_1 and H_2 antagonists have no effect on ureteric force (Bertaccini et al., 1983).

- **Serotonin (5-Hydroxytryptamine (5-HT)):** 5-HT has shown to have a spasmogenic effect in the pig ureter through $5-HT_{2A}$ receptors (Hernandez et al., 2003), and this effect was antagonised by $5-HT_2$ antagonists (Hauser et al., 2002). Also, 5-HT was suggested to be a ureteric SMs constrictor in human, via $5-HT_3$ receptors (Hauser et al., 2002).

- **Prostanoids (Prostaglandins (PG$_s$), Thromboxanes, Prostacyclins):** There is a controversy in the literature about the role of Prostanoids in the ureteric SMs. It appears that PGF_2 causes ureteric SMs contraction and PGE_1 and PGE_2 cause relaxation in both normal, non-obstructed animal and human ureter (Andersson and Forman, 1978), while causing contraction in the obstructed ureters of pigs (Lowry et al., 2005). Moreover, Prostanoids concentration has found to be increased in the obstructed rats and human ureter (Jerde et al., 2004), which was explained by the induction of the enzyme that synthesizes Prostanoids (the Cyclooxygenase (COX) enzymes), and the suppression of the enzyme that degrades Prostanoids (15-Hydroxyprostglandin dehydrogenase enzyme). Likewise, COX inhibitors such as Diclofenac or Indomethacin (the non-selective COX inhibitor) and NS-398 or Celecoxib (the selective COX-2 inhibitors), which are generally used because of their spasmolytic properties for renal colic, were shown to inhibit agonist induced ureteric contraction (Mastrangelo et al., 2000).

- **Neuropeptides (vasoactive intestinal polypeptide (VIP), Endothelins, tachykinins, angiotensins and calcitonin Gene-Related Peptide (CGRP):** They are synthesized in the ureter and urinary bladder, but their functions are not well known (Canda et al., 2007). It has shown that VIP causes SMs relaxation (Hernandez et al., 2004), whereas

Tachykinins (Bustamante et al., 2001) and CGRP (Maggi et al., 1994) cause SMs contraction in the pigs ureter.

1.1.4 Myogenic activity of the ureter:

The ureters' SMs belong to a single unit type of SMs, in which the smooth muscle cells (SMCs) are electrically coupled via gap junctions (points of close contact between ureter SMs) (Canda et al., 2007). This means that the action potential, triggered by the invading pacemaker potential, can propagate between SMCs and synchronize mechanical activity of a large group of cells to produce propagating rhythmic contraction known as peristalsis. Ureteric peristalsis is mainly controlled by a myogenic mechanism, through the electrical spread (Burnstock and Prosser, 1960, Canda et al., 2007).

The myogenic nature of the ureteric activity is supported by the observation of the occurrence of peristalsis in isolated ureters connected to the renal pelvis (Finberg and Peart, 1970, Malin et al., 1970, Santicioli and Maggi, 1998), after denervation of the ureter, and in autotransplanted ureters (Santicioli and Maggi, 1998). However, the neurogenic factors play a modulatory role (Santicioli and Maggi, 1998). The modulatory role of the neurogenic factors was suggested by the reduction in the amplitude and the frequency of force when the specific sensory nerve and prostaglandin synthesis blockers were used (Lang et al., 1998b). The precise coordination between ureteral SMs and the excitation produced by the diffuse release of neurotransmitters are the main regulator of ureteric peristalsis (Canda et al., 2007).

1.1.5 Pacemaker function of the Renal pelvis: Topology, Morphology, and Function of the pacemaker cells:

In the SMs of the unicalyceal renal pelvis animals, atypical SMs are found in their outer layer. They start from the renal papilla and end at the ureteropelvic junction. These cells have a similar spindle-shaped, but are smaller in size, with fewer contractile filaments than the typical SMs (Schmidt–Nielsen, 1987, Feeney and Rosenblum, 2014) that make the inner layer and extend to the ureter (Gosling and Dixon, 1974, Dixon and Gosling, 1990).

However, in the multicalyceal renal pelvis, all the minor calyces muscle layers are composed of the atypical SMs (Gosling and Dixon, 1974, Dixon and Gosling, 1990) and these atypical SMs continue to the major papilla and form its inner layer, but they don't extend to the pelvicalyceal junction (Dixon and Gosling, 1990). These cells were assumed to be the only pacemaker generating cells in the renal pelvis (Lang et al., 1998b). However, other pacemaker cells, the interstitial cells of Cajal (ICC) like cells, located in the renal pelvis, (Lang et al., 1998b, Santicioli and Maggi, 1998) are also considered by some researchers to be responsible for the autorythmicity (Klemm et al., 1999, Lang et al., 2006). These cells are found in the lamina propria of the pelvicalyceal junction and the renal pelvis of the guinea pigs, but absent in their ureter (Klemm et al., 1999). Whereas, in the rats, they are only present in a smaller number in the renal pelvis (Lang et al. 2001). They have a distinctive morphology. Their shape is irregular with multiple broad and branching processes. They contain large quantities of sarcoplasmic reticulum and mitochondria. The Golgi apparatus is well developed. Besides, a number of flask shaped caveolae exists in the plasma membrane with a patchy and incomplete basal lamina (Lang et al., 1998b).

The function of the pacemaker cells is to initiate and propagate the electrical activity in the renal pelvis and proximal ureter. They have a spontaneous activity and their peristaltic wave is only triggered with the coupling and synchronisation between the subunits, the multiple coupled oscillators model of several pacemaker subunits (Golenhofen & Hannappel, 1973). According to this model, a lonely single subunit is unable to trigger peristalsis, but the coupling and synchronisation of multiple subunits (multiple pacemaker cells) can trigger peristaltic waves.

1.1.6 Propagation of the pacemaker electrical signal:

The electrical activity, causing the development of the phasic contraction, differs in different SMs and in different SM layers in the same tissue (Parkington and Coleman, 1990). In contrast to the tonic muscle, which respond to excitatory stimuli with only a graded depolarization, the ureters' SMs are phasic muscles, wherein the contraction occurs as a result of the cell membrane depolarization, and hence the generation of action potential (Somlyo and Somlyo, 1994). The electrical and mechanical activities occurred as a result of the distal conduction of the renal pelvis pacemaker spontaneous activity by the proximal part of the ureter to the rest of the ureter, thus initiate a series of phasic contractions (Lang et al., 1998b, Santicioli and Maggi, 1998). This conduction is basically by the myogenic process, in which the pacemaker potential generated by the pacemaker cells in the renal pelvis propagate to the typical SMs of the main ureter to trigger an action potential discharge. Then the action potentials propagate distally through the gap junctions to trigger other typical SMs (Santicioli and Maggi, 1998, Uehara and Burnstock, 1970).

Normally, not all renal pelvis pacemaker contractions propagate to the ureter. Good coupling between cells is needed to overcome the conduction block. While the frequency of the pacemaker cells is independent of the urine output, the urine transportation is mainly regulated by the excitation conduction. In the presence of large amount of urine, each excitation is conducted from the renal pelvis to the ureter, whereas at low urine volume, a physiological conduction blocks occurs, i.e. many excitations are stopped myogenically in the region of the pyeloureteral junction. Thus, stretch applied by the accumulating urine in the pyeloureteral region increases the coupling power and permits the incoming pacemaker wave of excitation to travel along the ureter (Santicioli and Maggi, 1998, Constantinou and Yamaguchi, 1981).

The frequency of the contraction will continue to increase, till it reaches the frequency of the pacemaker. Then, any increase in the amount of the urine will be accommodated, up to the point where the ureter become as an open tube (Constantinou et al., 1974).

The frequencies of contraction varied in different studies. In one study, in man, the number ranges from 2-3 contractions per minute, (Bjork and Nylen, 1972). While in another study, it ranges from 0.5 - 10 contractions per minute (Kondo, 1970). Whereas in other species, as in dogs, it is almost two fold that of man (1-20), and in the commonly used model (the mouse explant kidneys), the number is 4-9 contractions per minute (Kondo, 1970).

The frequency is 4 to 6 in pigs (Djurhuus, 1977), 20 to 30 in hamsters, and 40 to 50 contractions per minute in rats (Schmidt-Nielsen et al., 1980). Moreover, the frequency of the contraction is the same in different muscle strips that cut circumferentially and have an equal distance from the renal pelvis (Constantinou and Yamaguchi, 1981, Yamaguchi and Constantinou, 1989). On the other hand, the frequency of contraction diminished at more distant sites (Potjer et al., 1992, Lang et al., 1998b, Lang et al., 2002).

Additionally, a regional difference in both the excitability and the propagation rate of peristalsis waves has been demonstrated in the guinea pigs, cats, and rats ureter (Kobayashi, 1965, Tindall, 1972, Tsuchiya and Takei, 1990, Weiss, 2002), which were independent of the site of application of the stimulus and the nature of the depolarizing stimulus (Santicioli and Maggi, 1998). As well, the propagation of the impulses wasn't altered by any changes in the action potential shape or duration, as well as the contractility (Santicioli and Maggi, 1998).

1.2 Ureters' ion channels and currents:

Ion channels are cellular proteins, through which ions move from one side of a membrane to the other. The resting membrane potential and the generation of the action potential are controlled by the distribution and the relative cell membrane permeability to certain ions.

1.2.1 Calcium (Ca^{2+}) channels:

In SMs, two models have been suggested for Ca^{2+} influx from the extracellular milieu to the intracellular cytosol, the capacitive Ca^{2+} entry and the non-capacitive Ca^{2+} entry (Fixman et al., 2005). The capacitive Ca^{2+} entry through Store operated Ca^{2+} channels (SOCCs) is activated by a store depletion, whereas the non-capacitive Ca^{2+} entry is stimulated by the activation of specific Ca^{2+} channels, such as the Voltage operated Ca^{2+} channels (VOCCs) and the receptor operated Ca^{2+} channels (ROCCs).

1.2.1.1 L- Type Voltage Operated Calcium Channels:

VOCCs are expressed in the SMs and are the main contributors of increasing Ca^{2+} concentration in the SM cells. VOCCs are activated by changes in transmembrane potential (depolarization) (Fixman et al., 2005). VOCCs are responsible for the Ca^{2+} influx during action potential or membrane depolarization (Lang et al., 1998b).

VOCCs composed of α_1 subunit and regulatory β, $\alpha_2\delta$, and γ subunits. The α_1 subunit act as pore-forming and is responsible for most of the their functions, the voltage sensitivity, Ca^{2+} permeability, Ca^{2+}-dependent inactivation, and Ca^{2+} blocker sensitivity. While, the β, $\alpha_2\delta$, and γ subunits act to regulate surface expression of the α_1 subunit (Hill-Eubanks et al., 2011). The voltage of activation of Ca^{2+} currents of the VOCCs is rapid and high, and the single channel conductance is large. The inward currents increase by increasing extracellular Ca^{2+} concentration, or by the use of Ca^{2+} channel agonist (BayK8644) or the addition of Barium (Ba^{2+}) (Lang, 1990) and completely abolish in Ca^{2+} free solutions (Reuter, 1979, Tsien et al., 1988). VOCCs are specifically inhibited by Ca^{2+} antagonist (Reuter, 1979, Tsien

et al., 1988). On the other hand, the inward Ca^{2+} current inactivation of the VOCCs, which is also voltage-dependent is slow (Hill-Eubanks et al., 2011). They decline over 200-400ms (Lang, 1989, Sui and Kao, 1997b), and it is even much slower in the guinea pig ureter than other SMs. The slow inward Ca^{2+} current inactivation is considered as a significant contributor in determining the plateau phase of the ureteric action potential (Sui and Kao, 1997a, Sui and Kao, 1997b). Moreover, the inward Ca^{2+} current inactivation in the guinea pig ureter does not increase with the increase of the extracellular Ca^{2+} concentration as seen in other SMs (Lang, 1990).

Furthermore, in the SMs, three Ca^{2+} signals mediated by VOCCs have been found, the global rises in intracellular Ca^{2+}, Ca^{2+} flashes, and Ca^{2+} sparklets (Hill-Eubanks et al., 2011). Global Ca^{2+} rise signals refer to the uniform changes in Ca^{2+} concentration all over the cell. Whereas, Ca^{2+} flashes refer to the optically seen Ca^{2+} flash (brightly and briefly lights up cells, loaded with fluorescent Ca^{2+} indicators) due to a massive Ca^{2+} entry through VOCCs that causing a rapid elevation of the global intracellular Ca^{2+}. While, Ca^{2+} sparklets refer to the visualized, highly localized, stationary Ca^{2+} influx through VOCCs, which is either from a single or group of channels.

1.2.1.2 Store-Operated Ca^2 Channels:

Store operated Ca^{2+} entry (SOCE) through SOCCs, is a capacitive model of Ca^{2+} entry. The capacitive Ca^{2+} entry is coupled to Ca^{2+} release from the SR. In the SMs cells, Ca^{2+} depletion of the SR, following agonists binding to G-protein coupled receptors, can induce entry of extracellular Ca^{2+} through SOCCs (Casteels and Droogmans, 1981, Marthan, 2004). This Ca^{2+} entry can refill the stores, directly activate SMs contraction, or indirectly activate VOCCs by SOCCs induced depolarization (Albert and Large, 2003).

The SOCE membrane current is known as Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) (Hoth and Penner, 1992). Two families members of proteins are required by SOCE, the stromal

interaction molecules (STIM), (the $STIM_1$ and $STIM_2$), and the Orais molecules ($Orai_1$, $Orai_2$ and $Orai_3$). STIM proteins present in the SR, arranged in a tubular structure, and act as Ca^{2+} sensors. Whereas, Orais molecules are present in the cell membrane and act as pore-forming subunits of SOCCs (Smyth et al., 2010).

When the SR Ca^{2+} store content decreases, STIM proteins sense that and move very close to the plasma membrane and re-arrange their shape into punctate structures and activate Orai Ca^{2+} influx channels (Smyth et al., 2010). SOCE is a reversible process. It terminates when Ca^{2+} store refilling occurs. In response to store refilling, the near-plasma membrane STIM puncta are reversed into tubular form and move away to their original place, and this is considered as the responsible factor of the SOCE termination (Liou et al., 2005, Varnai et al., 2007, Smyth et al., 2008).

1.2.1.3   Receptor-operated calcium channels:

ROCCs are cell membrane channels that are stimulated independently of the membrane depolarization. ROCCs are activated by ligand binding to a range of G-protein-coupled receptors without involvement of diffusible second messengers (McFadzean and Gibson, 2002). ROCCs are sometimes referred as receptor-operated cation channel because of their non-selective cation currents with variable degrees of Ca^{2+} selectivity in the SMs (McFadzean and Gibson, 2002). ROCCs were described in a number of SM types, the small intestine of the guinea-pigs and rabbits, the ear artery and portal vein of the rabbits, the vas deferens and the tracheal myocyte of the rats (McFadzean and Gibson, 2002), as well as the saphenous vein of human (Xiong et al., 1991). However, there were no published studies on the neurotransmitters and hormones that reported to activate a receptor-operated cation current in smooth muscle such as ATP, NA, Ach, Histamine, Endothelin, Neurokin A substance P and vasopressin were found in the rat ureter.

In the SMs, ROCCs are described as ligand operated ion channels, in which the ligand binding site and the ion channel are contained within the same macromolecular complex (McFadzean and Gibson, 2002). Different types of ligands are found in the SMs that can activate ROCCs and produce rapid, transient depolarization with significant Ca^{2+} permeability (McFadzean and Gibson, 2002).

ATP (Suzuki, 1985, Nakazawa and Matsuki, 1987, Friel, 1988, Xiong et al., 1991) can activate ROCCs following activation of purinoreceptor (P$_2$X) (Khakh et al., 2001), Noradrenaline, following activation of α-adrenoceptors (Trapani et al., 1981, Amedee et al., 1990, Byrne and Large, 1988, Amedee and Large, 1989, Wang and Large, 1991), Acetylcholine, following activation of M$_2$ muscarinic receptors (Benham et al., 1985, Vogalis and Sanders, 1990, Sims, 1992, Kim et al., 1995, Lee et al., 1993), as well as several other hormones such as Histamine, Endothelin- 1, Neurokinin A, Substance P and Vasopressin (McFadzean and Gibson, 2002).

Moreover, ROCCs current are augmented by the IP$_3$-mediated release of Ca^{2+} from the intracellular stores (Inoue and Isenberg, 1990, Pacaud and Bolton, 1991) and Ca^{2+} entering through VOCCs (Pacaud and Bolton, 1991).

Furthermore, ROCCs modify membrane potential, and therefore can activate or inhibit VOCCs (McFadzean and Gibson, 2002).

All these emphasize the degree of the interaction between different calcium entry pathways.

1.2.2 Potassium (K+) channels:

K^+ channels contribute to the regulation of the membrane potential in electrically excitable cells. There are four types of K^+ channels that have been identified in the SMs; the Voltage operated inactivating potassium channels (K_V), Calcium-activated potassium channels (BK_{Ca}), the inward rectifier potassium channels (K_{ir}), and the ATP-sensitive potassium channels (K_{ATP}).

In the SMs of the rat ureter, three types of potassium channels have been identified, the K_V (A-type) channels, the BK_{Ca} channels, and the K_{ir} channels (Smith et al., 2002). While in the guinea pig, only two K^+ channel subtypes, contributing to the action potential repolarization phase and maintaining the resting membrane potential, have been identified, the K_V (A-type) and the BK_{Ca} channels (Imaizumi *et al.*, 1989;Lang, 1989).

1.2.2.1 The Voltage operated inactivating potassium (K_V) channels:

K_v channels are Ca^{2+}-independent potassium channels modulated by membrane depolarisation. K_v channels are responsible for maintaining resting state after depolarization, thus maintaining resting SMs tone. In response to membrane potential depolarization, which lead to Ca^{2+} influx through L-type Ca^{2+} channels, K_V channels open and allow K^+ efflux, resulting in repolarization and return to the resting membrane potential. Therefore, reduction of the duration of the action potential, decline of the influx of Ca^{2+} by the voltage-dependent calcium channels (Andersson, 1992), and a subsequent SMs relaxation. These channels are blocked by 4-aminopyridine (4-AP), but not Tetra-ethyl-ammonium (TEA) (Lang et al., 1998;Smith et al., 2002a). K_v channels are divided into rapidly inactivating (Type-A) and delayed inactivating (Stühmer et al., 1989).

The rapidly inactivating voltage operated potassium channel (Type-A) are found in the rat (Smith et al., 2002) and the guinea pig (Imaizumi et al., 1989, Imaizumi et al., 1990, Lang, 1989) ureteric SMs. They are fast, transient inactivating potassium outward channels.

Potassium currents are promptly activated to the greatest amplitude within 5-15 milliseconds and then, rapidly declined within 200 milliseconds (Lang et al., 1998a). Under normal resting membrane potentials, the channels are inactive and have little contribution to the delay of the action potential. Nevertheless, in the spontaneously contracting ureter, at the peak of the after-hyperpolarisation, more channels will be activated and their opening would slow the depolarisation and contribute towards the refractory period between the contractions (Lang et al., 1998), which may be responsible for controlling the rate of the spontaneous contractions (Smith et al., 2002). They are inactivated rapidly by depolarization, allowing the production of the action potential (Smith et al., 2002).

On the other hand, the delayed inactivating channels are slower than the rapidly inactivating and the mechanism of inactivation remains insufficiently studied (Magura et al., 2004).

1.2.2.2 Calcium-activated potassium (BK$_{Ca}$) channels:

BKCa channels are one of the best characterised ion channels and are present in copious amount in the SMs (Toro et al., 1998). BKCa are large conductance calcium activated K^+ channels, having similar structure of the K$_V$ channels (Bao et al., 2002). They were first defined in the SMs in 1986 (Benham et al., 1986). These channels can be blocked by TEA (Toro et al., 1998) and activated by an increase in the intracellular Ca^{2+} concentration and membrane depolarization. BK$_{Ca}$ channels play a vital role in reducing the plateau component of the action potential and controlling the repolarisation of the membrane potential (Imaizumi et al., 1989;Lang, 1989) by enhancing K^+ efflux, thus counteract Ca^{2+} induced depolarization and decrease membrane excitability. BK$_{Ca}$ channel blockers can produce a significant prolongation of the plateau component of the action potential in ureteric SMs cells (Imaizumi et al., 1989;Lang, 1989). Furthermore, the action potential of the ureteric SMs is considerably longer than that of the other SMs. This was explained by the observation of the smaller actual

outward current, and the lower open probability in the range of membrane voltages achieved during a depolarisation episode in the ureter as compare to other SMs (Sui & Kao, 1997b).

BK_{Ca} can be activated by Ca^{2+} Sparks (the short, focal events of local Ca^{2+} release from the filled SR in close proximity to the plasma membrane) after an action potential (Imaizumi et al., 1989). Ca^{2+} sparks can activate BK_{Ca} channels to produce spontaneous transient outward currents (STOCs) in the guinea pig and the rat ureteric SMs, as well as other SMs (Benham et al., 1986). Ca^{2+} spark and the followed STOCs are assumed to be an essential mechanism in controlling the resting membrane potential. Furthermore, Burdyga and Wray study showed that the refractory period is determined by the negative feedback process through the activation of BK_{Ca} by Ca^{2+} sparks. However, when Ca^{2+} store reduced, as a result of Ca^{2+} spark, this negative feedback ceases and the refractory period terminated, and thus tissue excitability resumed and contraction occurred (Burdyga and Wray, 2005).

1.2.2.3 Type 1 and 2 delayed rectifier K^+ (K_{ir}) channels:

K_{ir} are present in the rat's ureter, but not in the ureter of the guinea pig (Smith et al., 2002). K_{ir} are not blocked by TEA (Imaizumi *et al.*, 1989). It was suggested that they contribute towards the repolarisation phase of the action potential and may be involved in the regulation of the resting membrane potential (Smith et al., 2002).

1.2.2.4 K_{ATP} channels:

K_{ATP} channels can be activated by the changes in the cellular metabolism (Nelson & Quayle, 1995). They are activated by K^+-channel opener, leading to the induction of potassium efflux, thus increase cellular polarization, when the cell is in a polarized state, or accelerate its repolarization after a depolarization (Andersson, 1992).

1.2.3 Chloride (Cl⁻) channels:

1.2.3.1 Calcium-activated Chloride Channels:

The calcium-activated chloride currents have been found in several SMs (Arnaudeau *et al.*, 1994;Greenwood & Large, 1996), including the rat ureters (Smith et al., 2002), but not the guinea pig ureters (Imaizumi *et al.*, 1989). They are activated by an increase in the intracellular Ca^{2+} concentration, when Ca^{2+} enters from extracellular and/or released from the store (Amedee *et al.*, 1990;Lamb *et al.*, 1994). However, in the rats ureters SMs, they are chiefly activated by Ca^{2+} entry via L-type Ca^{2+} channels (Smith et al., 2002). Calcium-activated Chloride Channels can potentiate force via membrane depolarisation and increase Ca^{2+} entry through L-type Ca^{2+} channels (Bolton et al., 1999;Burdyga & Wray, 2002a). Smith et al study proposed that calcium-activated chloride currents might be an important contributor to the maintenance of the myogenic tone and the longer lasting plateau component of the action potential, seen in the rat ureter SMs (Smith et al., 2002). They can be blocked by any agents, reducing Ca^{2+}, or by the use of Ca^{2+} blocker (Criddle et al., 2002). Phosphorylation of the chloride channel by calcium-calmodulin kinase II inactivates chloride current (Wang and Kotlikoff, 1997).They are sensitive to temperature. Cooling prolongs action potential and increases force in the rat ureter by potentiation of the Ca^{2+-}activated chloride currents (Burdyga and Wray, 2002).

1.3 Excitation – Contraction in the ureteric smooth muscle:

Elevation of intracellular Ca^{2+} concentration is the main trigger for ureteric SMs contraction. The precise coordination between ureteric SMs and the excitation produced by the diffuse release of the neurotransmitters are the main regulator of ureteric peristalsis (Canda et al., 2007). Ureteric SMs contraction is regulated by two mechanisms, the electromechanical and the pharmaco-mechanical coupling process.

1.3.1   Electromechanical coupling process:

The initiation of the contraction caused by a change in the membrane potential has been called electro-mechanical coupling (Somlyo & Somlyo, 1994). This process plays a principal role in the phasic SMs (McFadzean and Gibson, 2002). The electrical activity, initiating the phasic contraction, differ in different SMs, and also in different muscle layers in the same tissue (Parkington and Coleman, 1990). Membrane depolarization and, or action potential (Somlyo & Somlyo, 1994) with the subsequent opening of the VOCCs, as a result of neurotransmitters or hormones stimulation, is the chief drive for the increase in intracellular Ca^{2+} and the consequent contraction of the muscles (McFadzean and Gibson, 2002). Whereas membrane hyperpolarizing agents cause relaxation. The electromechanical coupling process is inhibited by Ca^{2+} entry blockers of the VOCCs (McFadzean and Gibson, 2002).

1.3.1.1 The action potential in the ureteric SMs:

Each contraction is preceded by a complex action potential, consisted of a single or series of spikes, superimposing a prolonged plateau phase that lasts hundreds of milliseconds (300–800ms) in the rats (Burdyga and Wray, 1997, Burdyga and Wray, 2002), guinea-pigs (Burdyga and Wray, 1999a, Lang, 1989, Shuba, 1977) and human (Santicioli and Maggi, 1998), with or without oscillations of the potential, and a rapid falling repolarization component ended by an after-hyperpolarization (Figure 1.3.1-1). The peak diastolic potential of after hyperpolarization is −66 mV, which then declines slowly over 5–20 seconds, till one

more threshold is achieved to produce another action potential (Exintaris and Lang, 1999, Lang et al., 1998b).

Moreover, the refractory periods of the ureter is extremely long (more than 10 s) to ensure the unidirectional flow of the urine from the kidney to the bladder, and hence preventing urine reflux and kidney damage (Burdyga and Wray, 2005).

At rest, the membrane potential of the ureter SMs is negative, ranging from -50 to -45 mV in human, cats, and rats (Kaplon and Nakada, 2011, Lang et al., 1998b). Whereas in the guinea-pigs, it is between -49 and -68 mV (Lang et al., 1998b).

Ca^{2+} is a vital element in generating action potentials. Raising extracellular Ca^{2+} concentration, increases the amplitude of the action potential initial spike (Vereecken et al., 1975). Furthermore, the action potential can be abolished by Ca^{2+} channel blockers, but not with the neurotoxins, which suggests that the generation and the propagation of the action potential is myogenic (Washizu, 1966, Maggi et al., 1995b).

The upstroke phase of the action potential causes the opening of the fast L-type VOCCs, hence the initial rapid rise in the intracellular Ca^{2+} (Lang et al., 1998). Whereas, the plateau component is due to the slow Ca^{2+} channel that is permeable to both Ca^{2+} and sodium and the Ca^{2+}-activated chloride channels (Burdyga & Wray, 1999b). It was observed that the plateau phase in the guinea pig ureter can be abolished by the removal of the extracellular Na^+ (Kuriyama & Tomita, 1970; Shuba, 1977b). The repolarization phase is chiefly caused by the outward Ca^{2+}-activated potassium channels (BK_{Ca}), which are activated in response to the rise in the concentration of the intracellular Ca^{2+}, following release from the store (Imaizumi et al., 1989). The BK_{Ca} were also described to play an important role in the control of the duration of the plateau component (Imaizumi et al., 1989). Both the smaller actual outward ion current and the lower open probability reached during depolarization in the membrane voltage range, were suggested to be the cause of the observed exceedingly prolonged action

potential in the ureteric SMs as compared to other SMs (Sui & Kao, 1997b). Furthermore, the rats' action potential is very sensitive to temperature change, while that of the guinea-pigs isn't. This was due to the modulation of Ca^{2+}-activated Chloride currents, which are present in the rat, but not in the guinea pig ureteric SMs (Burdyga and Wray, 2002).

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1.3.1.2 Temporal relationship between action potential, Ca^{2+} transient and force in the ureteric SMs:

The action potential produces a Ca^{2+} transient, consisting of three components: (i) a fast rise in Ca^{2+}, associated with the initial action potential spike, (ii) a slower rise lasts 200-300 ms, and this is associated with the succeeding spikes of the action potential and the initial part of the plateau component, (iii) and a steady state part (maintained peak Ca^{2+} signal), associated with the plateau phase (Burdyga and Wray, 1999b).

It was found that the amplitude and the duration of Ca^{2+} transient is correlated with the action potential plateau phase duration and any agent, producing a prolongation of the plateau component of the action potential, will cause a prolongation of the steady state part of Ca^{2+} transient and increase the amplitude and the duration of force (Burdyga and Wray, 1999b). Yagi's study on single cells showed that both Ca^{2+} and force rose after maximal electrical stimulus, but Ca^{2+} increased much more, before the earliest noticeable increase in force. The force showed maximal sensitivity between 150 and 500 nM of Ca^{2+} (Yagi et al., 1988). However, when Ca^{2+} was introduced rapidly, the force-Ca^{2+} relationship displayed hysteresis; more force was produced when Ca^{2+} declined than when it rose (Yagi et al., 1988). This observation was further investigated by the study of Burdyga and Wary in intact phasic guinea-pig ureteric SMs and the myosin phosphorylation was suggested to be the major contributor of the delay between Ca^{2+} and force, but not the mechanical properties. Moreover, this hysteresis was found to be increased by cooling and abolished by slowing of the rate of intracellular Ca^{2+} rise (Burdyga and Wray, 1999b).

1.3.2 Pharmaco-mechanical coupling process:

A combination of Ca^{2+} release from the intracellular stores and Ca^{2+} entry, through the non-voltage-operated channels, mainly ROCCs and SOCCs, is termed as pharmaco-mechanical coupling, which considered as another main drive of the rise in the intracellular Ca^{2+} and the SMs contraction regulation (McFadzean and Gibson, 2002). The pharmaco-mechanical coupling referred to the binding of the excitatory agonists to the hetrotrimeric G-protein-coupled receptors, leading to the generation of the second messengers, and hence the SMs contraction, without a change in the membrane potential (Somlyo *et al.*, 1969;Somlyo & Somlyo, 1968).

The Ca^{2+} signal produced, consisted of an initial rapid, transient rise in the intracellular Ca^{2+} concentration, followed by a sustained smaller rise, dependent on Ca^{2+} entry from the extracellular environment (McFadzean and Gibson, 2002).

The pharmaco-mechanical coupling process is linked to Ca^{2+} sensitization, in which the contraction is maintained for prolonged periods in the presence of the agonist, while the levels of the intracellular Ca^{2+} concentration is near-resting levels (McFadzean and Gibson, 2002).

1.4 Calcium homeostasis in the ureteric smooth muscle:

Calcium ions play a critical role in the SMs contraction. An increase in intracellular Ca^{2+} is the major stimulus for activating SMs contraction. During activation, the intracellular Ca^{2+} concentration in the SMs increases from about 140 nM (resting value) to 500-700 nM (Williams and Fay, 1986). The rise in intracellular Ca^2 can be from Ca^2 entry from the extracellular space and/or from Ca^2 release from the SR.

1.4.1 Calcium entry mechanisms:

The concentration of Ca^{2+} is about 10 thousand times higher outside than inside the cell (Meldolesi and Pozzan, 1998), thus Ca^{2+} moves from the extracellular to the intracellular space. It has been observed that in the ureteric SMs, Ca^{2+} influx into the cells is mediated by both L-type VOCCs and Na^+-Ca^{2+} exchanger in Ca^{2+} entry mode (Aickin et al., 1984).

1.4.1.1 Voltage-Operated L-type Ca^{2+} channels (VOCCs):

The Ca^{2+} entry via voltage operated Ca^{2+} channels is considered as the chief mechanism for the increased intracellular Ca^{2+} concentration in the ureteric SMs during action potential generation (Sui and Kao, 1997a).

1.4.1.2 Sodium/ Calcium (Na^+-Ca^{2+}) exchanger:

The Na^+-Ca^{2+} exchanger is a plasma membrane transporter, playing a vital role in the maintenance of Ca^{2+} homeostasis in numerous cell types. It was proposed to be present in the SMs of the ureter (Aickin et al., 1984;Aickin et al., 1987).

It can operate in two modes, depending on the electrochemical gradient across the plasma membrane, (i) the forward Ca^{2+} mode, in which it extrudes intracellular Ca^{2+} from the cell in exchange of extracellular Na^+, and (ii) the entry Ca^{2+} mode (the reverse mode), which extrudes intracellular Na^+ in exchange of extracellular Ca^{2+} (Michel et al., 2014).

In the forward mode, the Na^+-Ca^{2+} exchanger maintain intracellular Ca^{2+} homeostasis by carrying three sodium ions into the cell and removing one Ca^{2+} ion outside the cell, using

energy from the electrochemical gradient, after SMs contraction (Reuter, 1991). However, when the intracellular Ca^{2+} concentration decrease, the Na^+-Ca^{2+} exchanger's entry (the reverse mode) becomes active and calcium influx occurs.

When the Na-K^+ pump inhibitor (Ouabain) was used in the guinea pig ureter, it caused an increase in the intracellular sodium concentration and transient contraction, when an extracellular sodium free solution was added. This observation was explained to be as a result of the activation of the Ca^{2+} entry mode of the sodium/ calcium exchanger (Aickin et al., 1984). Furthermore, this transient contraction was resistant to Ca^{2+} channel blockers, but sensitive to the sodium-calcium exchanger blocker (Manganese) that used in the cardiac muscle (Aickin et al., 1984;Aickin et al., 1987).

1.4.2 Calcium release from the store:

SR is the predominant intracellular Ca^{2+} store in the ureteric SMs. Ca^{2+} is stored in the SR, bound to the high capacity-low affinity Ca^{2+} buffering proteins (the Calreticulin and Calsequestrin) (Fixman et al., 2005). The high capacity Ca^{2+} binding reaches to 10 to15 mmol/l of Ca^{2+} (Fixman et al., 2005, van Breemen and Saida, 1989).

Two types of receptors were described in the membrane of the SR of the ureteric SMs responsible for Ca^{2+} release from the store; the IP_3 and the Ryanodine receptors.

In the rat's ureter, the SR contains only IP_3 receptors. IP_3 receptors can be activated by IP_3 and blocked by heparin (Burdyga et al., 1998). Agonists activating G-protein linked receptors cause the formation of IP_3, by the activation of PLC enzyme. The activation of IP_3 receptors can cause the release of Ca^{2+} from the SR (Patel et al., 1999), in a process called as IP_3-induced-calcium release (IICR) and the release of Ca^{2+} from a cluster of IP_3 receptors is called calcium puffs.

On the other hand, in the guinea pig ureters, only Ryanodine receptors (RyR) were found (Burdyga et al., 1995, Maggi and Giuliani, 1995). Ryanodine receptors can be activated by Ryanodine or caffeine (Hymel et al., 1988). They are activated by the rise in the intracellular Ca^{2+}, resulting from Ca^{2+} entry by VOCCs or Ca^{2+} release from the store. Release of Ca^{2+} from the store by the activated Ryanodine receptors is called calcium-induced-calcium release (CICR) (Maggi et al., 1995a) and the release of Ca^{2+} from a cluster of Ryanodine receptors is known as calcium sparks (Zholos et al., 1991;Zholos et al., 1992).

Both Ca^{2+} sparks and Ca^{2+} puffs produce the spontaneous transient outward currents (STOCs), which were seen in the guinea pig and the rat ureteric myocytes (Smith et al., 2002), as well as in a number of other SMs (Benham & Bolton, 1986). These spontaneous Ca^{2+} release events, the Ca^{2+} sparks and Ca^{2+} puffs, and the subsequent STOCs are thought to be an essential mechanism in maintaining the resting membrane potential (Smith et al., 2002).

Ca^{2+} release from the store by the activation of IP_3 or Ryanodine receptors is considered as the active (the stimulated) process of Ca^{2+} release. However, the passive (the leak) process of Ca^{2+} release is less well recognized and occurs by inhibition of the Sarco/Endoplasmic Reticulum calcium-ATP$_{ases}$ (SERCA) pump (Catterall, 2011).

SERCA pump is the membrane surrounding the SR and the calcium-ATP$_{ases}$, which act to maintain a 10.000 fold concentration gradient of Ca^{2+} between the lumen of the SR and the cytosol using ATP. It is the main mechanism of SR Ca^{2+} uptake.

The SERCA pump is normally bound to and inhibited by a small transmembrane protein (the phospholamban) (Arkin et al., 1997). Phosphorylation of phospholamban by protein kinase A (PKA) or protein kinase G (PKG) leads to the dissociation of the protein from the SERCA pump, and as a result, the SERCA pump become active (Raeymaekers et al., 1990).

Drugs such as Cyclopiazonic acid and Thapsigargin can inhibit SERCA pump leading to Ca^{2+} leak into the cytosol. Ca^{2+} leak causes emptying of the store and increases the intracellular Ca^{2+} concentration (Kuriyama et al., 1995). As well, this Ca^{2+} leak was shown to produce a transient rise in the amplitude and the duration of the phasic contractions.

Conversely, Ca^{2+} uptake from the cytosol into the store possibly plays a role in the relaxation of SMs (Maggi et al., 1995a).

1.4.3 Calcium sparks:

Ca^{2+} Sparks are the short, focal events of local Ca^{2+} release from the filled store in close proximity to the plasma membrane (Imaizumi et al., 1989) after an action potential.

Ca^{2+} sparks are produced mainly by the activation of the Ryanodine receptors. They produce the spontaneous transient outward currents (STOCs) (Bolton et al., 1999). Activation of Ryanodine receptors, causing the release of Ca^{2+} from the store is termed as calcium-induced-calcium release (CICR) (Maggi et al., 1995a). However, Ca^{2+} sparks is not sufficient to produce a global rise in Ca^{2+} concentration that could activate the contractile machinery and produce a contraction. Therefore, Ca^{2+} sparks can activate nearby Ca^{2+} sensitive ion channels located in the plasma membrane to get a sufficient Ca^{2+} level.

Application of Ryanodine receptor agonists in the guinea pig ureter SMs causes an increase in the amount of Ca^{2+} sparks and the number of STOCs recorded, by stimulating the BK_{Ca} channels on the surface membrane (Imaizumi *et al.*, 1989).

BK_{Ca} channels were described to have a vital role in the control of the action potential (Lang, 1989, Imaizumi et al., 1989) by causing hyperpolarisation and shortening the duration of the plateau component of the action potential. Furthermore, Burdyga and Wray study showed that the refractory period is determined by the negative feedback process, through the activation of BK_{Ca} by Ca^{2+} sparks. However, when Ca^{2+} store reduced as a result of Ca^{2+} sparks, this negative feedback ceased and the refractory period terminated, thus tissue excitability resumed and contraction occurred (Burdyga and Wray, 2005).

Calcium sparks may also activate calcium-activated chloride channels in other SMs types, such as the guinea pigs tracheal myocytes (Kotlikoff and Wang, 1998) and the rabbits corpus cavernosum (Craven et al., 2004), and this activation could initiate chloride ions efflux, thus causing membrane depolarisation and contraction.

1.4.4 Calcium puffs and oscillations:

- **Calcium puffs:**

The localized Ca^{2+} events released from the store, through the activation of IP_3 receptors is called calcium puffs (Boittin *et al.*, 2000;Kotlikoff, 2003;Lee *et al.*, 2002b;Morel *et al.*, 2003). As well as the calcium released by this process is called IP_3-induced-calcium release (IICR). Similar to the Ca^{2+} sparks, they also produce STOCs. However, in the rat ureter, IICR acts to potentiate force via membrane depolarisation and an increase in calcium entering the cell, through L-type VOCCs (Bolton et al., 1999;Burdyga & Wray, 2002a).

- **Calcium oscillations:**

Calcium oscillations (fluctuations) are sequential regenerative discharges of the stored Ca^{2+}. This fluctuation in Ca^{2+} concentration inside the cell occurs either spontaneously or after activation of the receptor by a ligand binding (Parkash and Asotra, 2012, Smedler and Uhlén, 2014), such as ACh by stimulating IP_3 receptors or caffeine by stimulating Ryanodine receptors (Coxhead, 2000). Ligand binding increases Ca^{2+} sensitivity of the SR, causing calcium-induced calcium release (CICR) from the SR (Coxhead, 2000). However, after the SR Ca^{2+} get depleted, Ca^{2+} -uptake increases and CICR decreases. Therefore, the SR refills again with Ca^{2+}. The process is repeated as long as the agonist is attached (Coxhead, 2000).

1.5 Calcium dependent activation of force:

In the SMs, Ca^{2+} is the chief second messenger. Stimulation of the SMs by different agonists or depolarization produces an increase in the free myoplasmic $[Ca^{2+}]$.

Free Ca^{2+} binds to Calmodulin. Ca^{2+}-CaM produced activates the Myosin light chain kinase enzyme (MLCK). As a consequence, activated MLCK catalyses phosphorylation of Myosin (II) at serine-19 of each of the two 20-kDa light chains. This allows Actin-Myosin interaction, resulting in force development.

Termination of the Ca^{2+} transient leads to the inactivation of MLCK. Thus, Myosin is dephosphorylated by the Myosin light chain phosphatase (MLCP), so it dissociates and remains detached from the Actin filament and the muscle relaxes.

1.5.1 Regulatory proteins:

Two major contractile proteins are identified in the cytoplasm of all muscle types, the Myosin (thick) and the Actin (thin) filaments (Broderick & Broderick, 1990). Nevertheless the amount of Actin is almost 3-5 times more in the SMs than in the striated muscles (Marston and Taylor, 1980, Malmqvist and Arner, 1990). As well, in contrast to the skeletal muscles, the Actin and Myosin myofilaments do not form myofibrils and the dense bodies that are either attached to the sarcolemma or disseminated in the cytoplasm and responsible for anchoring the thin Actin and the intermediate filaments (Desmin and Vimentin) are correspondent of the Z-lines, existed in the striated muscles.

When the muscle is activated, Myosin and Actin filaments overlap over each other, therefore tension is generated causing either shortening or lengthening of the muscles. This mechanism of contraction is called the sliding filament model (Gestrelius and Borgström, 1986).

1.5.1.1 Myosin:

Myosin II is the chief component of the thick filament. It is an elongated two headed protein, composed of three pairs of peptides. (i) Two heavy chains of 230kDa that contain a folded N-

terminal half in the globular head region having ATP and Actin binding site, and a helical C-terminal half forming a long coiled-coil tail (Craig and Woodhead, 2006). (ii) Two pairs of a regulatory light chain (RLC-20) of 20kDa to regulate the heavy chain structure and (iii) two pairs of an essential light chain (ELC-17) of 17kDa to stabilize the heavy chain (Craig and Woodhead, 2006, Vicente-Manzanares et al., 2009).

The two light chains (the RLC-20 and ELC-17) bind to the neck region, which act to amplify rotation of the head, when ATP hydrolysis is occurred (Craig and Woodhead, 2006). Phosphorylation of the two pairs of the MLC_{20} is needed for the Actin-activated Mg^{2+}-ATP_{ase} activity of the SMs Myosin (Higashihara and Ikebe, 1988). When MLC_{20} is phosphorylated, the Myosin undergoes conformational changes, leading to the activation of the Actin-activated Mg^{2+}-ATP_{ase} enzyme. However, when MLC_{20} is un-phosphorylated, it inhibits the Actin-activated Mg^{2+}-ATP_{ase} enzyme.

1.5.1.2   Actin:

Actin, the thin filament, is made up of polymeric filaments called F-actin. The F-actin is composed of many monomeric globules called G proteins. Each G protein molecule consists of 375 amino acids of 43kDa and contains ATP /ADP nucleotide binding site, involved in the polymerization, and a high-affinity Myosin head--binding site.

The conformation of Actin is depending on whether ATP or ADP is in the nucleotide-binding site. When it is bound to ATP, more F-actin is formed (Craig and Woodhead, 2006).

A pair of parallel F-actin strand, rotated by 166º in the filaments, forms the double helix microfilaments structure, existing in the cytoskeleton (Dominguez and Holmes, 2011), and this is called polymerization. In the polymerized condition, the Actin is capped and the thin filament stabilized by a protein known as β-Actinin (Ono, 2010).

It has been postulated that the regulation of the thin-filament may be responsible for the regulation of the muscle contractions (Haeberle, 1999). The strong-binding or the rigor-like

cross bridges of the thin filaments has a capacity of turning on the Actin filament, even in the absence of Ca^{2+}, as well as a role in the activation of both phosphorylated and un-phosphorylated SM Myosin, which suggests a probable mechanism for the activation of the slow cycling unphosphorylated cross bridges during tonic contractions of the SMs.

1.5.1.3 Caldesmon

Caldesmon is a thin filament and microfilament associated protein, or sometimes called as Calmodulin and Actin binding protein. It is the most abundant (l0-12µM) thin filament protein in the SMs, after Actin and Tropomyosin (Ngai and Walsh, 1985).

There is one Caldesmon protein for every 26 Actin monomers (Marston and Smith, 1984). It binds to Calmodulin, Actin, and Tropomyosin at the carboxyl terminal domain, whereas to Myosin at the N-terminal end of the muscle cells (Shukla et al., 2004).

It has high affinity for Actin binding and act as Acto-Myosin ATP_{ase} activity inhibitor, a process required for cross-bridge cycling by cross-linking Actin and Myosin filaments. However, this inhibition is reversed by its binding to Ca^{2+}-CaM (Pritchard and Moody, 1986).

Caldesmon can be phosphorylated by PKC, which is activated by the binding of the agonists to G- protein coupled receptors and Calmodulin-kinase II (CaM-kinase II), causing inhibition of its activity and thus, a reversal of Myosin ATP_{ase} inhibitory effect. Therefore, activation of G-protein-coupled receptors can lead to phosphorylation of Caldesmon, resulting in SMs contraction, which is Ca^{2+} independent. Thus, Caldesmon is considered as the large inhibitory protein (Marston and Smith, 1984) that prevent the uncontrolled activation of the contraction cycle (Horowitz et al., 1996).

1.5.1.4 Calponin

Calponin, a Troponin-T like protein, is present at the same concentration as Tropomyosin in the SMs (Winder et al., 1991). The h_1 isoform is expressed only in the SMs. It binds to

Actin, Myosin, Ca^{2+}-binding proteins and Tropomyosin. It also inhibits the Acto-Myosin ATP$_{ase}$, which is Calcium and Tropomyosin independent and inhibits the in-vitro movement of Actin filaments over Myosin.

Similar to Caldesmon, the inhibitory effect on MgATP$_{ase}$ can be reversed by the phosphorylation of the Calponin protein. Calponin can be phosphorylated at Ser$_{175}$ (Winder & Walsh, 1990) or Thr$_{184}$ (Nakamura et al., 1993) by PKC and CaM-kinase II and dephosphorylated by MLCP (Winder et al., 1992). When the inhibitory effect of Calponin is removed, an increase in the contractile force in a calcium independent manner occurs.

1.5.1.5 Calmodulin:

Calmodulin, a Ca^{2+} modulating protein, is a low molecular weight (15kDa) Ca^{2+}-binding protein, existed in the cytosol (Vetter and Leclerc, 2003). It is considered the chief transducer of Ca^{2+} signals in the mammalian cells (Van Eldik and Watterson, 1998) by regulating the activity of MLCK and the subsequent contraction (Allen and Walsh, 1994).

At rest, where the levels of the intracellular Ca^{2+} concentration are low, Calmodulin exists in a free form. When the intracellular Ca^{2+} concentration rises as a result of the influx of Ca^{2+} into the muscle, either from the SR or from the extracellular environment, four Calcium ions bind to one molecule of Calmodulin leading to a conformational change of Calmodulin molecule (Filenko et al., 1997). Therefore, permitting Ca^{2+}-CaM interaction with its target proteins. Myosin light chain kinase (MLCK) is one of the target proteins for Ca^{2+}-CaM.

The Ca^{2+}-Calmodulin-MLK complex formed can catalyse the two 20kDa of the Myosin light chain (MLC$_{20}$), via the phosphorylation of Myosin at Ser $_{19}$ (Allen and Walsh, 1994).

In the same way, Calmodulin can also regulate the thin filament-associated proteins (Caldesmon and Calponin) that inhibit the actin-activated MgATP$_{ase}$ enzyme activity of the SM Myosin, either by the direct binding of Ca^{2+}-CaM or indirectly by promoting phosphorylation of the two proteins by Ca^{2+}-CaM kinase II enzyme. This leads to

conformational changes in the head of Myosin. Thus, permits the activation of the myosin Mg-ATP$_{ase}$ activity by actin.

This activation causes actin-myosin interaction (the sliding of myosin cross-bridges along the actin filaments), and consequently SMs contraction (Jude et al., 2008).

Besides, calmodulin can also control proteins, which control the Ca^{2+} transport across the plasma and SR membranes to modulate SMs contractility. For instance, the plasma membrane Ca^{2+} pump and the Ryanodine receptor Ca^{2+} release channels, as well as other proteins that indirectly control intracellular Ca^{2+} concentration via cyclic nucleotide synthesis and breakdown, such as the NO synthase and the cyclic nucleotide phosphodiesterase (Walsh, 1994).

1.5.1.6 Myosin light chain kinase (MLCK):

MLCK is a serine/threonine-specific protein kinase, responsible for the phosphorylation of the regulatory light chain of myosin II (MLC$_{20}$), increasing Mg ATP$_{ase}$ enzyme activity, and the subsequent contraction in the SM tissues (Kamm and Stull, 1985).

It is mainly activated by Ca^{2+}-CaM complex.

MLCK has multiple ordered conserved domains; (i) the actin-binding domain at the N-terminus, which binds weakly to actin and Ca^{2+}-CaM, (ii) the proline rich repeat regions domain, which has unknown function, (iii) the Ig$_1$ and Ig$_2$ domains that can also bind to actin, (iv) the fibronectin (Fn) type 3 domain, which also has unknown function, (v) the catalytic domain (the kinase domain) that binds to ATP, as well as to the N-terminal phosphorylation domain of the myosin RLC that phosphorylate Ser$_{19}$ (Hong et al., 2011) and also Thr$_{18}$, when there is a higher level of MLCK or higher muscle activation (Ikebe et al., 1986).

Two other domains, which are to some extent, overlap with each other, (vi) the autoinhibitory domain and (vii) the calmodulin-binding domain. The autoinhibitory domain's amino acid sequence are similar to the amino acid sequence around Ser$_{19}$ of the MLC$_{20}$. This enables the

polypeptide chain of the MLCK to get folded, so that the autoinhibitory domain can bind to the myosin-binding site and prevent the access of the myosin-binding site to myosin. However, binding of calcium-calmodulin induces a conformational change, thus unfold the structure of the polypeptide chain, and as a consequence, the Myosin-binding site get exposed and can interact with Myosin (Islam, 2012). The last domain is (viii) the IgT domain at the C-terminal end, which binds to the smooth muscle Myosin (Hong et al., 2011).

Furthermore, MLCK can be phosphorylated at Ser_{815} by CaM kinase II (Stull et al., 1990;Stull et al., 1993) or PKC or PKA (Horman et al., 2008) resulting in desensitization of MLCK to the activation by Ca^{2+}-CaM complex, thus higher Ca^{2+} concentration would be required for the Myosin light chain phosphorylation (Stull et al., 1993).

1.5.1.7 Myosin light chain phosphatase:

Myosin light chain phosphatase, Myosin phosphatase, is also a serine/threonine-specific protein phosphatase, responsible for the dephosphorylation of the RLC of Myosin II (MLC_{20}) and relaxation in the SMs.

MLCP has three subunits: (i) a 37kDa catalytic (Protein Phosphatase 1 catalytic (PP_{1c})), (ii) a 110-130kDa regulatory Myosin-binding subunit (Myosin Phosphatase Targeting Protein 1($MYPT_1$)), and (iii) a 20kDa variable third subunit, which was identified to bind to the C-terminal end of MYPT (Hirano et al., 1997).

The $MYPT_1$ subunit binds MLCP to MLC_{20} and enhances dephosphorylation, thus allowing the light chain of Myosin to remain dephosphorylated, and thereby promoting relaxation (Hartshorne et al., 1998, Hartshorne, 1998). Additionally, the Myosin-binding subunit that binds to the phosphorylated MLC_{20} also promotes the activity of the catalytic subunit to dephosphorylate the $MLC_{20.}$

MLCP activity can be controlled by RhoA/ROCK pathway, thru receptor coupled to G proteins (Somlyo and Somlyo, 2000) and the phosphatase inhibitor protein (the PKC),

through receptor coupling with $G_{q/11}$ and PLC (Kitazawa et al., 2000). PKC is activated by DAG. Activated PKC phosphorylates protein phosphatase-1 inhibitory protein (CP1-17) at Thr38, and thus, enhancing its potency of inhibition MLCP (Eto et al., 1995, Eto et al., 1997).

1.6 Calcium independent modulation of force:

Besides to the Ca^{2+}- dependent activation of MLCK, MLCP is another important factor controlling muscle contraction via Ca^{2+} sensitization.

Ca^{2+} sensitization is the phenomenon of enhanced myosin light chain phosphorylation by inhibiting the activity of MLCP. The ratio of the activities of Calcium-Calmodulin dependent MLCK to MLCP is the major determent of the level of myosin light chain phosphorylation, and hence myosin Ca^{2+} sensitivity and the subsequent activation of the muscle contraction (Somlyo and Somlyo, 2003a).

1.6.1 Rho-Kinase:

RhoA, a small (~20 kDa) monomeric GTP_{ase} and its target Rho associated protein kinase (ROCK) have a vital role in the regulation of MLC phosphatase activity. Phosphorylation of MYPT1 at Thr_{695} by ROCK, inhibits MLCP activity and triggers Ca^{2+} sensitization (Uehata and Ishizaki, 1997).

Agonist activating GPCR ($G_{\alpha q}$) can stimulate not only Phospholipase C-β, but also RhoA (Somlyo and Somlyo, 2003a). RhoA is activated by guanine nucleotide exchange factors (GEFs) (Pelaia et al., 2008). Activated RhoA causes activation of ROCK by pathway known as the RhoA/ROCK pathway. Activated ROCK causes an increase in the force of the SMs, via inhibiting MLCP, through phosphorylation (Kitazawa et al., 1991a, Kitazawa et al., 1991b).

Bacterial toxins and Exoenzyme affecting RhoA are one of the ROCK inhibitors being used to explore the intracellular physiological role of RhoA (Wilde et al., 2000).

Clostridium difficile toxins A (Enterotoxin) and B (Cytotoxin) are high molecular weight glucosyltransferases, known to inhibit RhoA (Lucius et al., 1998). They have identical C-terminals, but different N-terminals, which constitute two-thirds of the toxins and contain the

glucosyltransferase activity that induce glucosylation of the threonine residue, causing inhibition (Hofmann et al., 1997).

They are nonspecific because they are not only deactivating RhoA, but also the small GTP_{ases} (Rac and Cdc_{42}) (Dillon et al., 1995, Aktories and Just, 1995).

Conversely, The C_3-Exoenzyme (C_3) (one of the ADP-ribosyltransferase bacterial toxins) can only inactivate RhoA. Therefore, it has been used as a specific inhibitor of RhoA to define the significance of the RhoA/Rho-kinase pathway (Wilde et al., 2000). However, because C_3-Exoenzyme is relatively impermeable to the cell plasma membranes, it was restricted to the experiments involving the use of permeabilised SMs. Moreover, formation of cell permeable chimeric protein (DC_3B) by genetically fusing of the Clostridium botulinum exoenzyme C_3 with the diphtheria toxin allowed to use the C_3-Exoenzyme as a specific inhibitor of RhoA, and thus evaluating its roles (Aullo et al., 1993).

Y-27632 dihydrochloride, HA-1077 and H-1152P can inhibit both Rho-kinase and PKC. However, they are considered as selective inhibitors of ROCK (Sasaki et al., 2002) because they are 100-fold more selective for Rho-kinase than PKC, which makes them valuable, when used in low concentrations. Moreover, Their PKC inhibition depends on the level of the expression of PKC and CPI-17 in various SMs.

Y-27632, (R-[+]-Trans-N-[4-pyridyl]-4-[l-amino ethyl]-cyclohexane carboxamide) is a selective pharmacological inhibitor of Rho-associated coiled protein kinase p160ROCK. It aid in the identification of RhoA-ROCK induced Ca^{2+} independent contraction and sensitization by inhibiting the kinases, via binding to the ROCK-1 and ROCK-2 catalytic sites (Amano et al., 1999, Fu et al., 1998, Kishi et al., 2000, Nakahara et al., 2000, Narumiya et al., 2000, Yoshii et al., 1999, Ishizaki et al., 2000). It is a highly potent cell-permeable pyridine derivative with IC_{50} of 700nM, and K_i of 140nM (Ishizaki et al., 2000). It has a 50 to 100 times more affinity for Rho-kinase than PKC_α, and an even more than 2000 fold affinity

than MLCK. Furthermore, the H-1152P is more specific for Rho-kinase, with K_i of 0.0016 μM for Rho-kinase and K_i of 9.27 μM for PKC (Somlyo and Somlyo, 2003b).

1.7 Bradykinin (BK) and its physiological effects:

1.7.1 Historical Overview:

In 1926, Frey and his colleagues characterized hypotensive substance, found in the urine in 1909, and they named it Kallikrein, as it was believed that the substance was produced mainly by the pancreas (Kallikreas in a Greek language) (Bader, 2011).

By 1937, Werle, et al had established that Kallikrein is an enzyme that generates a pharmacologically active substance from an inactive substance existed in the plasma, and later in 1948, they named the active substance Kallidin (KD) and the inactive one Kallidinogen (Bader, 2011).

On the other hand, the discovery of bradykinin (BK) was incidental. When the venom of Bothrops Jararaca and trypsin incubated with the pseudo-globulin fraction of the plasma by Rocha et al, they produced a potent vasodilator substance, having a slower SMs stimulating effect than that of the histamine and acetylcholine on the guinea pig ileum. The substance was not antagonised by atropine or antihistamine, therefore they named it bradykinin. Brady means slowly contracted and Kinein is the name of the guinea pig ileum in Greek language (Rocha et al., 1949). BK took many years to be characterised, yet a complete characterization was achieved in 1961 (Elliott, 1970).

Moreover, in 1980, Marin-Grez and his colleagues found that the addition of the rat's urine to the rat's ureter specimen caused ureteric contraction and this was prevented by the prior addition of aprotinin (a competitive serine protease inhibitor) or acid or heating (Figure 1.7.1), and they attributed this effect to the presence of kallikrein or kallikrein enzymes in the urine (Marin-Grez et al., 1980). However, in 1995, this urinary kinin was purified and identified as BK (Hagiwara et al., 1995).

1.7.2 Bradykinin physiological effects:

Bradykinin is one of the pharmacologically active ingredient of the vasoactive kinins, which have either pro-inflammatory and/ or protective effect in different tissues (Moreau et al., 2005). The pro-inflammatory effect of Kinins occurs after their release in response to tissue injury and a subsequent initiation of inflammation by activating endothelial cell, thereby causing vasodilatation, increase vascular permeability, NO production, and mobilization of Arachidonic acid (Moreau et al., 2005).

Kinins inflammatory effects manifest as the classical parameters of inflammation (the redness, hotness, swelling, and pain) (Kaplan et al., 2002). Burning dysesthesia is one of the pain manifestation, caused by kinins and it is due the stimulation of the sensory nerve endings (Moreau et al., 2005).

On the other hand, kinins defensive or protective effect can be seen in some organs, such as the heart and the kidney and it is manifested in controlling blood pressure, as well the angiogenesis-promoting effect (Moreau et al., 2005). Kinins also aid in the tissue repair and healing (Moreau et al., 2005).

Moreover, Kinins can affect other biological processes, such as contraction of the airways, intestine and uterine SMs. Besides, they have a role in cell proliferation, sperm motility and cytokine release (Eggerickx et al., 1992, Bhoola et al., 1992). In addition, it was proposed that Kinins have a possible role as a neurotransmitter, after observing the intracerebral bradykinin injections hypertensive, analgesic and hyperthermic effects (Eggerickx et al., 1992).

Kinins of human and most mammals include Bradykinin (the nona-peptide) (Arg-Pro-Pro-Gly-PheSer-Pro-Phe-Arg) and the Kallidin (KD) (the deca-peptide) (lysyl-bradykinin (LysBK)), with the sequence lysarg-pro-pro-gly-phe-ser-pro-phe-arg, and their carboxy-

terminal des-Arg metabolites. However, in the rats, T-kinin ((IIe-Ser-BK) and Met-T-kinin have only been described (Moreau et al., 2005).

1.8 Biochemistry of BK Formation:

1.8.1 Pathways of BK production:

BK is generated from two types of Kininogens; (the high molecular weight Kininogens (HK) and the low molecular weight Kininogens (LK)) by two serine protease pathways (the tissue and plasma Kallikreins) (Figure 1.8).

The Kininogens are formed from a structural gene, localized to chromosome 3q26-qter and made up of 11 exons. The first 9 exons encode the heavy chain, whereas the 10th exon encodes the BK and the light chain of HK. While the light chain of LK is coded by exon 11 (Moreau et al., 2005).

HK and LK have the same heavy chain amino acid sequence (the N-terminus), however the light-chain moieties (the C-terminus) is different.

HK is an α-globulin, existing in human plasma as a single-chain glycoprotein of 88 to 120kDa molecular mass and at a concentration of 70 to 90 µg/ml. However, LK is a β-globulin, found at a concentration ranging from 170 to 220 µg/mL and has a molecular mass of 50 to 68 kDa (Moreau et al., 2005).

Tissue Kallikrein (the glandular) is an acid glycoprotein, found in almost all cells throughout the body (the kidney, blood vessels, brain, pancreatic exocrine gland, gut, spleen, neutrophils, salivary, sweat and adrenal glands) (Bhoola et al., 1992, Mahabeer and Bhoola, 2000). Tissue Kallikrein produces KD from the low molecular weight kininogen (Mahabeer and Bhoola, 2000). Then, the N-terminal lys of the KD is cleaved by the plasma aminopeptidase to produce BK. The tissue kallikrein, identified in the plasma, has been proposed to be originated from the exocrine glands. It is produced as a proenzyme (prokallikrein), of which

neither the plasmin nor the plasma kallikrein could activate it successfully (Kaplan et al., 1998).

The plasma Kallikrein (The contact system of plasma) is synthesised in the liver and secreted as an inactive single chain α-globulin molecule called Prekallikrein (Moreau et al., 2005, Mandle et al., 1976, Bhoola et al., 1992). Prekallikrein is mostly circulated in plasma of humans and other animal species and generally bound to HK at a concentration of $35 - 50$ µg/mL (Moreau et al., 2005, Mandle et al., 1976). Formation of BK by this process is more complex than the tissue Kallikrein (Moreau et al., 2005). This process involves the activation of the intrinsic coagulation pathway. It starts by binding of Hageman factor (factor XII) to certain negative charge macromolecules, such as collagen, basement membrane, bacterial liposaccharides, urate crystals, etc., leading to the formation of an active form of factor XII (the XIIa). Plasmin facilitates contact activation of factor XII. At that point, The XIIa, activates Prekallikrein to Kallikrein, which consequently cleaves HK and releases BK (Kaplan et al., 1997).

In human, the plasma Kallikrein cleaves HK in two stages (Mori et al., 1981). Initial, HK is cleaved at the Arg^{389}-Ser^{390} bond of the carboxy-terminal portion of the BK sequence, causing the production of BK, attached to the carboxy-terminal end of the heavy chain. Next, the sequence Leu^{378}-Met-$LysArg^{381}$ is cut at the Lys^{380}-Arg^{381} bond liberating BK from the heavy chain.

Other mechanisms, by which BK is formed, is by binding of HK to the endothelial cells in the presence of zinc, leading to activation of prekallikrein to kallikrein (Moreau et al., 2005, Zhao et al., 2001, Motta et al., 1998), and subsequently, the release of BK from HK (Moreau et al., 2005, Motta et al., 1998). Moreover, the serum and tissue proteases, such as plasmin, can release both BK and des-Arg^9-BK from HK (Moreau et al., 2005, Molinaro et al., 2002).

1.8.2 Pathways of BK breakdown:

Plasma BK is degraded by three kinases, (Figure 1.8). (i) The kininase I (carboxypeptidase N (CPN; EC 3.4.17.3)), which degrades BK to Des-Arg^9-BK ((1–8)BK), (ii) the kininase II (Angiotensin-Converting Enzyme (ACE) (EC 3.4.15.1)), which degrades BK to (1–7)BK and (iii) the Neutral Endopeptidase ((NEP) (EC 3.4.24.11)) that degrades the (1–8)BK to (1–5)BK. Furthermore, Kininase II (ACE) degrades both (1–8) BK and (1–7) BK to (1–5) BK (Murphey et al., 2000).

On the other hand, Angiotensin-converting enzyme inhibitors inactivate the kinins-degrading enzyme (kininase II), resulting in accumulation of the kinins (Toda et al., 1987).
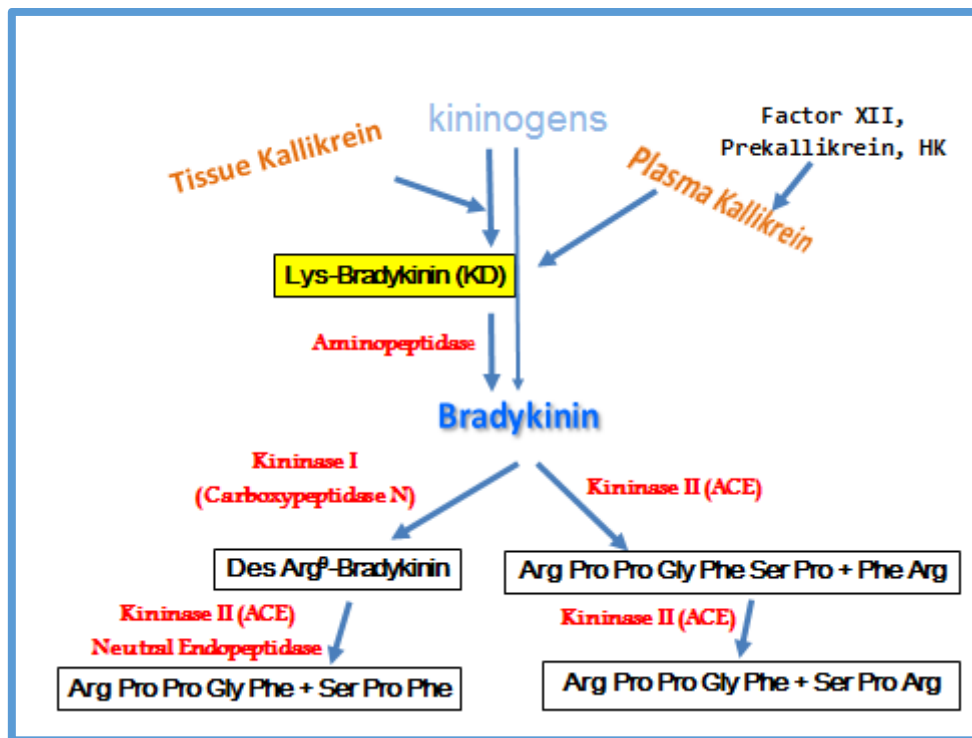
Figure 1.8. *Schematic diagram showing how BK is formed and degraded. HK, High molecular weight kiniongin; KD, Kallidin; ACE, Angiotensin-Converting Enzyme.*

1.9 BK receptors:

BK receptors are cell surface G-protein coupled receptors (GPCRs) of the seven transmembrane domain family (AbdAlla et al., 1996). They have been categorised into two different categories (BK receptor-1 (B_1) and BK receptor-2 (B_2) subtypes) by receptor cloning and expression studies (Hall, 1997b).

B_1 and B_2 receptors are encoded by a single-copy gene localized to chromosome $14q_{32}$ (Ma et al., 1994) and have 36% whole sequence homology (Hall, 1997b). B_2 subtypes are the chief receptors in human and rats (Eggerickx et al., 1992). However, in mice , both B_1 (McIntyre et al., 1993) and B_2 (Eggerickx et al., 1992) receptors were similarly detected. While in dogs preparations, the properties of B_1 receptors were seen more prominently (Toda et al., 1987).

B_1 receptors were characterized initially in the rabbit vascular SM preparations and the selective B_1 receptor antagonists (des-Arg^9-BK and des-Arg^{10} –Kallidin) were found to be a strong constrictors by antagonizing the effect of B_1 receptor agonists (BK and Kallidin) that cause vasodilation (Eggerickx et al., 1992). They are expressed at very low level in the cardiovascular, urinary, and intestinal systems and also in the vascular, endothelial, mesangial, tracheal, bone and fibroblast cultured cells (Hall, 1997b). Their role is mostly in chronic inflammatory responses and this was revealed by the in-vivo use of B_1 receptor antagonists (Hall, 1997b). They are normally absent, but are usually induced, following tissue injury, to enhance the inflammatory responses (Duchene et al., 2007) by increasing intracellular Ca^{2+} concentration in endotoxin induced inflammation in the rats (Bascands et al., 1998).

However, human B_2 receptors are expressed in most human tissues (Ma et al., 1994). They have a high affinity for the intact kinins (BK and KD), which mediate the majority of their effect. They are involved in more classical acute inflammatory events, such as oedema and inflammatory pain (Hall, 1997b).

B_2 receptors are further divided into presynaptic (neuronal) and postsynaptic (SM) subtypes according to their pharmacological response to the BK analogue ([Thi^{5,8},D-Phe^7]-BK) (Eggerickx et al., 1992).

BK is a B_2 receptor agonist (Figure 1.9) causing vasodilation of the arteries and veins of the gut, aorta, uterus and urethra (Golias et al., 2007) by the release of prostacyclin (Barrow et al., 1986), and NO (O'Kane et al., 1994) in human. However, in canine, it causes coronary artery vasodilation by the effect of endothelium-derived hyperpolarizing factor (EDHF) (Mombouli et al., 1992). In addition, BK causes visceral SMs contraction of some organs, such as the guinea pig ileum, trachea and urinary bladder, as well as the rat uterus and vas deferens (Calixto, 1995). Moreover, BK can also induce pain hypersensitivity by enhancing spinal cord glutamatergic synaptic transmission (Wang et al., 2005), as well it has a natriuretic effect (Willis et al., 1969). BK has a little affinity for B_1 receptors.

The intracellular second messengers that mediate BK responses are complicated, and not clearly identified in most tissues (Calixto, 1995). The effector tissue activated by BK depends on the enzymes or the channels that coupled to in each tissue (Calixto, 1995).

BK stimulates a variety of intracellular events, such as activation of phospholipase C (PLC) and phospholipase A_2 (PLA$_2$), opening of ion channels (Bhoola et al., 1992) and accumulation of cyclic nucleotide, adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) (Hall, 1992).

Activation of PLC and the generation of IP$_3$ cause an increase in intracellular Ca^{2+} concentration by stimulation of IP$_3$ receptors in the SR, leading to mobilizing of Ca^{2+} from the intracellular store to the cytoplasm, which subsequently increase Ca^{+2} influx from the extracellular environment in human bronchial epithelial cell line (Ricciardolo et al., 1998), nasal epithelial cells (Paradiso et al., 1991) and mesothelial cells (Andre et al., 1998).

Increased intracellular Ca^{2+}, induced by BK, caused SMs contraction in the guinea pig ileum, trachea, urinary bladder, and the rat uterus and vas deferens (Calixto, 1995).

The contractions in the guinea pig ileum, urinary bladder, as well as the rat uterus and vas deferens were proposed to be through extracellular Ca^{2+} influx, through L-type voltage-sensitive dihydropyridine channels (Calixto, 1995). However, in tracheal preparations, the BK contractile response was due to the Ca^{2+} release from the store.

Moreover, in other studies, BK relaxed the guinea pig tracheal SMs, with intact epithelium, and contract it by the removal of the epithelium (Figini et al., 1996), or by keeping the epithelium and using a protocol mimicking epithelial removal, such as pre-incubation with a combination of indomethacin and phosphoramidon that inhibit the neutral endopeptidase (an enzyme responsible for bradykinin metabolism) (Frossard et al., 1990) or a combination of indomethacin, propranalol, and phentolamine (Noor et al., 2011). In these studies, BK was hypothesized to induce bronchoconstriction by either directly or indirectly by leukotrienes release (Noor et al., 2011).

Furthermore, Bk acts on endothelial cells and has a powerful vasodilator effect by activating PLA_2, leading to the release of the vasodilator prostaglandins (PGs) such as PGI_2 in the rabbit, cat, guinea-pig, rat arteries, and the dog mesenteric veins (Toda et al., 1987) and the NO in the renal arteries of dogs (Toda et al., 1987) and both PGI_2 and NO in the coronary arteries of dogs.

Finally, BK may also increase the cAMP or cGMP levels secondary to the release of prostanoids (Hall, 1992). BK can induce a rapid increase in the level of cAMP and cGMP in the guinea pig ileum by Ca^{2+} dependent mechanism (Wei and Wei, 1992). In addition, BK relaxed bovine intrapulmonary artery and vein (Ignarro et al., 1987) through the action of cGMP and cAMP that is stimulated by NO and prostacyclin, respectively.
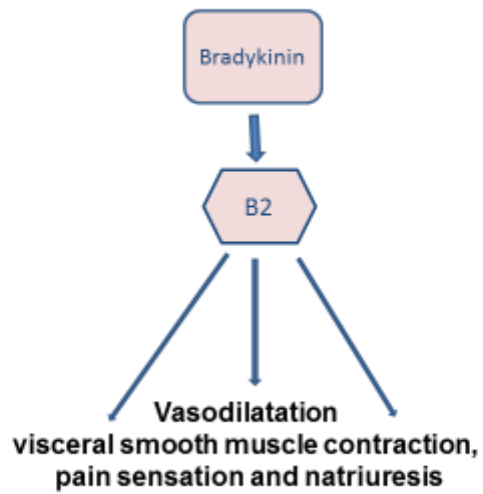
Figure 1.9. *The schematic diagram illustrating the biological action of bradykinin via activation of Bradykinin receptor-2 (B$_2$)*

1.10 Summary:

In summary, the ureters are vital tubes, made up mainly from SM fibres and their main role is chiefly the unidirectional transport of the urine from the kidney to the urinary bladder. Any disorders in the ureters, causing an obstruction to the passage of the urine, would have a detrimental effect on the kidney function.

Although the ureters' mechanisms of function have been investigated for a long time, much still remains elusive and unclear.

Bradykinin was reported to be present in the urine and cause stimulant action on the ureter more than 30 years ago (Marin-Grez et al., 1980). However, the mechanisms of its action on the ureteric SMs have not been studied.

The work in this thesis was carried out to investigate the mechanism, by which bradykinin induces ureteric SMs contraction. Overall, this study could serve as a basis for development of drugs, which could be used to treat ureteric disorders, caused by BK, in an infected ureter during reflux.

1.11 Aims:

The major goal of my study was to examine the Ca^{2+}-dependent and Ca^{2+}-independent mechanisms, underlying stimulant action of BK, on the smooth muscles of the rat ureter.

   The specific aims of this study were:

1- To examine the effect of different concentration of BK on the contractile activity of the SMs of different parts of the rat ureter.

2- To determine the functional role, expression and distribution of B_2 receptors along the rat ureter.

3- To investigate sources of Ca^{2+} and contribution of Ca^{2+} entry via voltage gated and store operated Ca^{2+} channels and Ca^{2+} release from the SR to Ca^{2+} signalling and force induced by BK in the rat ureter.

4- To investigate the possible role of Rho-associated kinase-induced Ca^{2+} sensitization as a component of Bradykinin (BK)–induced pharmaco-mechanical coupling in the rat ureteric SMs.

Chapter 2

General Material and Methods

Chapter 2

General Materials and Methods

2.1   Animals

Wistar rats of either sex were humanely killed, in accordance with UK legislation (Schedule 1 procedure; Animals (Scientific Procedure) Act 1986, UK). The ureters were removed and cleaned of any fat and connective tissue, under a dissection microscope. Subsequently, the ureters were cut into three segments: the upper, the middle, and the lower, (Figure 2.1) and each segment was cut into two strips, accordingly. The cut strips were placed in different plastic cups clearly marked for further use.
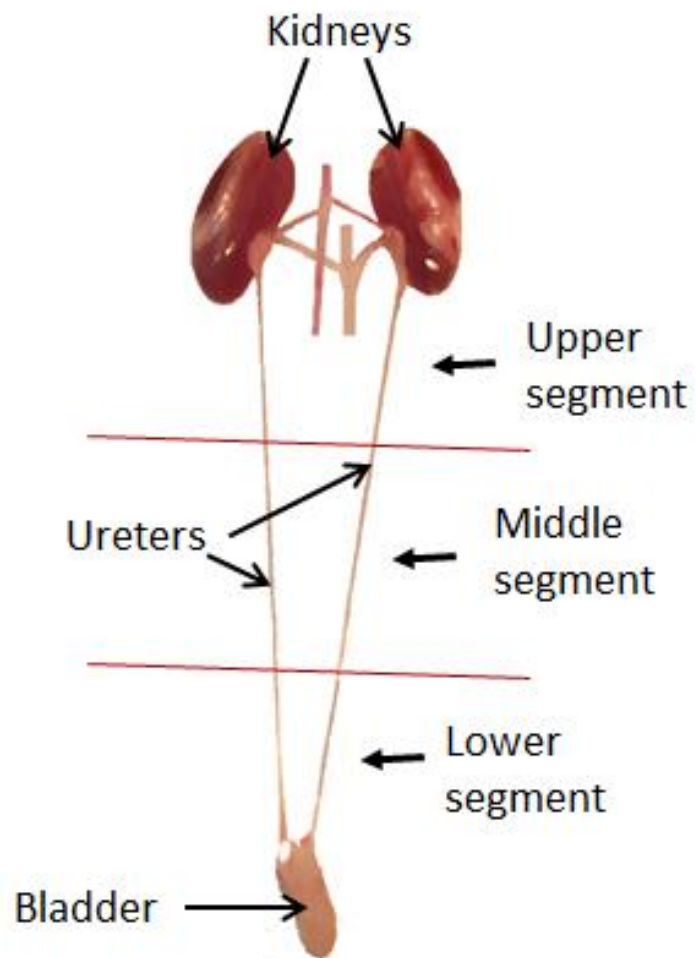
Figure 2.1. *A colour image showing the upper, middle and lower segments of the rat ureters, after dissection and removal of the connective and fat tissue, used in the present experiments.*

2.2   Solutions and chemicals

In all the experiments, the muscle strips were superfused with a modified physiological salt solution, buffered to pH 7.4 and containing (mM): 124 NaCl; 5.4 KCl; 1.2 $MgSO_4$; 11 HEPES; 11.7 Glucose and 2 $CaCl_2$. The high potassium solution (120mM) was prepared by isosmotically substituting 120mM of NaCl for KCl.

Bradykinin, Carbachol, Nifedipine, HOE 140, SKF 96365 were used in some experiments. Details of these and other stock solutions are given in the individual chapters. The vehicles used to dissolve the chemicals (e.g. ethanol, DMSO) did not alter the contractile properties of the ureter to the agonists. All chemicals and materials were acquired from Sigma (Dorset, UK), unless otherwise stated.

2.3 Measurements of mechanical activity and intracellular calcium

2.3.1 Measurements of force

Measurement of force was obtained by the use of a special apparatus, designed by Prof. Theodor Burdyga (University of Liverpool). A schematic diagram of the apparatus is given in Figure 2.3.1.

The apparatus consisted of a twelve wells plate, mounted on a moveable spring system, to enable easy transfer of the tissue between wells and filled with different solutions.

Ureters were cut into small segments (5 - 6 mm in length), clipped at both ends, using aluminum foil clips (Laser Services, USA), and attached to a force transducer (FORT 100,WPI) at one end and a stainless steel hook, fixed to the supporting rod, at the other end.

Force was measured with the force transducer and recorded, using DIGIDATA 132x data translation board (Axon instruments, USA) and AxoScope 8.2 data acquisition software (Axon instruments, USA).

The force transducer was attached to a 3-d manipulator (Narashige, Japan), allowing movement of the strip in the X-Y-Z directions, to align the strip and apply an optimal stretch, which did not exceed 40% of the active maximal force, induced by high $[K^+]$ depolarization that was also used as reference.

The force transducer was calibrated by using 200mg weight. The signal obtained was converted to force, using the equation $N=kg.ms^{-2}$ where 1kg is 9.8N.
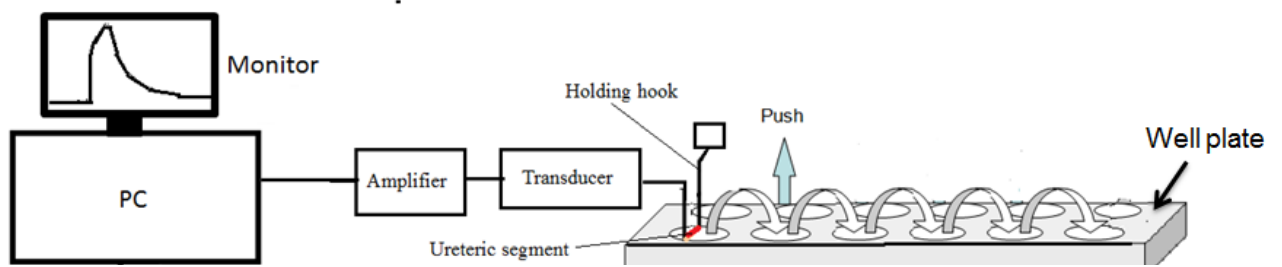
Figure 2.3.1. *Schematic diagram of the experimental set-up, used to measure mechanical activity of the ureteric segments. See text for detail.*

2.3.2 Fluo 4 loading

To load the tissue with fluo 4, strips of the ureter were placed in a plastic cuvette, containing 1ml of HEPES-buffered Krebs solution with 15 µM-fluo 4-AM dissolved in dimethyl sulphoxide premixed with Pluronic F127 (final concentration of 0.01 %).

Loading was performed at room temperature for 3 h, with the cuvettes wrapped in a black tape and rotated at 30 rpm. Tissue samples were then removed from the loading medium and placed in a normal Krebs solution for at least 30 min to allow cleavage of fluo 4-AM to fluo 4 by intracellular esterases.

2.3.3 Confocal imaging of intact rat ureteric strips combined with force measurement

We used fast Nipkow disc-based confocal imaging system (Figure 2.3.3). The images were captured by a high sensitivity (iXon Andor) CCD camera. Data acquisition was performed, using Andor iQ software (Andor, UK).

Images were acquired, using an inverted microscope, equipped with dry x2 (N.A. 0.04), x4 (N.A. 0.08), x10 (N.A. 0.4) and x20 (N.A. 0.72), as well as water immersion x40 (N.A. 1.2) and x60 (N.A. 1.2) objectives (Olympus, UK).

To simultaneously measure Ca^{2+} transients and force, ureters were cut into small segments (5 - 6 mm in length), clipped at both ends, using aluminum foil clips (Laser Services, USA), and attached to a force transducer (FORT 10,WPI) at one end and a stainless steel hook, fixed to the bottom of the experimental chamber at the other end. The force was measured by transducer using the same procedure explained in previous experimental set up.

The preparation, which was loaded with fluo-4, was mounted in a plastic chamber with a volume of 500µl that was placed on a stage of an inverted microscope. The bottom of the chamber was sealed with a glass coverslip, using high vacuum grease. This allowed the transmission of light to and from the tissue, and also allowed the coverslip to be easily changed when necessary.

The tissue was superfused at a flow rate of $2.5ml.min^{-1}$ with the buffered physiological Krebs solution (pH 7.4). However, in those experiments, in which High K^+ solution or agonists were used to stimulate the muscle, a flow rate of $5ml.min^{-1}$ was used to ensure that the solution exchange in the organ bath occurred as rapidly as possible.
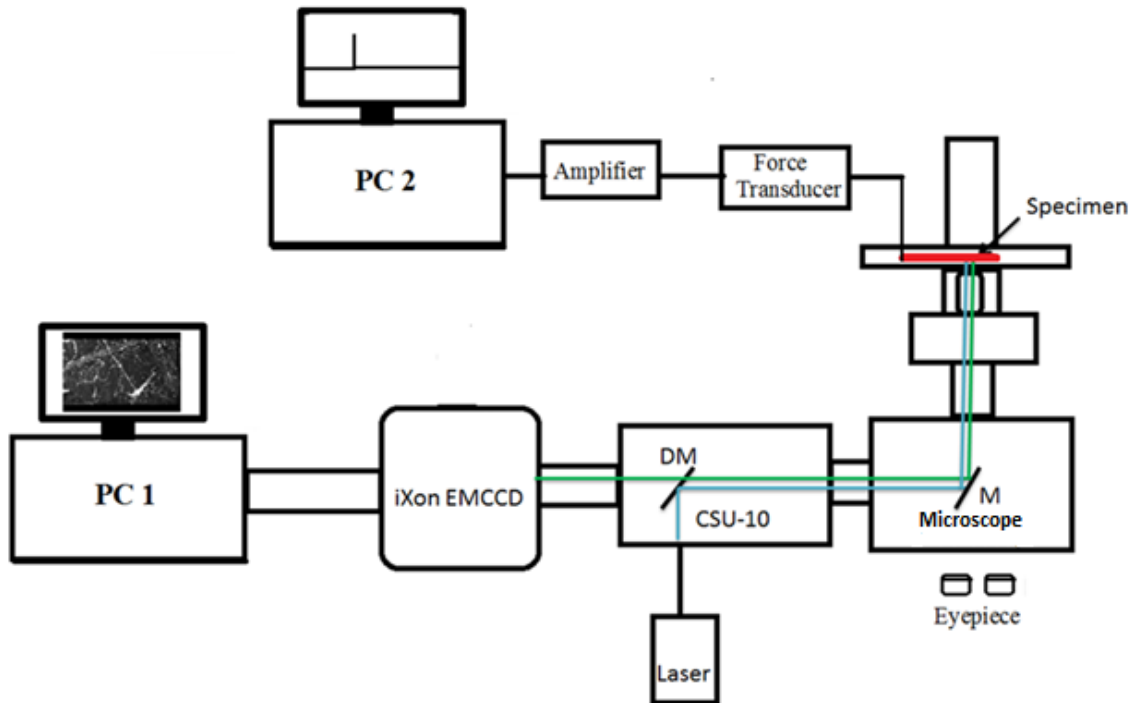
Figure 2.3.3. *Schematic diagram of confocal Nipkow disc based confocal imaging system combined with the system of force measurement. Pale blue and green lines show the excitation and emission light path, respectively.*

2.4   Immunohistochemsitry.

The ureters from the rats were freed from the fat and the connective tissues. The ureters were left attached to small parts of the kidney and the urinary bladder to help in recognizing ureter's parts. Then, the cleaned ureters were fixed with 4% Paraformaldehyde (PF) in phosphate buffer saline (PBS) at room temperature for 2 hours, after which they were washed with PBS three times for 5 minutes per time. Subsequently, the ureters were put in a PBS containing 20% sucrose in the refrigerator. On the second day, the ureters were cut into three pieces, upper, middle, and lower sections, after removing parts of the kidney and the urinary bladder, and embedded in 75% gelatine, put in 15% sucrose in PBS and incubated for 1 hour at 37C° in square weighing boats.

The embedded sections were then removed from the weighing boats, once solidified, and mounted with cryo-M-bed embedded compound (Bright Instruments, UK) on a cork disc labelled with a histology number. The specimens were covered with glue and frozen with Isopentane from -80C° freezer and stored at -30C°. Afterwards, sections of different specimens were prepared in a Cryostat at a thickness of 7µm. The cryosections were then placed on a chrome alum gel coated slide (Kiernan, 1999) and stored at -20C°.

Frozen slides were taken out the refrigerator and left to thaw for about 15-20 minutes. Then, a Dako pen was used to draw around and mark each section to prevent cross-contamination between them.

The anti-bradykinin-2 (B_2) receptor antibody (1:1000 dilution) (ABR-012, Alomone Labs) was used. A mixture of 0.2% triton and 5% goat serum in PBS was added to the diluent primary antibody. The triton was added to permeabilise the cells, while the goat serum to block the nonspecific protein binding. The primary antibody mixture was added across the sections and left for 2 hours.

After being rinsed for 3-4 times in PBS, the sections were incubated in a secondary antibody diluent (1:200 dilution), Alexa Fluor ® 488 Goat anti-Mouse IgG (H+L) secondary antibody (A-11001, Invitrogen), in a mixture of 0.2% triton and 5% goat serum in PBS at the appropriate concentration for one hour at room temperature. The Secondary antibody was selected in that they should correspond to the animal, in which the primary antibody was raised in.

After washing four times in PBS, the nuclei were counterstained with 4', 6 Diamidino-2-phenylindole, Dihydrochloride (DAPI) (D1306, Invitrogen) for 7 minutes at room temperature. The DAPI was used at 1 in 100,000 dilution of 1mg/ml stock in PBS.

Finally, following a five minutes wash in PBS, a few drops of Dako cytomotion, a fluorescent and mounting media, were placed on the slide and cover slips of (50 x24 mm) were mounted on the slides.

Upper, middle, and lower samples were stained at the same time, to avoid any variation in the results. Slides stained with the antibody were left in the fridge, until they were ready for imaging.

The sections were viewed under a fluorescence microscope. A Nikon Eclipse 50i microscope with NIS- elements-F software (Nikon instruments) was used to observe and photograph the sections that used for analysis. The results taken under the same settings were compared, using Image J software (image J 1.34s public domain downloaded from rsb.info.nih.gov/ij/).

Furthermore, high quality images of the sections were taken by the confocal microscope. Besides, confocal imaging was also used to quantify the level of the expression of B_2 receptors in the ureteric smooth muscle samples. The ratio of the fluorescence, measured in the ureteric smooth muscle layer and the urothelium was taken as a relative measure of the level of the expression of B_2 receptors in the ureteric smooth muscle cells.

2.5　Analysis and statistics

The software used to analyse the force and calcium recorded from the force -calcium rig was Microcal Origin (Massachusetts, USA). The data are given as mean ± s.e.m with 'n' represents the number of experiments on different animals. The statistical significance of the results was measured using the appropriate t-test (paired or unpaired). P values <0.05 were taken as statistically significant.

Most of the results were given as a percentage relative to the High-K peak contractile response, unless otherwise stated in the individual chapters.

Chapter 3

Expression and distribution of bradykinin B$_2$ receptor in the rat ureter

Chapter 3

Expression and distribution of Bradykinin B_2 receptors in the rat ureter

3.1   Introduction

Bradykinin, which is one of the pharmacologically active ingredients of kinins, is present in the urine and induces inflammatory and contractile responses in the ureteric smooth muscle (Catacutan-Labay 1966; Ribero et al., 2016). BK is involved in many physiological and pathological processes in many organs (Moreau et al., 2005).

BK effects are mediated mostly by the high affinity B_2-subtype receptors, which are one of the G-protein-coupled receptors, and considered as the chief receptors in human and rats (Eggerickx et al., 1992).

The presence of B_2 receptors has been established by the pharmacological, radioligand binding studies and receptor cloning and expression analysis in different cells of the rats, guinea pigs, human and mice (Hess et al., 1994, Hall, 1997a, Ma et al., 1994, Calixto, 1995), including SM cells (Calixto, 1995), in which BK is a potent stimulant (Catacutan-Labay and Boyarsky, 1966a) via activation of the smooth muscle B_2 receptors (Calixto, 1995, Dray and Perkins, 1993).

B_2 receptors are expressed in many SMs of the rats, including the uterus and vas deferens (Calixto, 1995), as well as the urinary bladder (Chopra et al., 2005). Moreover, recent data indicate that B_2 receptors are also expressed in the smooth muscle of the pig ureter (Ribero et al., 2016). However, there are no data exists, illustrating the expression and the distribution of B_2 receptors along the length of the rat ureter.

Mapping the distribution of B_2 receptors can help in understanding the probable role of B_2 receptors in the physiological, as well the pathophysiological processes of the ureter.

The aim of these experiments was to study the expression and the distribution of B_2 subtype receptors along the rat ureter.

3.2   Material and method

To see the expression and the distribution of B_2 receptors along the rat ureters, we performed a highly sensitive immunofluorescence staining protocol of the ureteric cross sections, prepared from the upper, middle and lower rat ureter.   Frozen sections produced from different segments of the rat ureters were immunostained with, Anti-B_2 receptor antibodies. The method was discussed in details in Chapter 2.

3.3   Results

3.3.1   Expression and distribution of bradykinin B_2 receptors along the ureter

To observe the expression of B_2 receptors in the ureter, 100 sections from 4 rats were immunohistochemically stained for B_2 receptor using Anti- B_2 receptor antibody. Immunohistochemical studies from the upper, middle and the lower parts of the ureter revealed the presence and a homogenous distribution of B_2 receptors in the urothelium (Figure 3.3.1, left panel). In marked contrast, the expression and the distribution of B_2 receptors in the smooth muscle layer was non-homogenous. Thus, the anatomic distribution of B_2 receptors in the rat ureteric smooth muscle was significantly higher in the smooth muscle cells of the proximal upper ureter (Figure 3.3.1, Left panel). The corresponding nuclei staining with DAPI of each section is shown in figure 3.3.1 (Middle panel) and the merged images of anti- B_2 receptor and DAPI are also illustrated in figure 3.3.1 (Right panel).
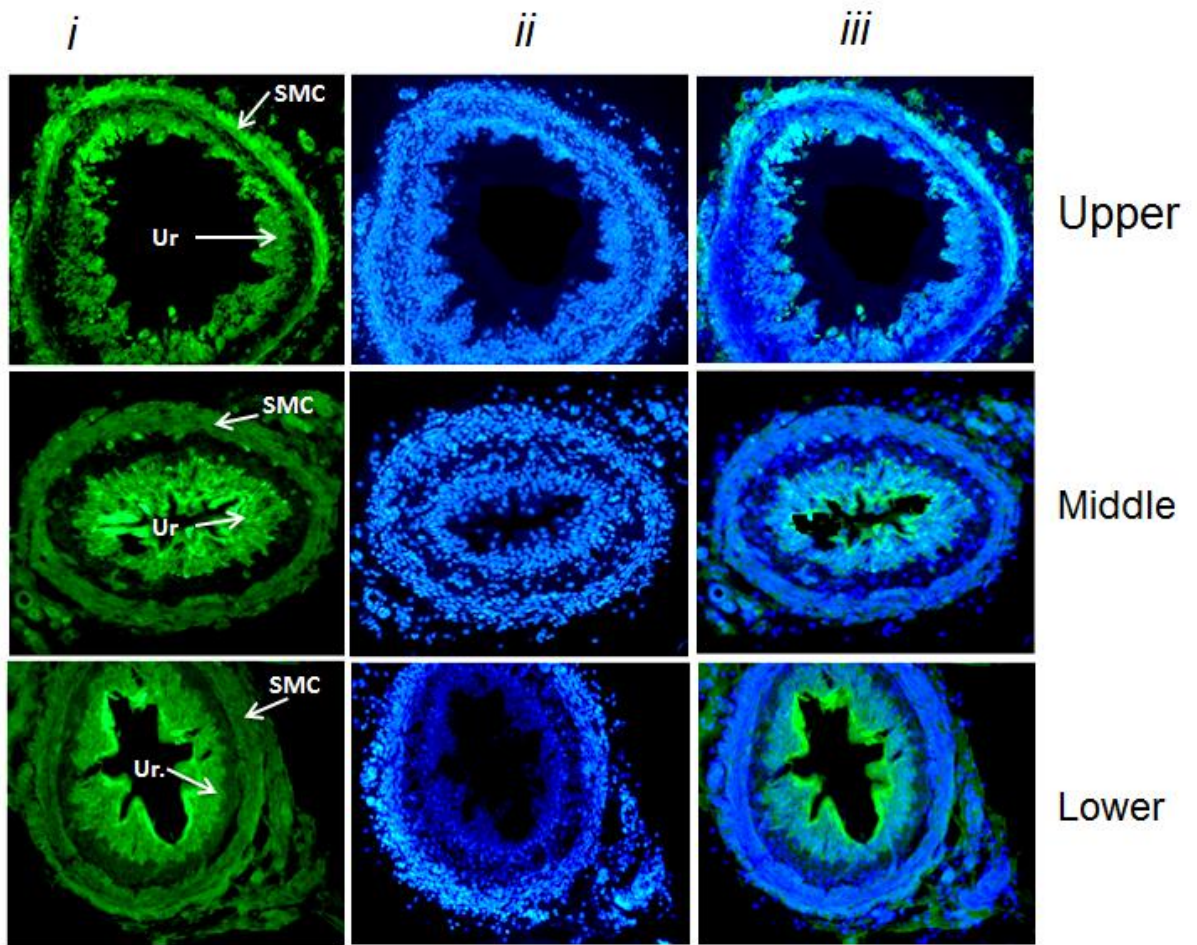
Figure 3.3.1. *Immunohistochemical localization of B$_2$ receptors in the upper, middle and lower rat ureter. Immunohistochemistry shows a homogenous expression of B$_2$ receptors in the urothelium and a non-homogenous in the smooth muscle layer of the rat ureter. Shown are Alexa Fluor 488 immunostaining (i, green), DAPI staining (ii, blue) and overlay of Alexa Fluor 488 and DAPI (iii). Urothelium (Ur) and smooth muscle cell layer (SMC) are shown by white arrows.*

3.3.2 Quantification of level of the expression of B_2 receptors in the smooth muscle cells using confocal imaging

Further analysis of level of the expression and the distribution of the B_2 receptor was done by the use of confocal imaging. The relative measure of the level of the expression of B_2 receptors, in the ureteric smooth muscle cells, is calculated by measurement of the ratio of fluorescence intensity in the ureteric smooth muscle layer relative to the urothelium (Figure 3.3.2A and B).

As can be seen in figure 3.3.2B, the percentage of the expression of B_2R was highest in the upper part of the ureteric smooth muscle layer, ($93\% \pm 0.06$, n=7), lowest in the middle part ($41\% \pm 0.06$, n=9), and modest in the lower part, ($51\% \pm 0.06$, n=11) relative to the urothelium.
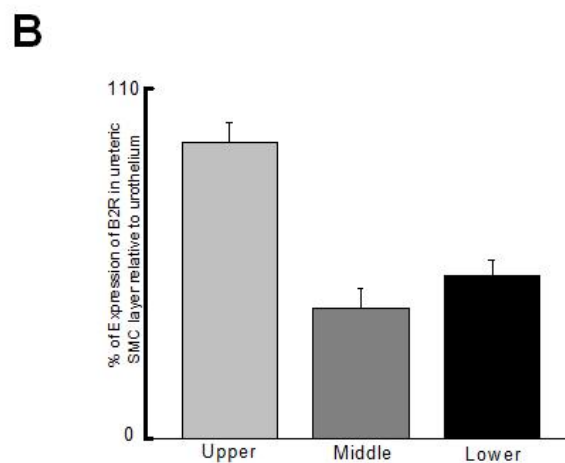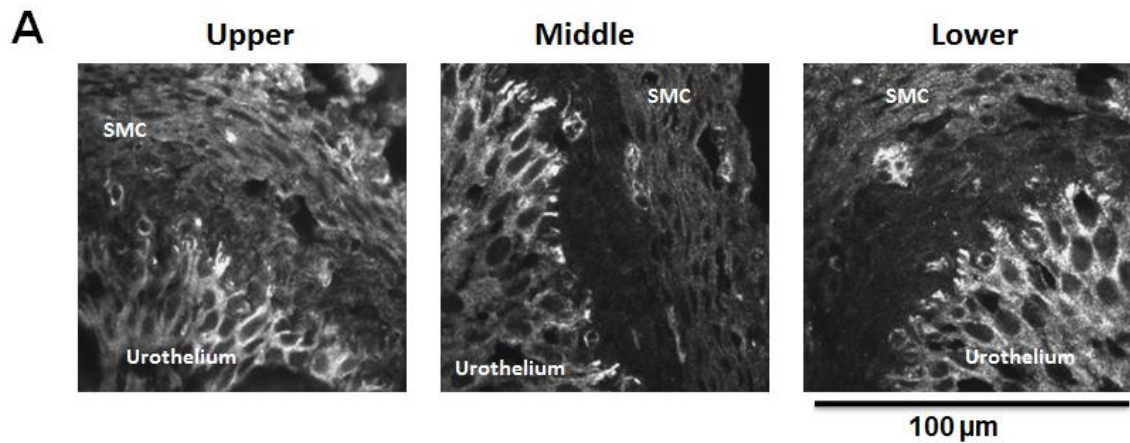
Figure 3.3.2. *Immunofluorescence staining of B_2 receptors in the upper, middle and lower rat ureter. A: Confocal grey scale images showing B_2 receptors expression in the urothelium and the smooth muscle of different parts of the rat ureter. B: Quantification of B_2 receptors expression in the ureteric smooth muscle of different parts of the rat ureter. The B_2 receptors' level of expression was quantified relative to the level of expression in the urothelium taken as control. n=7-11, p<0.005.*

3.4   Discussion

The expression and the distribution of B_2 receptors had not previously been characterised in the rat ureters, although, this was determined in other tissues (Manning and Snyder, 1989, Monsees et al., 2002). Previous immunohistochemical studies showed the presence of B_2 receptors in the rat urinary urothelium and the smooth muscle cells of the bladder (Chopra et al., 2005), as well as the uterus, vas deferens, kidney, lung, heart, ileum, testis, and brain (Adrienne et al., 1991). This is the first study showing the presence of bradykinin B_2 receptor in the rat ureter.

Our data reveal that B_2 receptors are present in the urothelium and the smooth cell layer of the rat ureter, which agrees with the recent data obtained on the pig ureter (Ribeiro et al., 2016). The present results provide evidence for the first time, which indicates that the anatomic distribution of B_2 receptors in the smooth muscle cells of the rat ureter is not homogenous and that the receptor is more predominantly expressed in the proximal upper part of the ureter. On the basis of the data obtained, one can conclude that the rat ureteric SMs express B_2 receptors, which are differentially distributed along the length of ureter.

The higher level of B_2 receptor expression in the SM layer of the upper ureter could be expected to facilitate a greater potential for stronger stimulant action of BK on the ureteric smooth muscle.

Chapter 4

Effects of BK on the contractile activity of the rat ureteric smooth muscles

Chapter 4

Effects of BK on the contractile activity of the rat ureteric smooth muscles

4.1   Introduction

Bradykinin (BK) is a potent mediator of inflammation causing pain, vasodilatation, increased vascular permeability, and smooth muscle contraction. BK is reported to cause contraction of different types of visceral smooth muscles, in different species. Thus, BK was shown to cause stimulant action on the SMs of the guinea pig gall bladder (Stasiewicz et al., 1977), ileum, trachea and urinary bladder (Calixto, 1995), as well as the rat uterus and vas deferens (Calixto, 1995), the rabbit gall bladder (Stasiewicz et al., 1977), the cat jejunum (Ferreira et al., 1973) and the pig intravesical ureter (Ribeiro et al., 2016). However, in some types of the smooth muscles e.g. guinea pig *taenia coli*, BK produces dual effect, an increase in force followed by a decrease (Aarsen and Van Caspel-De Bruyn, 1970).

BK caused graded contraction in the guinea pig ileum, trachea and urinary bladder, as well as the rat uterus and vas deferens in vitro (Calixto, 1995). The contractile responses to bradykinin in the guinea pig ileum and urinary bladder as well as the rat vas deferens and uterus were markedly attenuated in Ca^{2+} -free medium with or without EGTA or by Nicardipine, whereas those in the guinea pig trachea, they depended almost exclusively on the intracellular Ca^{2+} sources, which were sensitive to Ryanodine (Calixto, 1995, Ricciardolo et., al 1998).

On the basis of these data, it was proposed that in most visceral smooth muscles, the main mechanism involved in the elevation of the intracellular Ca^{2+} is mediated by Ca^{2+} influx via L-type voltage-sensitive dihydropyridine channels (Calixto, 1995). In contrast, in the smooth muscle of the airways, the BK contractile response was assumed to be due to the Ca^{2+} release from the store.

BK has been identified in the urine and the stimulant action of BK on the ureteric smooth muscle of the dog and the cat have been reported as early as 1966 (Catacutan-Labay and Boyarsky, 1966b). However, there was no systematic study, investigating the sites and the mechanisms of action of BK on the ureteric SM.

BK effects are mediated mostly by the high affinity B_2-subtype receptors, which are one of the G-protein-coupled receptors and are considered as the chief receptors in human and rats (Eggerickx et al., 1992). The immunohistochemical data presented in the previous chapter indicated a non-homogenous distribution of B_2 receptors in the rat ureter. Yet, the site and the mechanism of the action of BK on the rat ureteric smooth muscle is still unknown.

Based on these anatomical data presented in the previous chapter, one could hypothesize that the BK effect on the rat ureter smooth muscle should be site specific and more prevalent in the upper ureter.

For those reasons, we have aimed to investigate and compare the effect of BK in different regions of the rat isolated ureter, and also to explore the mechanisms underlying the BK mediated ureteric effect by using the selective B_2 receptor antagonist (HOE- 140) (Hock et al., 1991, Wirth et al., 1991), the selective blocker of the voltage gated L-type Ca^{2+} channel (Nifedipine) and the non-selective blocker of receptor/ and store-operated cation channels (SKF 96365) (Merritt et al., 1990). Also, the role of the SR Ca^{2+} release and Ca^{2+} release/ Ca^{2+} entry coupling mechanism in the contractile responses, elicited by bradykinin in the rat ureteric smooth muscle, were also investigated.

4.2.   Materials and methods

Measurement of force was done by the use of the apparatus described in details in Chapter 2. The ureter was cut into 3 segments: upper, middle and lower. The ureteric segments from each part of the ureter were exposed to BK (0.1, 0.2, 0.5, 1, 2.5, and 5 μM), which were added cumulatively with 5 minutes increments. In most of the experiments, three concentrations of BK were used (0.2, 0.5, and 5μM), which allowed to get more reproducible data and get larger number of experiments. The effects of CCh on the contractile activity of the ureteric smooth muscle of all parts of ureter were also investigated.

To study the role of B_2 receptors, the effects of the selective blocker of B_2 receptors (HOE-140) on the contractile responses induced by BK at maximal concentration (5μM) were explored. To study role of the L-type voltage gated Ca^{2+} channels, the effects of the selective blocker (Nifedipine) on the contractile responses of the ureteric smooth muscle to 5μM BK was studied. Nifedipine was added before and during BK stimulation.

To study the role of SR Ca^{2+} release, the effects of BK on the contractile responses of the ureteric smooth muscle in Ca^{2+}- free solution containing 2 mM EGTA was investigated.

The non-selective blocker of store operated channels (SKF96365) was also used, to study the possible role of the capacitive Ca^{2+} entry, in the stimulant action of BK on the ureteric smooth muscle.

For statistical analysis, the amplitude of the tonic contraction, induced by BK, was measured and expressed as a percentage relative to the high-K peak contractile response. In addition, the frequency of mechanical oscillation was measured and expressed as a number of oscillations per 1 minute. The data were analysed with students (paired or unimpaired) t-test; differences between means were assumed to be significant at $P<0.05$. Data are given as mean ± s.e.m.; n is the number of samples.

4.3   Results

4.3.1   The effects of BK on the contractile responses of the smooth muscles of the upper, middle and lower ureter

The first aim of these experiments was to define the sensitivity of the SM of the upper, middle and lower segments of the rat ureter to BK. Figure 4.3.1 shows an example of the contractile responses of the ureteric SMs of different parts of the ureter from the same rat, induced by brief (40s) application of high-K^+ (used as reference) and different concentrations of BK (0.1, 0.2, 0.5, 1, 2.5, and 5µM) added in a cumulative way (n=3).

These experiments showed that the ureteric SM of the upper segments was much more sensitive to BK than those of the middle and the lower parts of the ureter. Also, in the upper part of the ureter, the threshold concentration, which evoked spontaneous activity, was 0.5 µM, while in the middle and the lower segments, 2-5µM was required to initiate spontaneous activity in the majority of the experiments. Based on these data, in the subsequent experiments, the concentrations of BK used have been limited to 0.2, 0.5, and 5µM, which allowed studying the effects of BK on the contractile activity of the ureteric SMs in each segment of the ureter in more details and get quantitative results.
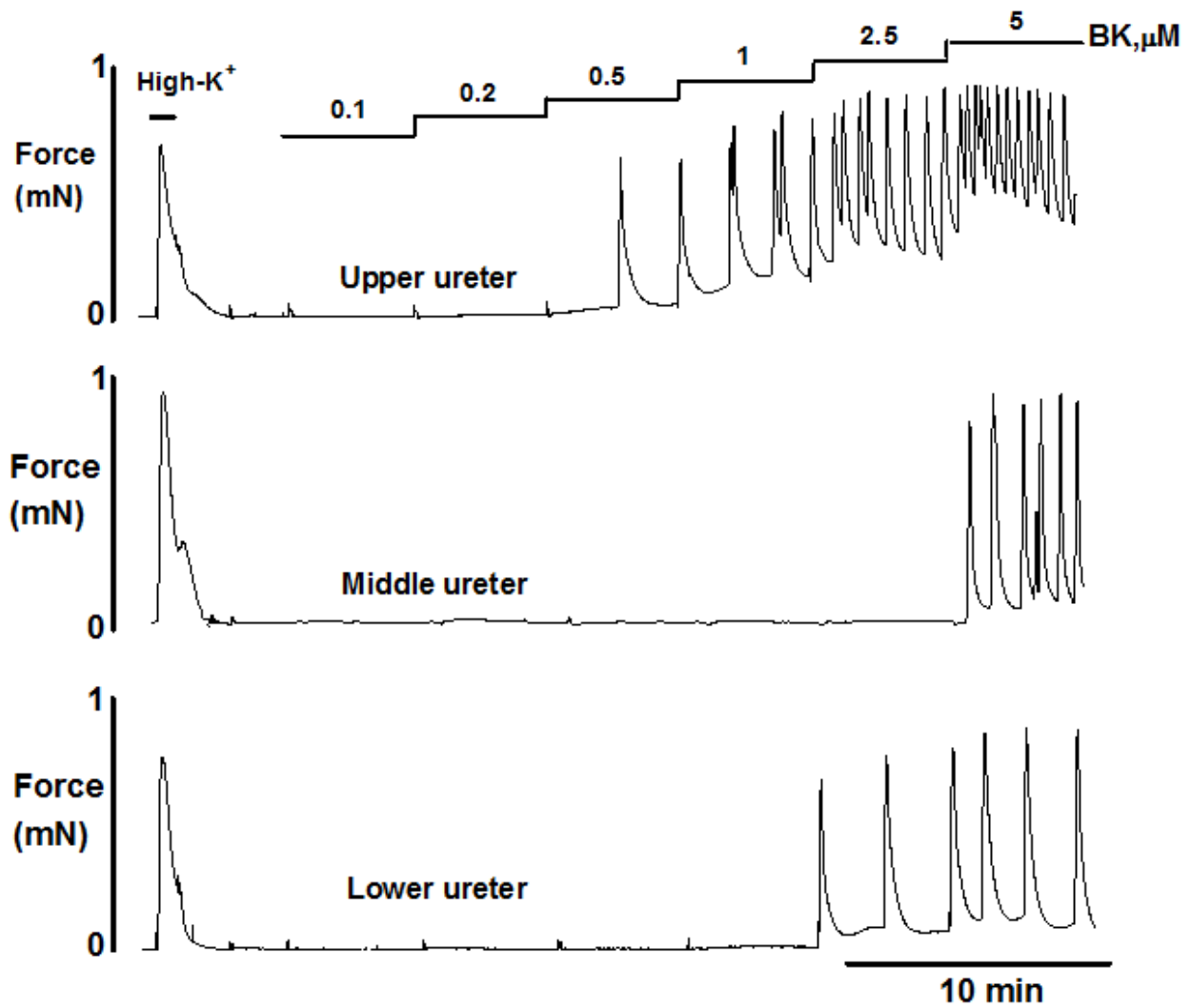
Figure 4.3.1. *Effects of 5 minutes application of different concentrations of BK on the contractile responses of the smooth muscle of the upper (Top trace), middle (Middle trace) and lower (Bottom trace) ureteric segments.*

4.3.2 Effects of BK on the contractile activity of the smooth muscles of the upper ureter

The effects of BK on the SMs of the upper ureter were studied on 13 ureteric upper segments. Figure 4.3.2 shows typical examples of the contractile responses of the ureteric SMs evoked by 0.2, 0.5 and 5µM added cumulatively.

Typically, BK at 0.2µM induced small elevation of the baseline force (7.5±0.5% relative to the peak high-K induced contraction, taken for 100%), which was not superimposed by phasic contractions (Figure 4.3.2). At 0.5µM, BK induced elevation of the baseline force to 16.4±0.3%, superimposed by spontaneous phasic contractions, with an average frequency of 1.06±0.6 cycles/min (n=9).

At 5µM, BK further increased the baseline force to 38.4± 0.6%, superimposed by spontaneous contractions, which appeared either as single phasic contractions (Figure 4.3.2, Top and Middle trace) or long lasting groups of fused phasic contractions (Figure 4.3.2, Bottom trace). The average frequency of the phasic contractions was 3.4±0.4 cycles/min. Within the individual group of the phasic contractions, the frequency of the phasic contractions could increase up to 10-20 cycles/min.
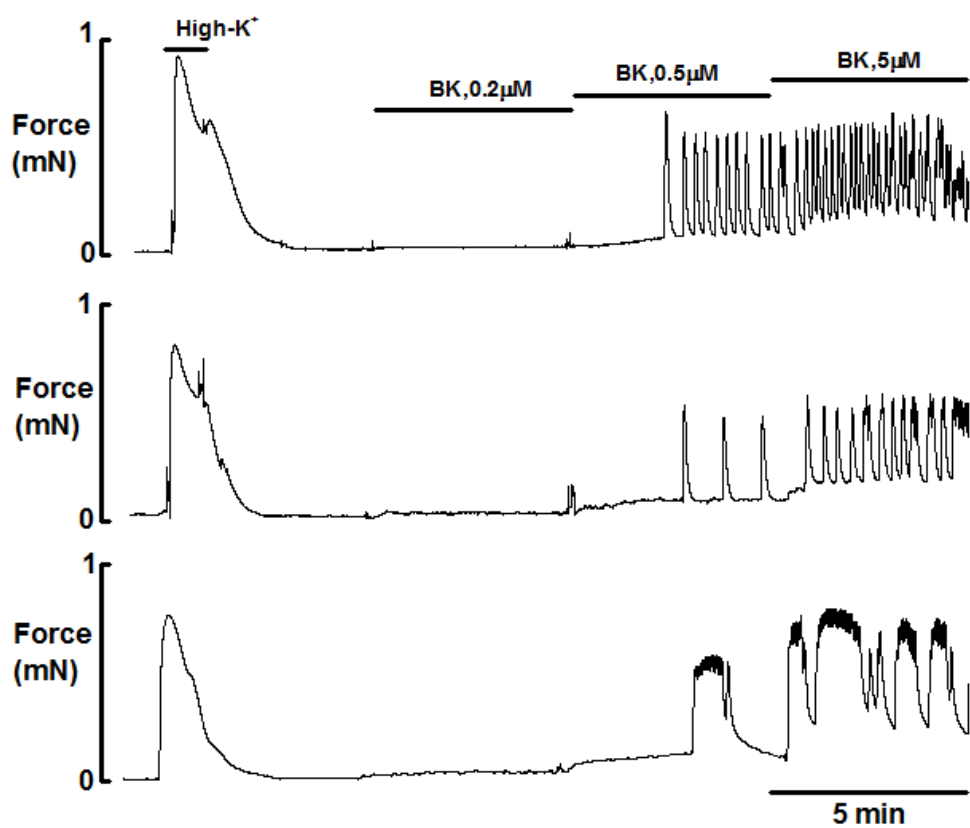
Figure 4.3.2. *Patterns of the contractile responses of the* smooth muscle of the upper ureter *to three different concentrations of BK, added cumulatively and applied for 5 minutes.*

4.3.3 Effects of BK on the contractile activity of the smooth muscles of the middle ureter

The SMs of the middle ureter were significantly less sensitive to BK. In about 30% of the strips tested, BK at 0.2 and 0.5µM had little or no effect on the SMs of the middle ureter, and at 5µM, BK produced an elevation of the baseline force, but failed to initiate spontaneous phasic contractions (Figure 4.3.3, Top trace). In 50-60% of the samples tested, only high concentration of BK (5µM) produced elevation of the base line force to 6.8 ±0.3% (n=7), which was superimposed by the phasic activity, with an average frequency of 0.9 ±0.1 cycles/min (Figure 4.3.3, Middle trace). In less than 30% of the strips tested, spontaneous activity was evoked by 0.5µM BK (Figure 4.3.3, Lower trace).
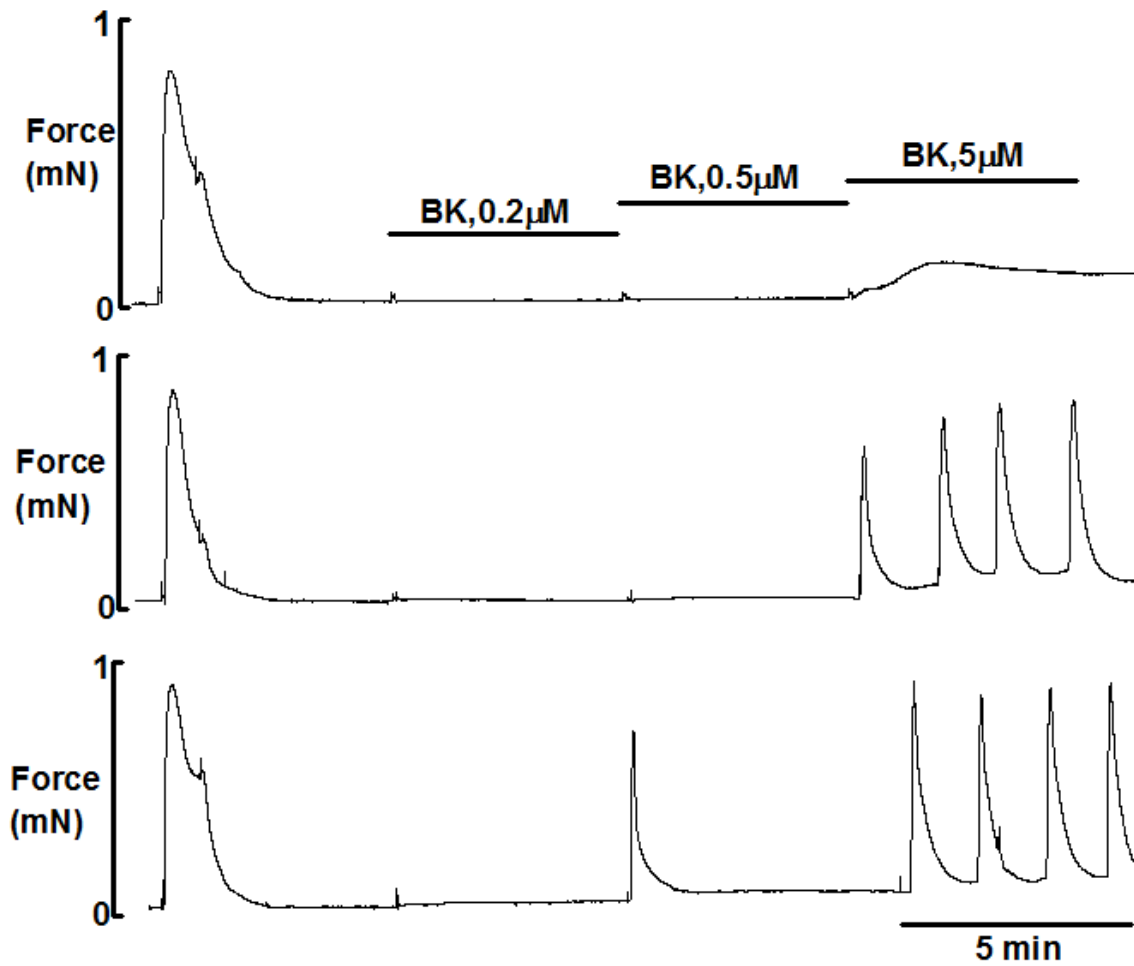
Figure 4.3.3. *Patterns of contractile responses of the smooth muscle of the middle ureter to three different concentrations of BK, added cumulatively and applied for 5 minutes.*

4.3.4 Effects of BK on the contractile activity of the smooth muscles of the lower ureter

The SMs of the lower ureter were also significantly less sensitive to BK than the smooth muscle of the upper, but more sensitive than those of the middle ureter (Figure 4.3.4).

At 0.2μ, BK had no or little effect on the baseline force, but in the presence of 0.5μM of BK, the base line force was increased to 3.7±0.2%, which was superimposed by phasic contractions of low frequency (0.7 cycles/min) (Figure 4.3.4, Middle and Bottom trace). At 5μM, BK increased both the tonic contraction to 11.9±0.3% and the phasic contractions to 1.6±0.4 cycles/min (n=6).

These data demonstrate a higher sensitivity of the smooth muscle of the upper ureter to BK in relation to those of the lower and the middle segments.
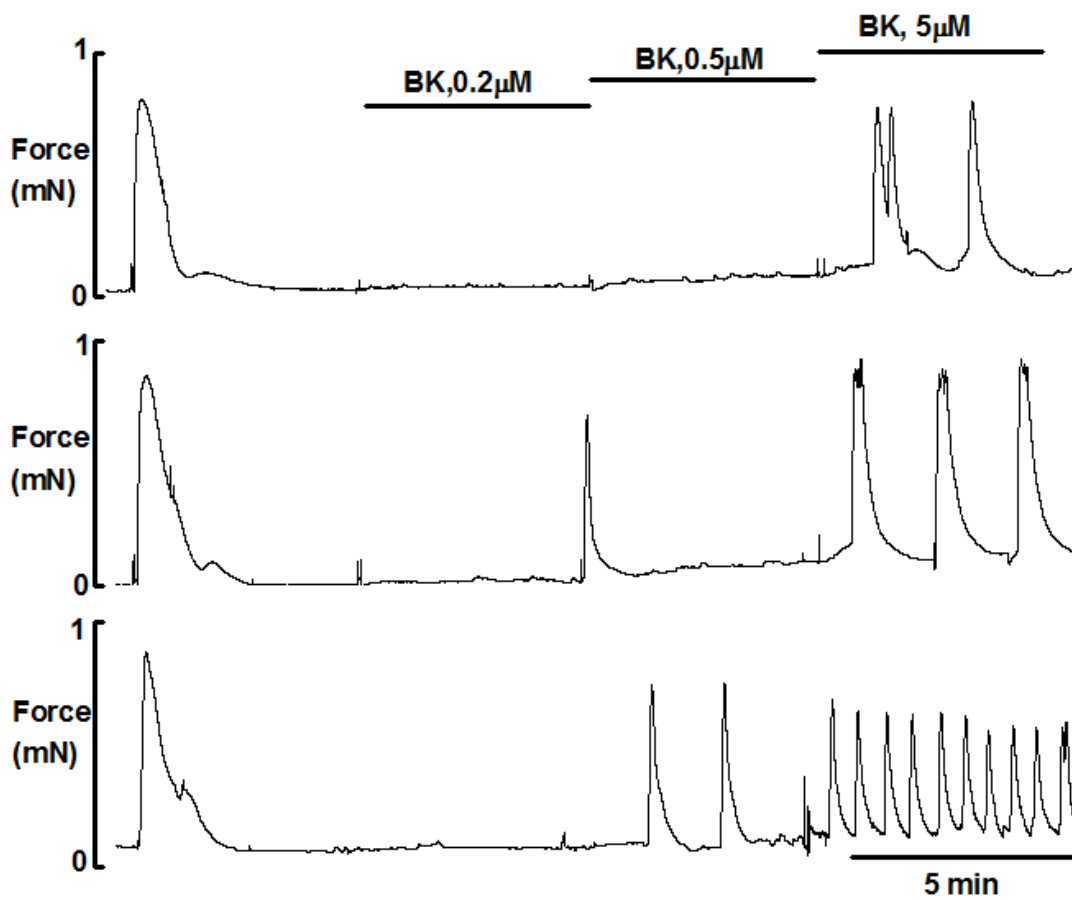
Figure 4.3.4. *Patterns of the contractile responses of the smooth muscle of the lower ureter to three different concentrations of BK, added* **cumulatively and applied for 5 minutes.**

4.3.5 Effects of CCh on the contractile activity of the SM of the upper, middle and lower ureter

The aim of these experiments was to compare the stimulant action of BK with that of the muscarinic receptor activator, Carbachol (CCh), in all parts of the rat ureter. Figure 4.3.5 shows typical contractile responses of the ureteric SMs of the upper, middle and lower ureter to CCh (5µM).

The data obtained indicated that unlike BK, CCh was causing elevation of the baseline force, superimposed by spontaneous phasic contractions in all segments of the ureter (n=3, Figure 4.3.5).
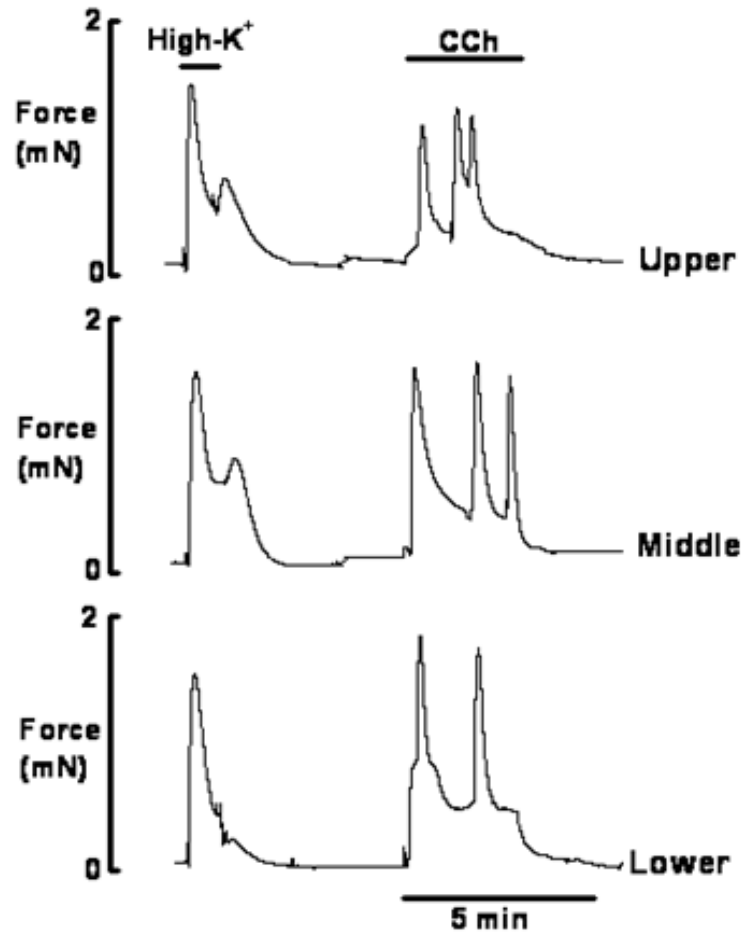
Figure 4.3.5. *Contractile responses of the ureteric smooth muscle of the upper, middle and lower* ureter to 120mM high-K for 40 seconds and 5µM CCh for 5 minutes.

4.3.6 Effects of B$_2$ receptor blocker, HOE-141 on the contractile responses of the ureteric smooth muscles induced by BK

In these and the subsequent experiments, maximal concentration of BK (5µM) was used, which allowed to get the contractile responses in the smooth muscle of all parts of the ureter. At this concentration, BK caused a complex contractile response of the smooth muscle of all parts of the ureter, which consisted of an initial transient phasic contraction that was followed by a sustained tonic component, superimposed after 30-40s delay by burst of phasic contractions (Figure 4.3.6-1) and (Figure 4.3.6-1, Top traces).

Pre-treatment of the rat ureteric smooth muscle with the selective B$_2$ receptor blocker (HOE-140) (10µM) had no effect on the contractile responses induced by high-K depolarisation, but fully and selectively blocked the stimulant action of BK on the ureteric smooth muscles (n=5, Figures 4.3.6-1). Even short application of HOE-141 (30-60s) fully abolished the contractile responses of the ureteric smooth muscle to BK (Figure 4.3.6-2, bottom trace).
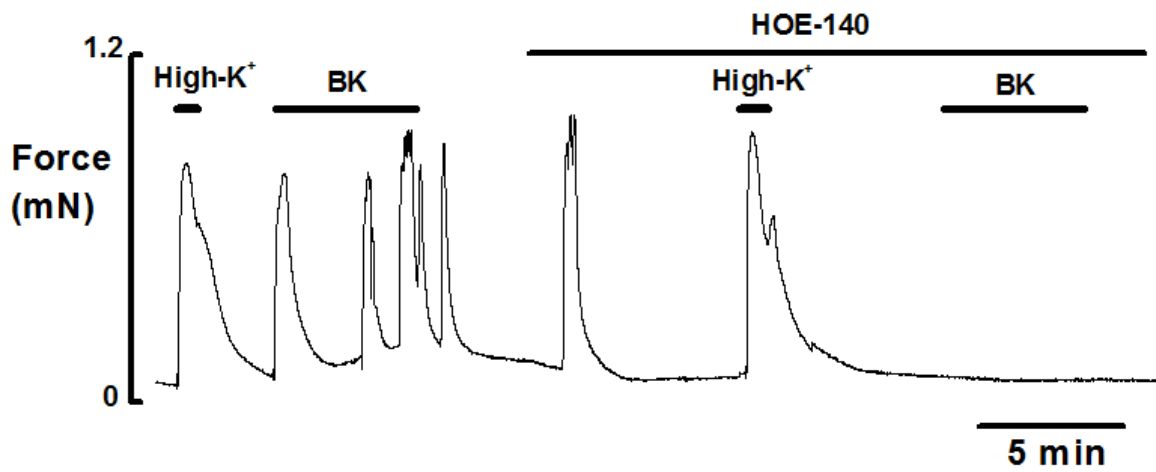
Figure 4.3.6-1. *Contractile responses evoked by 40 seconds application of high-K (120mM) and 5 minutes application of BK (5μM) on the rat smooth muscles of the upper ureter under control conditions and after 10 min pre-treatment with B_2 receptor selective antagonist (HOE-140) (10 μM).*
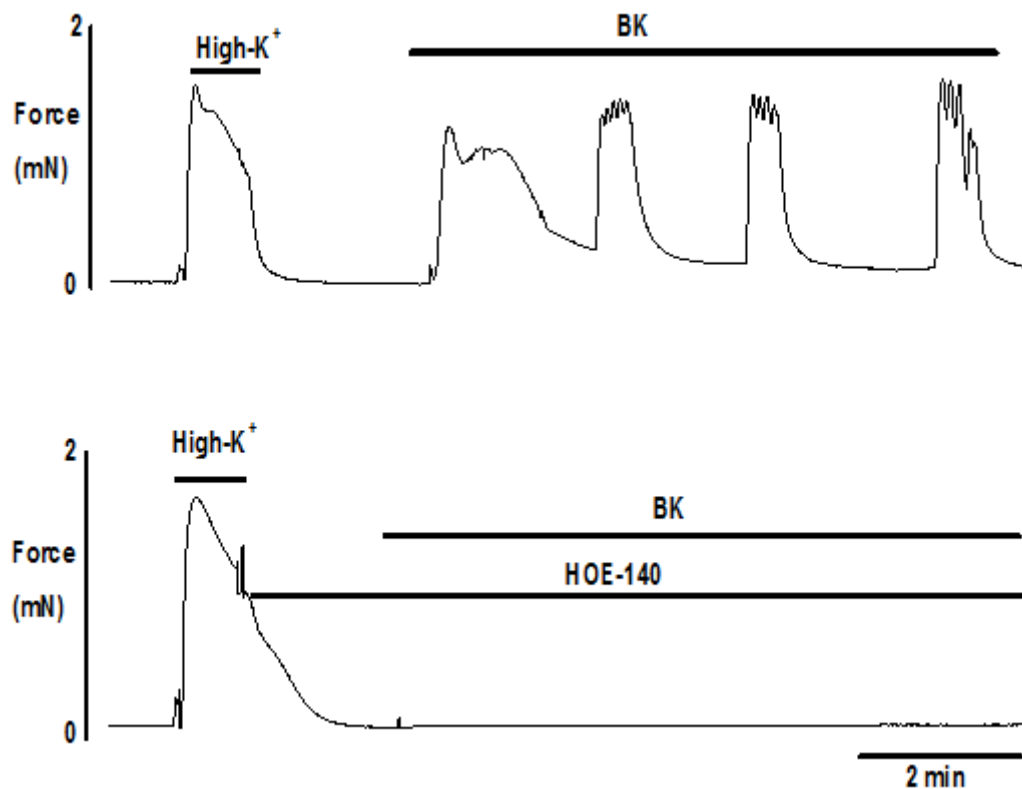
Figure 4.3.6-2. *Complex contractile responses of the smooth muscles of the upper ureter evoked by 5 minutes application of BK (5μM) in the absence (Top trace) and the presence of 10 μM HOE-140 (Bottom trace)* The tissues were preincubated with HOE-140 for 2 minutes and then HOE-140 was continued throughout the experiment.

4.3.7   Effects of Nifedipine

In the next series of experiments, the effects of the selective blocker of L-type voltage Ca^{2+} channels (Nifedipine) on the complex contractile response induced by 5 µM BK was investigated. The aim of these experiments was to define the functional role of the L-type Ca^{2+} channels in the control of BK induced complex contractile response in the ureteric smooth muscle. Figure 4.3.7-1 shows typical contractile responses of the upper ureter to 5µM BK in the absence (Figure 4.3.7-1, Top trace) and the presence (Figure 4.3.7-1, Bottom trace) of 10µM Nifedipine. The data obtained indicated that Nifedipine had little effect on the initial transient phasic and the subsequent tonic components of the initial contractile response, but fully and irreversibly blocked phasic mechanical oscillations superimposing the tonic contraction (Figure 4.3.7-1, Bottom trace). The amplitude of the initial phasic contractions under control conditions, expressed as a percentage of the peak high-K response, was 87.4 ±7.7 % (n=11), while in the presence of Nifedipine, it was reduced to 67.9 ±7.7 %. Besides, the amplitude of the tonic component of the BK –induced contraction in the absence of Nifedipine was 34.6.5 ±0.9 % (n= 11), while in the presence of Nifedipine was only slightly reduced to 32.4±0.4% (n=7). These data indicate that the initial phasic and the followed tonic component are only slightly sensitive to Nifedipine, suggesting that Ca^{2+} entry via L-type Ca^{2+} channels have small contribution to these two components. This was in marked contrast to the phasic mechanical oscillations, superimposing tonic component. Figure 4.3.7-2 shows that Nifedipine caused a quick inhibition of the spontaneous mechanical phasic oscillations induced by BK in the smooth muscle of the upper ureter superimposing tonic contraction.
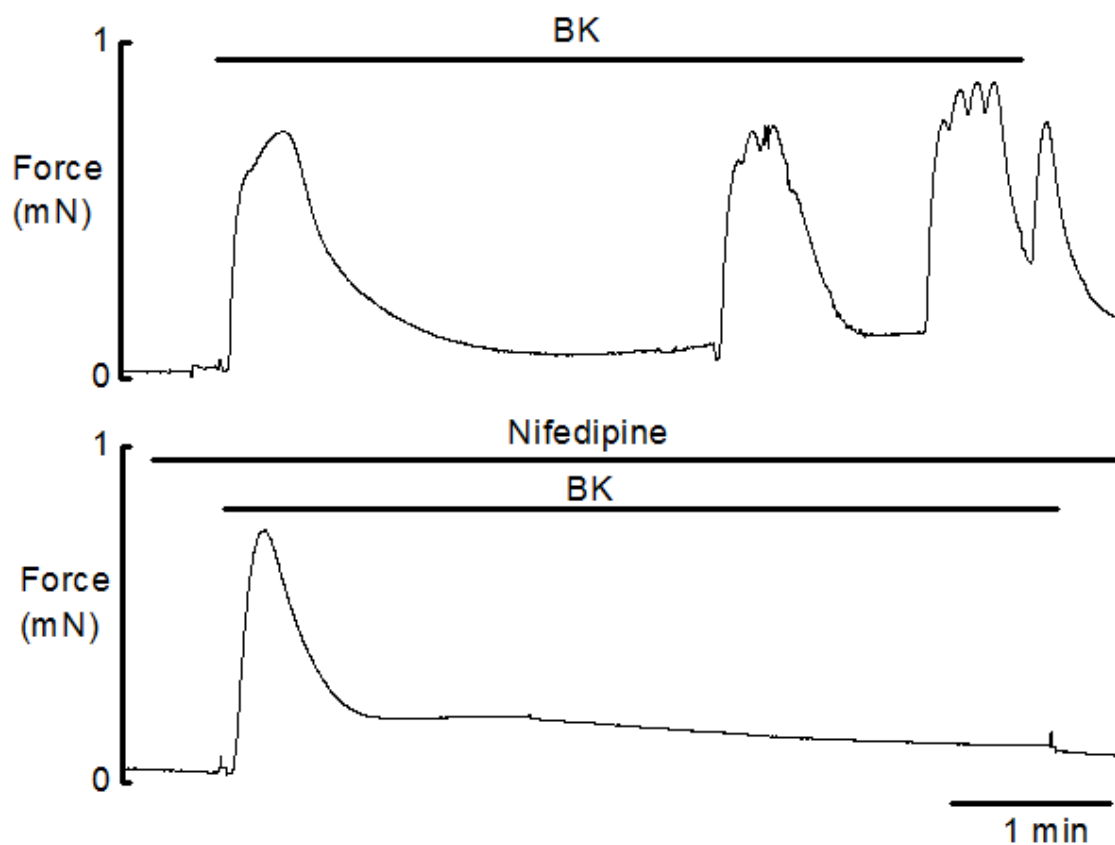
Figure 4.3.7-1. Effects of pre-treatment of the ureteric smooth muscle with Nifedipine (10μM) on the contractile responses induced by 5 minutes application of BK. The tissues were preincubated with Nifedipine for 2 minutes and then Nifedipine was continued throughout the experiment. Top and bottom traces illustrate the effects of BK on the smooth muscles of the upper ureter in the absence and the presence of Nifedipine, accordingly.
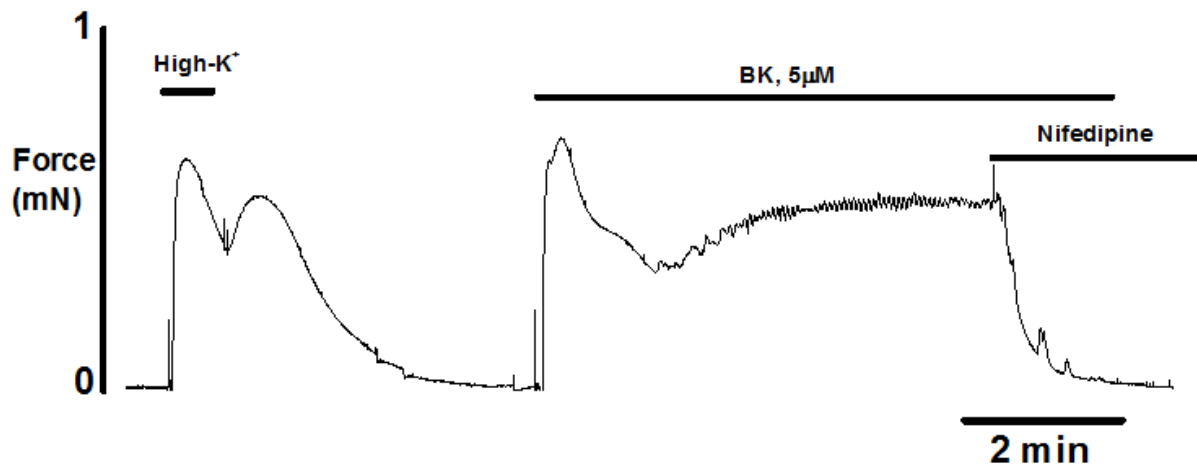
Figure 4.3.7-2. *Inhibition of the phasic mechanical oscillations induced by 5 minutes application of BK (5µM) in the smooth muscle of the upper ureter by Nifedipine (10µM).*

4.3.8 Effects of SKF 96365

In the next series of the experiments, the effects of the non-selective blocker of the receptor- and store-operated Ca^{2+} channels (SKF 963650 (10-30µM) on the contractile responses of the ureteric smooth were studied.

The aim of these experiments was to define the functional role of ROCCs/SOCCs in the control of BK induced complex contractile response in the ureteric smooth muscle.

Figure 4.3.8-1 shows typical contractile responses of the upper ureter to 5µM BK in the absence (Figure 4.3.8-1, Top trace) and the presence (Figure 4.3.8-1, Bottom trace) of 30µM SKF 96365. The data obtained indicated that SKF 96365 had little effect on the initial transient phasic, but significantly, reduced Nifedipine resistant tonic, and fully, blocked the burst of phasic contractions. The amplitude of the initial phasic contractions under control conditions, expressed as a percentage of peak high-K response, was 76.4 ±9.7 % (n=5), while in the presence of SKF 96365, it was reduced to 61.2.±9.9 %.

The amplitude of the tonic component of the BK –induced contraction in the smooth muscle of the lower ureter under control conditions was 34.6±0.9% (n=11), in the presence of Nifedipine 32.3±0.4% (n=7), and in the presence of SKF 96365, it was reduced to 5.19±0.2 % (n=4) (Figure 4.3.8-2).

These data indicate that SKF 96365 selectively blocks both Nifedipine resistant tonic and Nifedipine sensitive phasic oscillations, suggesting that the initial phasic component resistant to Nifedipine and SKF 96365 is likely to be caused by Ca^{2+} release from the SR.

To verify this suggestions in the next series of experiments, the effects of BK in Ca^{2+}-free solution was studied.
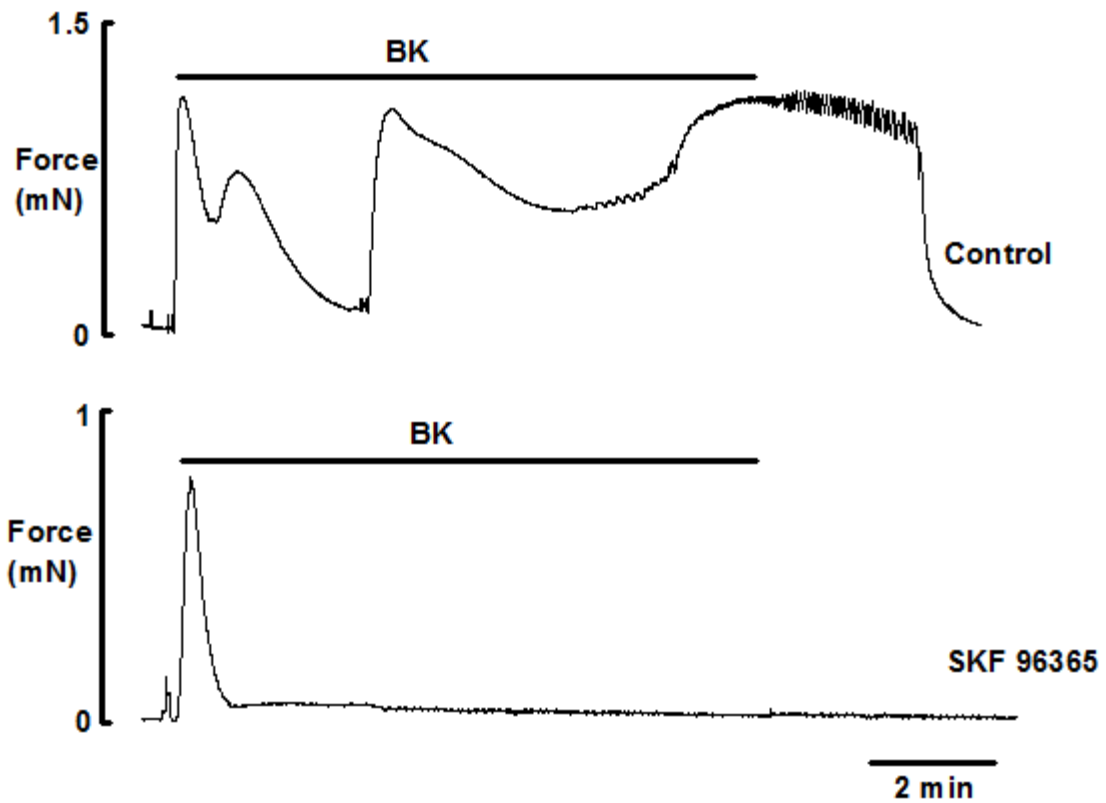
Figure 4.3.8-1. *Effects of treatment of the ureteric smooth muscles with SKF 96365 (30μM) on the contractile responses induced by 5 minutes application of BK (5μM) in the smooth muscles of the upper ureter. The SKF 96365 was introduced 2 minutes prior to BK application and then continued throughout the experiment. Top and Bottom traces illustrate the effects of BK in the absence and the presence of SKF 96365, accordingly.*
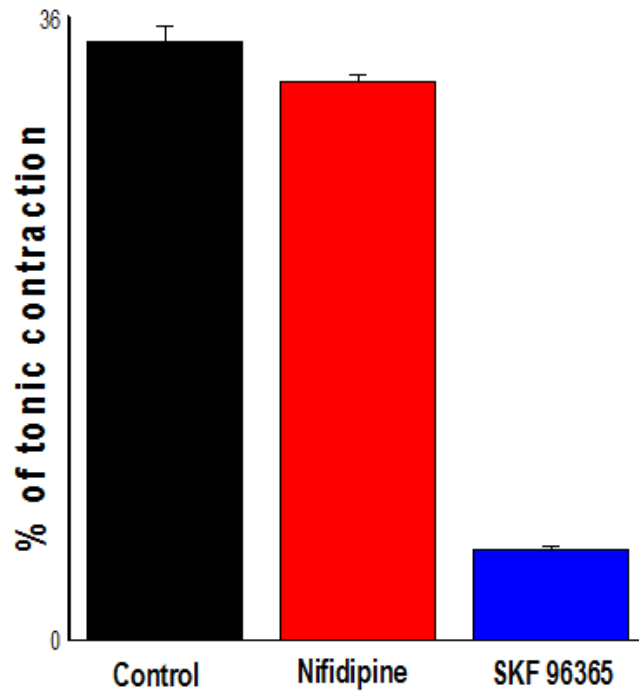
Figure 4.3.8.2. *Bar chart is showing the average values of the tonic component of BK-induced contractile response of the smooth muscles of the lower ureter, expressed as a percentage of the peak high-K contraction, taken for 100% under control conditions (Black bar, n=11), in the presence of Nifedipine (10µM) (Red bar, n=7, P>0.05), and SKF 96365 (30µM) (Blue bar, n=4, P<0.005).*

4.3.9 Effects of BK in Ca^{2+}-free solution

When ureteric smooth muscle of all parts of ureter were placed in Ca^{2+}- free solution with 2mM EGTA, BK caused only transient phasic contraction (Figure 4.3.9-1).

It should be noted that readmission of external Ca^{2+} to Ca^{2+}-free solution, following application of BK in Ca^{2+} -free solution caused a significant rise of the tonic contractions in all parts of the ureter (Figure 4.3.9-1), which in the smooth muscle of the upper and the lower ureter were superimposed by spontaneous phasic mechanical oscillations (Figure 4.3.9-1, Top and Bottom trace, respectively).

These data suggest that BK was likely to cause full depletion of the SR, which led to the activation of Ca^{2+} entry via store operated Ca^{2+} channels.

To verify this suggestions, the effects of the consecutive application of CCh and BK in Ca^{2+}-free solution in different orders have been studied. Also the effect of the non-selective blocker of SOCCs (SKF 96365) on the contractile responses induced by the re-admission of Ca^{2+} to Ca^{2+}-free solution, following store depletion by BK in Ca^{2+} -free solution was studied. The experiments were performed on the upper ureter. Figure 4.3.9-2 shows typical mechanical responses induced by a consecutive application of CCh (5µM) and BK (5µM) in different order. This figure shows that irrespective of the order of application, contractile response to either agonist was only observed when they were applied first. Subsequent application of both agonists, following the first contractile response induced by either of them, were fully abolished (Figure 4.3.9-2).
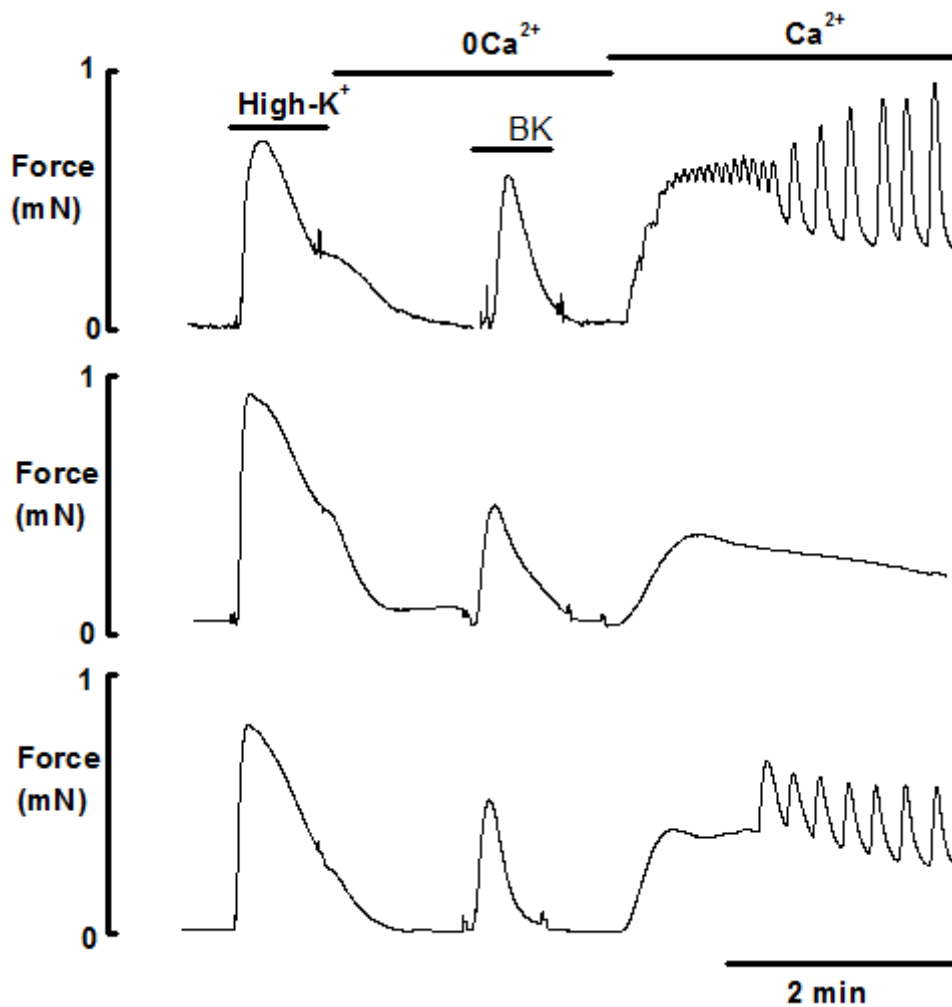
Figure 4.3.9-1. *Contractile responses of the smooth muscles of the upper (Top trace), middle (Middle trace) and lower (Bottom trace) rat ureter induced by 40 seconds application of BK (5μM) in Ca^{2+}-free solution with 2mM EGTA.*
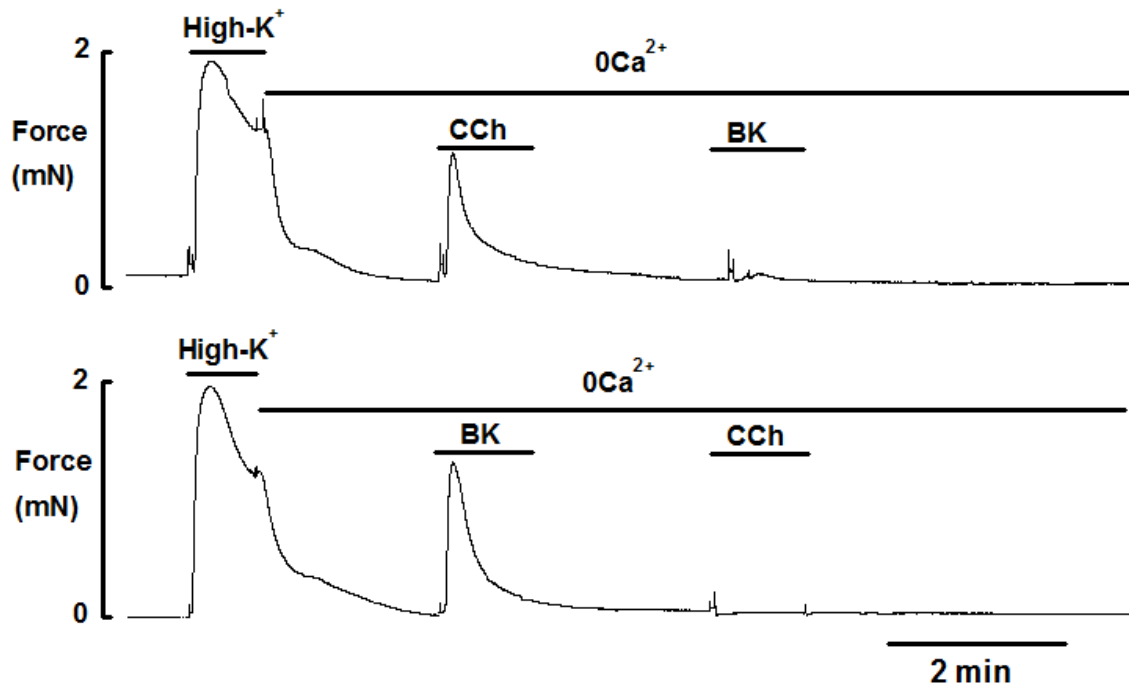
Figure 4.3.9-2. *Effects of the consecutive application of 40 seconds CCh (5μM) and BK (5μM) in different order of applications on the contractile responses of the rat ureteric smooth muscles of the upper ureter in Ca²⁺-free solution.*

4.3.10 Effects of SKF 96365 on the rebound contractile responses

To investigate the possible role of the SOCCs in the control of the rebound contractile responses induced by the readmission of external Ca^{2+} to Ca^{2+}- free solution, following Ca^{2+} store depletion by BK, the effects of the non-selective blocker (SKF 96365) on the rebound contractile responses in the upper ureter have been investigated (Figure 4.3.10A and B). Figure 4.3.10A shows that the tonic contraction induced by the readmission of external Ca^{2+} was significantly reduced and the phasic mechanical oscillations were fully abolished in the presence of 30μM SKF 96365. The amplitude of the tonic contraction under control conditions was 51.1±3.1% (n=9) of the peak high-K contraction and in the presence of 10 and 30 μM SKF 96365, it was reduced to 23.8±1.5% (n=5) and 3.8±1.1% (n=7), respectively.
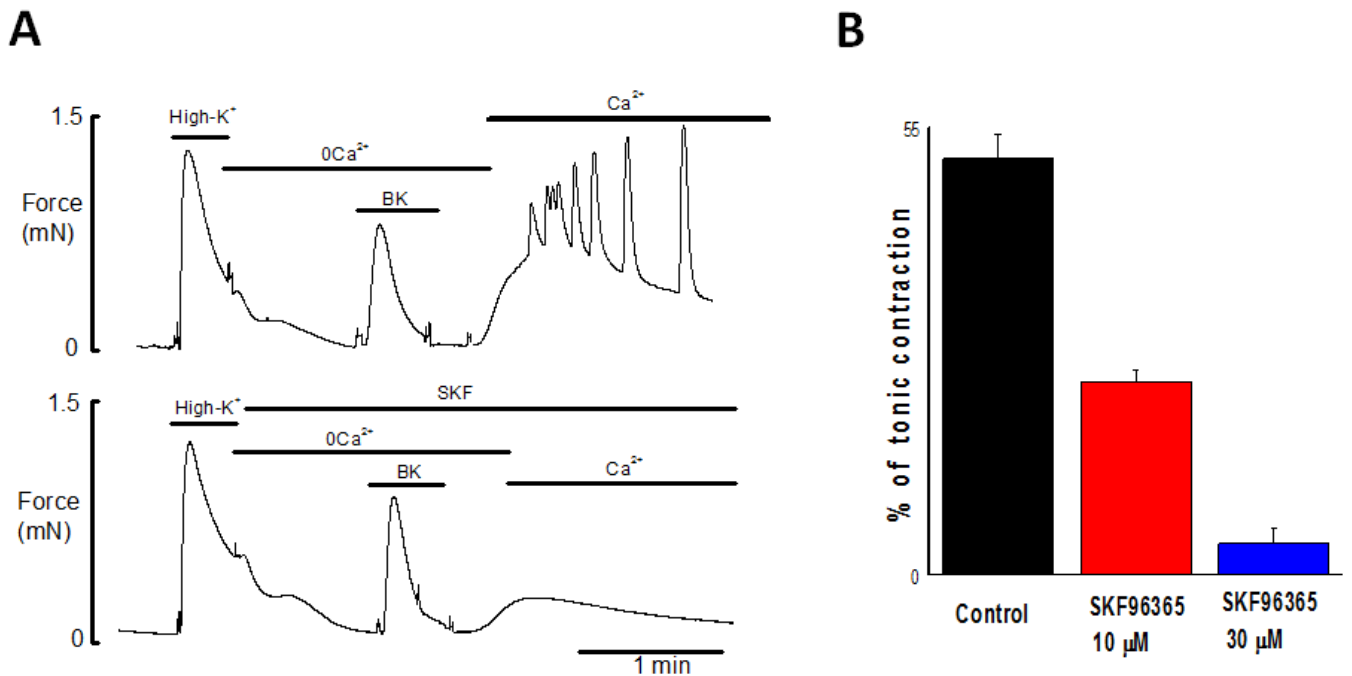
Figure 4.3.10. *Effects of pre-treatment of the smooth muscles of the upper ureter with SKF 96365 applied for 5 minutes on the contractile responses, induced by the re-admission of external Ca^{2+} to Ca^{2+}- free solution, following Ca^{2+} store depletion by 40 seconds application of BK (5μM). A: Top and bottom traces illustrate the effects of BK on the contractile responses of the smooth muscles of the upper ureter in the absence and the presence of SKF 96365, respectively. B: Bar chart showing the average values of the tonic component of the rebound contraction induced by the re-admission of external Ca^{2+} to Ca^{2+}- free solution, following Ca^{2+} store depletion by BK, expressed as a percentage of the peak high-K contraction, taken for 100% under control conditions (Black bar, n=9), and in the presence of 10 μM (Red bar, n=7, p<0.05) and 30 μM SKF 96365 (Blue bar, n=5, p<0.005), respectively.*

4.4   Discussion.

The results of the previous and the present chapters provide morphological and functional evidence, which indicates that the anatomic distribution of B_2 receptors in the rat ureter corresponded to regions of high sensitivity of the ureteric smooth muscle to BK, more specifically the proximal upper ureter.

Ureteral segments taken from the proximal ureter showed larger rises of the base line force and frequency of phasic contractions than the middle and the lower areas of the ureter and corresponded to increased levels of B_2 receptor expression. The frequency of the spontaneous phasic contractions, superimposing tonic component of the contractile response induced by BK, were significantly higher in the proximal segments of the rat ureter and could appear as a continuous burst of phasic contractions. Alterations in the contractility patterns also occurred. The number of contractions within the "signature" for each segment was greater in the most proximal segments. Thus, higher level of expression of B_2 receptors in the proximal ureter corresponded to areas of increased sensitivity of the rat ureteric smooth muscle to BK and higher frequencies of phasic oscillations. This was in contrast to the contractile responses of the rat ureter to high-K and CCh, which were even along the length of the ureter.

Our data demonstrate that BK effects are due to activation of B_2 receptors, since they were blocked by the selective bradykinin B_2 receptor antagonist HOE-140 (Hock et al., 1991). B_2 receptor is a member of G-protein coupled receptor that activates PLC (Hess et al., 1994). In several tissues, the action of BK on B_2 receptors involves the activation of PLC, leading to an increase in IP_3 concentration and subsequently of the intracellular Ca^{2+} (Den Hertog et al., 1988, Hess et al., 1994, Hall, 1992, Field et al., 1994). The IP_3 receptors cause Ca^{2+} release from the store (Patel et al., 1999). Ca^{2+} depletion can induce the entry of Ca^{2+} via the SOCCs (Casteels and Droogmans, 1981, Marthan, 2004). SOCE can refill the stores, activates SMs contraction directly or by activation of the VOCCs (Albert and Large, 2003).

In the current investigation, the potent inhibition of the BK-induced phasic contractions by the pre-treatment with Nifedipine (a L-type voltage gated Ca^{2+} channel blocker) suggests that BK induced burst of spontaneous phasic contractions occur through Ca^{2+} influx via voltage dependent L-type Ca^{2+} channels. In addition, a non-L-type Ca^{2+} entry mechanism also contributes to the BK-elicited tonic contraction. This mechanism could include voltage-independent store- and/or receptor operated channels.

In the rat ureter, the inhibition of Nifedipine resistant tonic contraction, produced by the non-selective blocker of ROCCs/SOCCs (SKF 96365), indicates that other Ca^{2+} channels different from the L-type VOCCs also contribute to BK-induced Ca^{2+} entry in the ureteric smooth muscles.

The transient contraction exhibited by BK in a Ca^{2+}-free medium suggests that an intracellular Ca^{2+} mobilization is also playing an important part in triggering initial transient phasic contraction, resistant to Nifedipine, SKF 96365, and the removal of the external Ca^{2+}. Furthermore, CCh has been reported to cause a release of Ca^{2+} from the SR through IP$_3$ production (Kobayashi et al., 1989), causing depletion of Ca^{2+} store (Shmygol and Wray, 2005, Coleman and Parkington, 1988, Shmigol et al., 2001, Noble et al., 2014). Abolition of BK –induced contraction after CCh application in Ca^{2+} -free conditions indicates that both agonists release Ca^{2+} from the same intracellular store.

Thus, the physiological and the pharmacological data, obtained in this Chapter, suggest that in the ureteric smooth muscle, B$_2$ receptors activation is coupled to Ca^{2+} release Nifedipine resistant and SKF 96365 sensitive Ca^{2+} entries via SOCCs/ROCCs and Nifedipine sensitive voltage gated L-type Ca^{2+} channels.

This conclusion is supported by the following data: (i) The anatomic distribution of B$_2$ receptors in the rat ureter corresponded to the regions of high sensitivity of ureteric smooth muscle to BK, more specifically the proximal upper ureter; (ii) The involvement of B$_2$

receptor is also supported by the selective inhibition of BK-induced contractile responses by the selective blocker of B_2 receptors (HOE-140); (iii) In the presence of Nifedipine, there was a selective inhibition of the burst of phasic contractions, which are known to depend on Ca^{2+} entry via L-type voltage gated Ca^{2+} channels, responsible for the generation of the plateau type action potential in the rat ureteric smooth muscle (Burdyga and Wray, 1999b); (iv) There was profound reduction of the Nifedipine resistant tonic component of BK induced tonic contraction as a consequence of SOCCs/ROCCs channel blockade by SKF 96365; (v) There was a transient contractile response of the ureteric smooth muscle to BK in Ca^{2+} – free solution. Thus, bradykinin-mediated responses involves both mobilization of the extracellular Ca^{2+} influx, which enters the cell through the voltage dependent and independent Ca^{2+} channels, and mobilization of intracellular Ca^{2+} stores as was suggested for other types of smooth muscles (Wray and Burdyga, 2010).

The mechanism, by which bradykinin mobilizes intracellular Ca^{2+} in most tissues, involves the activation of phosphatidylinositol-specific phospholipase C, resulting in an increases in the cellular levels of inositol 1, 4, 5-trisphosphate and diacylglycerol. While inositol 1, 4, 5-trisphosphate releases intracellular Ca^{2+} from SR, diacylglycerol directly activates protein kinase C (Farmer and Burch, 1992).

Based on the data obtained, it is suggested that the spontaneous phasic contractions, induced by BK, are activated by a membrane depolarisation, induced by the activation of Ca^{2+} entry, via SOCCs, caused by the activation of Ca^{2+} release/ Ca^{2+} entry coupling mechanism and/or ROCCs. SOCCs is one of the important Ca^{2+} entry mechanism, stimulated by Ca^{2+} store depletion, to increase Ca^{2+} influx (Szteyn et al., 2015, Choi et al., 2001). They mediate different physiological actions in different tissues (Zhang et al., 1999, Dobrydneva et al., 1999, Liu and Gylfe, 1997, Geiszt et al., 1997).

Collectively these data indicate that BK produces contraction of the rat ureter smooth muscle via B_2 receptors, coupled to Ca^{2+} release from the SR, mediated by IP_3R channels, responsible for the generation of the initial phasic contraction, and Ca^{2+} entry via the SOCCs/ROCCs, responsible for the generation of the tonic contraction, and the voltage gated L-type Ca^{2+} channels responsible for the generation of the phasic contractions, controlled by the action potentials (Shuba 1977; Brading et al., 1983; Burdyga and Wray, 1997).

Although the mechanical data in combination with the pharmacology provide valuable indirect data on the mechanisms of Ca^{2+} mobilisation by BK in the smooth muscle, the direct measurement of Ca^{2+} has not been done in a single study. Thus the next aim of this study was to investigate the temporal and spatial characteristics of Ca^{2+} signalling, associated with each of the mechanical component of the complex contractile response of BK on the ureteric smooth muscle, using live confocal imaging combined with force measurements.

Chapter 5

Effects of BK on Ca^{2+} signalling and force in the rat
ureteric smooth muscle cells

Chapter 5

Effects of BK on Ca^{2+} signalling and force in the rat ureteric smooth muscle cells

5.1   Introduction

Ca^{2+} is the primary activator of the smooth muscle contraction (Johansson and Somlyo, 1980). An increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) can be achieved by the activation of Ca^{2+} entry, via voltage-operated and/or receptor-operated or store-operated channels, Ca^{2+} release from the intracellular stores, or a combination of these mechanisms.

In smooth muscle, including the ureter, a global rise in $[Ca^{2+}]_i$ activates Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK), which phosphorylates the 20-kDa regulatory light chains of myosin (LC_{20}), thereby triggering cross-bridge cycling and contraction (Borysova et al., 2011).

The type of Ca^{2+} signaling varies, depending on the type of the smooth muscle and the type of the stimuli. In the phasic smooth muscles, such as the ureter, Ca^{2+} signaling is mainly controlled by a single plateau type action potentials (Burdyga and Wray, 1999b).

The action potential in the main ureter is normally triggered by the invading pacemaker potentials, initiated by the pacemaker cells, located in the renal pelvis (Lang et al., 2010, Hashitani et al., 2017). In the ureteric smooth muscle, Ca^{2+} transient is produced mainly by the entry of Ca^{2+} into the cell from the extracellular space, via L-type VOCCs. These channels are responsible for the generation of both components of AP (the spike and the plateau component) in the ureteric smooth muscle. The AP, inward Ca^{2+} current, Ca^{2+} transient and the twitch contraction are all fully blocked by the selective L-type Ca^{2+} channel blocker (Nifedipine) (Lang, 1989, Floyd et al., 2008).

Ca^{2+} entry through VOCCs, coupled to the activation of BKCa channels via Ca^{2+} sparks (e.g. guinea pig ureter) (Burdyga and Wray, 2005) or Cl_{Ca} channels (e.g. rat ureter) (Shabir et al.,

2004) provides a negative/ positive feed-back/-forward mechanism, respectively to the control of the excitability and the duration of the plateau component of the AP and Ca^{2+} transient, and therefore the amplitude and the duration of the twitch contraction.

The ureter, isolated from the pelvis, is not spontaneously active. However, when it is stimulated by the neurotransmitters (e.g. Noradrenaline, Acetylcholine) (Shuba, 1977) or the inflammatory agents (e.g. Bradykinin) (Catacutan-Labay and Boyarsky, 1966b) and (the present study) it becomes spontaneously active.

In the presence of agonists, the smooth muscles cells depolarise to the threshold for the AP firing and some of them begin to act as pacemaker cells, initiating intercellular AP-mediated Ca^{2+} waves that may propagate in both directions from the point of initiation to produce both antegrade and retrograde peristalsis of the ureter.

Agonist induced depolarisation can be caused by increasing Na^+ or Cl^- conductance or decreasing K^+ conductance (Shuba, 1977). In the rat ureteric smooth muscle, Cl^- conductance can be activated by Ca^{2+} entering the cell, via voltage gated Ca^{2+} channels or released from the SR (Smith et al., 2002).

Bradykinin-2 (B_2) receptor is a GPCR. Association of bradykinin leads to the activation of phospholipase C, which results in the production of DAG and IP_3, through the hydrolysis of PIP_2. IP_3 activates IP_3R Ca^{2+} release channels, present in the intracellular Ca^{2+} stores, such as the SR, causing an increase in Ca^{2+} release from the store.

The mechanical data, obtained on different types of visceral smooth muscle, suggest the involvement of different mechanisms of Ca^{2+} mobilisation by BK (Wray and Burdyga, 2010). However, the contractility data, presented in Chapter 4, suggest that BK-mediated contractile responses in the rat ureteric smooth muscle involve both Ca^{2+} release from the SR and Ca^{2+} entry from the extracellular space, via voltage-independent (SOCCs/ROCCs) and voltage dependent (L-type Ca^{2+} channels).

The aim of the next series of experiments was to study the effects of BK on Ca^{2+} signalling, in the rat ureteric smooth muscle, using live confocal imaging of fluo-4, loaded ureteric smooth muscle *in situ,* with or without simultaneous mechanical recordings. The major aim of this study was to investigate the temporal and spatial properties of Ca^{2+} signal, associated with Ca^{2+} release, responsible for the activation of the initial phasic contraction and the Ca^{2+} signalling, associated with Ca^{2+} entry via VOCCs, responsible for the generation of the intercellular Ca^{2+} signalling, associated with the phasic contractions. Also, the possible role of the capacitive Ca^{2+} entry in the control of the spontaneous activity, elicited by BK in the rat ureteric smooth muscle, was studied.

5.2   Methods

5.2.1   Tissue preparation

Rats (~200 g) were humanely killed using CO_2 anaesthesia, followed by cervical dislocation, in accordance with UK legislation. The ureters were dissected, carefully cleaned of the connective and paraureteric adipose tissue, using fine curved scissors, while keeping the sharp edges away from the tissue to avoid physical damage of the ureteric bundles.

5.2.2   Fluo -4 loading

To load the tissue with fluo 4, strips of ureter were placed in a plastic cuvette, contained 1ml of HEPES-buffered Krebs solution, containing 15 μM-fluo 4-AM, dissolved in DMSO, premixed with Pluronic F127 (final concentration of 0.01 %).

Loading was performed at room temperature for 3 h, with the cuvettes wrapped in a black tape and rotated at 30 rpm. Tissue samples were then removed from the loading medium and placed in a normal Krebs solution for at least 30 min to allow cleavage of fluo 4-AM to fluo 4 by intracellular esterases.

5.2.3   Confocal imaging of intact rat ureteric strips

We used fast Nipkow disc-based confocal imaging, attached to a high sensitivity (iXonAndor) CCD camera, which allow acquisition of the images at 60 - 200 fps, and thereby accurate measurement of the temporal and spatial characteristics of Ca^{2+} signaling in individual smooth muscle cells and bundles of the intact ureteric strips.

To measure temporal and spatial characteristics of Ca^{2+} signaling in individual smooth muscle cells, a x60 water objective (NA 1.2) was used, while low-power dry objectives (x4, NA 0.08 and x20, NA 0.72) were used to measure the temporal and spatial characteristics of Ca^{2+} signaling in the whole ureteric muscle bundles.

Data acquisition was performed using Andor iQ software.

5.2.4   Combined Confocal imaging with force measurement

To simultaneously measure Ca^{2+} transients and force, ureters were cut into small segments (5 - 6 mm in length), clipped at both ends, using aluminum foil clips (Laser Services, USA), and attached to a force transducer (WPI) at one end and a stainless steel hook, fixed to the bottom of the experimental chamber at the other end. The force transducer was attached to a 3-d manipulator (Narashige, Japan), allowing movement of the strip in the X-Y-Z directions to position it in the focal plane of the objective and apply an optimal stretch, which did not exceed 40% of active maximal force induced by high $[K^+]$ depolarization.

5.2.5   Analysis and statistics

Data analysis was performed using Andor iQ software and Origin 8.6 (Origin Labs). The data were analysed with students (paired or unimpaired) t-test; differences between means were assumed to be significant at $P<0.05$. Data are given as mean $\pm$ s.e.m.; n is the number of samples.

5.3   Results

5.3.1   Effects BK on force- Ca^{2+} relationship in the ureteric smooth muscle.

Confocal imaging of fluo-4 loaded ureteric segments, combined with force measurements, showed that BK caused a complex Ca^{2+} signalling, which consisted of an initial phasic component, followed by a sustained plateau component, superimposed by intercellular Ca^{2+} wave oscillations (Figure 5.3.1B, Bottom trace).

This multicomponent Ca^{2+} transient, induced by BK, was correlated well with the complex contractile response, which consisted of an initial transient phasic, followed by sustained tonic contraction, superimposed by multiple phasic contractions (Figure 5.3.1B, Top trace).
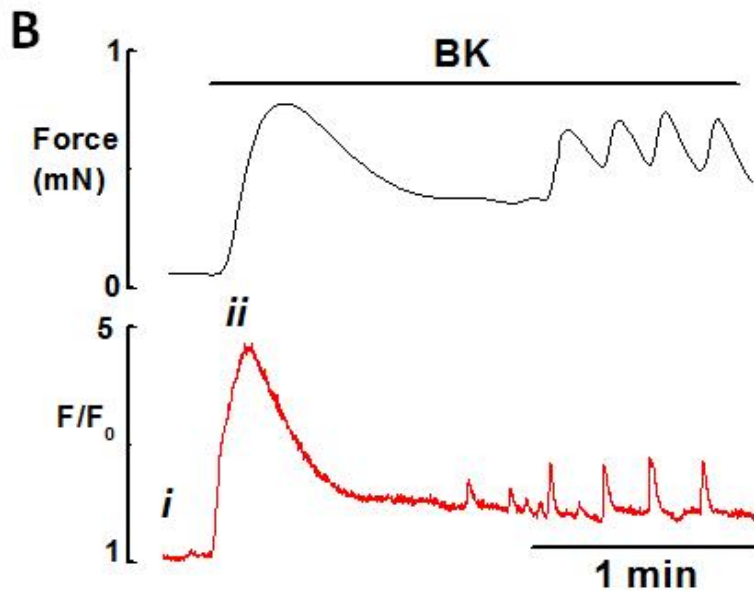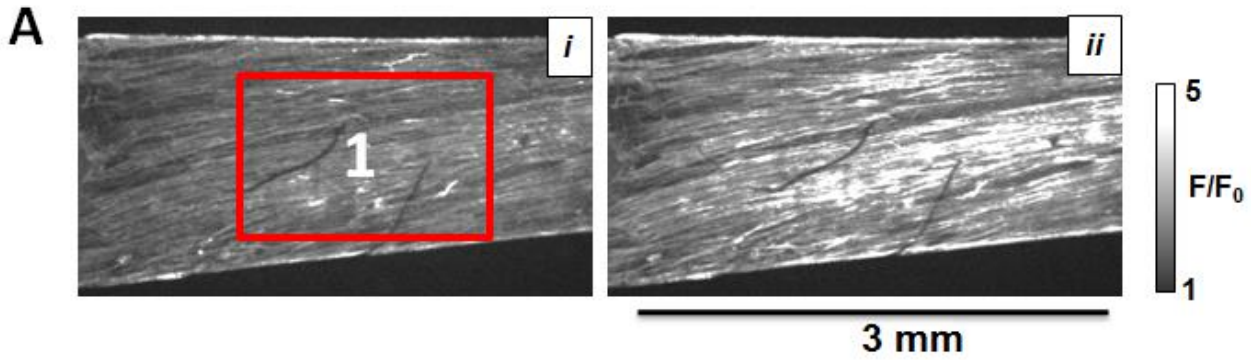
Figure 5.3.1. *The effect of 5 minutes application of BK (5μM) on Ca^{2+} transient and force in the smooth muscle of the lower ureter. A: Grey scale images showing fluo-4 loaded ureteric smooth muscle at rest (i) and during BK stimulation (ii). B: Graph showing force (Top trace) and Ca^{2+} transient (Bottom trace) measured in region 1 from A.*

5.3.2 Temporal and spatial properties of Ca^{2+} signal responsible for the initial phasic contraction.

The contractility data, presented in Chapter 4, suggested that the initial component of the BK induced contractile response was associated with a transient release of Ca^{2+} from the store, as it was immune to Nifedipine , SKF 96365 and the removal of external Ca^{2+} .

In the next series of experiments, the effects of BK on Ca^{2+} signalling of the ureteric smooth muscle in the presence and the absence of external Ca^{2+} was studied in more details.

Live confocal imaging of fluo-4 loaded ureteric smooth muscles *in situ* revealed that the initial phasic component of the Ca^{2+} transient was observed as transient elevation of the intracellular Ca^{2+} in the ureteric smooth muscle cell, which appeared as intracellular Ca^{2+} waves. The Ca^{2+} waves were asynchronous between smooth muscle cells and were initiated in different cells at different times (Figure 5.3.2-1). The amplitude of the Ca^{2+} transient in individual cell was normally higher and had steeper rising phase than the average signal measured from the whole region (Figure 5.3.2-1B).
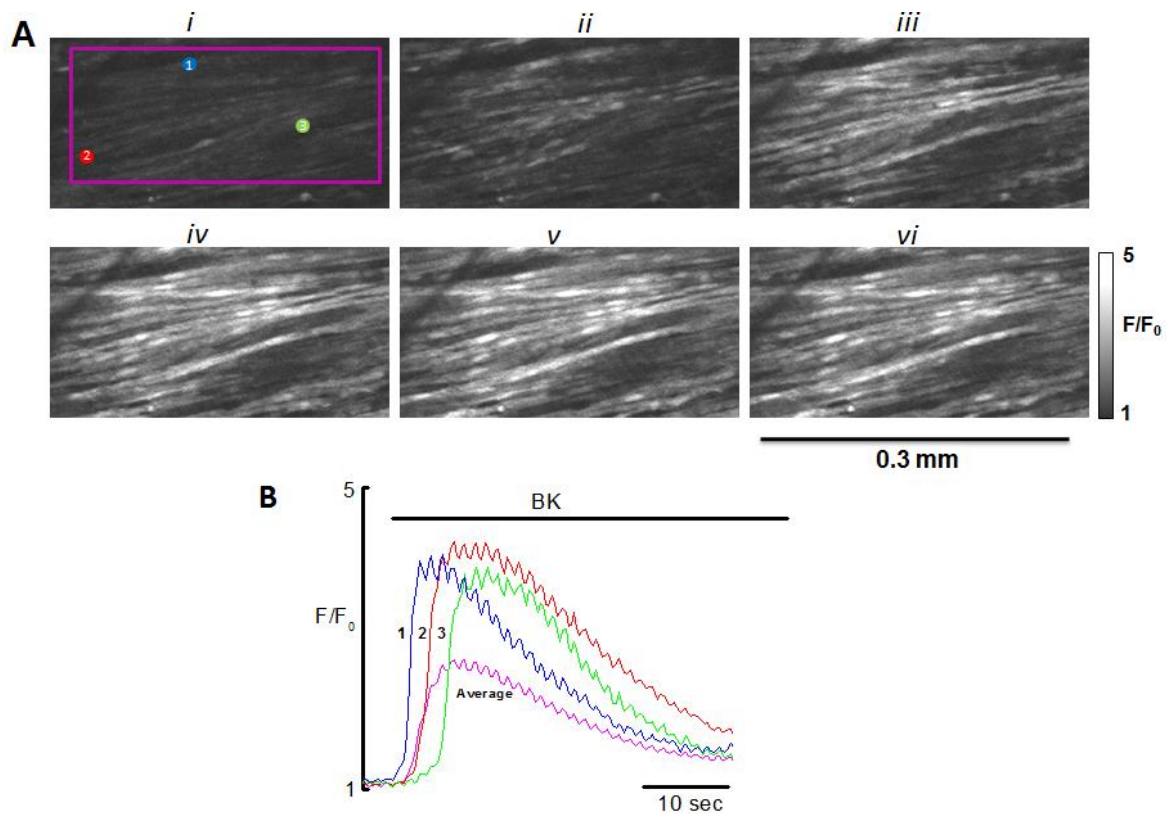
Figure 5.3.2-1. *Temporal and spatial properties of the initial component of BK induced complex Ca^{2+}transient in the rat ureteric smooth muscle in situ. A: Stack of images showing smooth muscle cells loaded with fluo-4 at rest (i) and during stimulation with BK (5µM), recorded with 120 msec time interval, (ii-vi) showing time dependent effect of BK on Ca^{2+}signalling in the ureteric smooth muscle. B: Graph showing Ca^{2+}transient measured in three region of interests shown in Ai, placed over three random cells (regions 1-3) and an average signal measured from the whole area (Region 4, Ai).*

In Ca^{2+}-free solution with 2mM EGTA, BK (5µM) induced a transient rise of intracellular $[Ca^{2+}]_i$, which was associated with a brief phasic contraction (Figure 5.3.2-2B, Right panel). This was in marked contrast to the effects of the high-K depolarisation, which caused a homogenous rise of the intracellular Ca^{2+}, associated with force, which reached a steady state and were limited by the time of the exposure of the ureteric smooth muscle to the high-K solution (Figure 5.3.2-2B Left panel).

Figure 5.3.2-3 shows an example of the ureteric smooth muscle cell, in which Ca^{2+} wave, induced by BK in Ca^{2+}- free solution, was initiated at one end and propagated regeneratively to another end of the cell. An average speed of propagation of intracellular Ca^{2+} waves was 47.7±7.6µm/sec (n=17 cells).
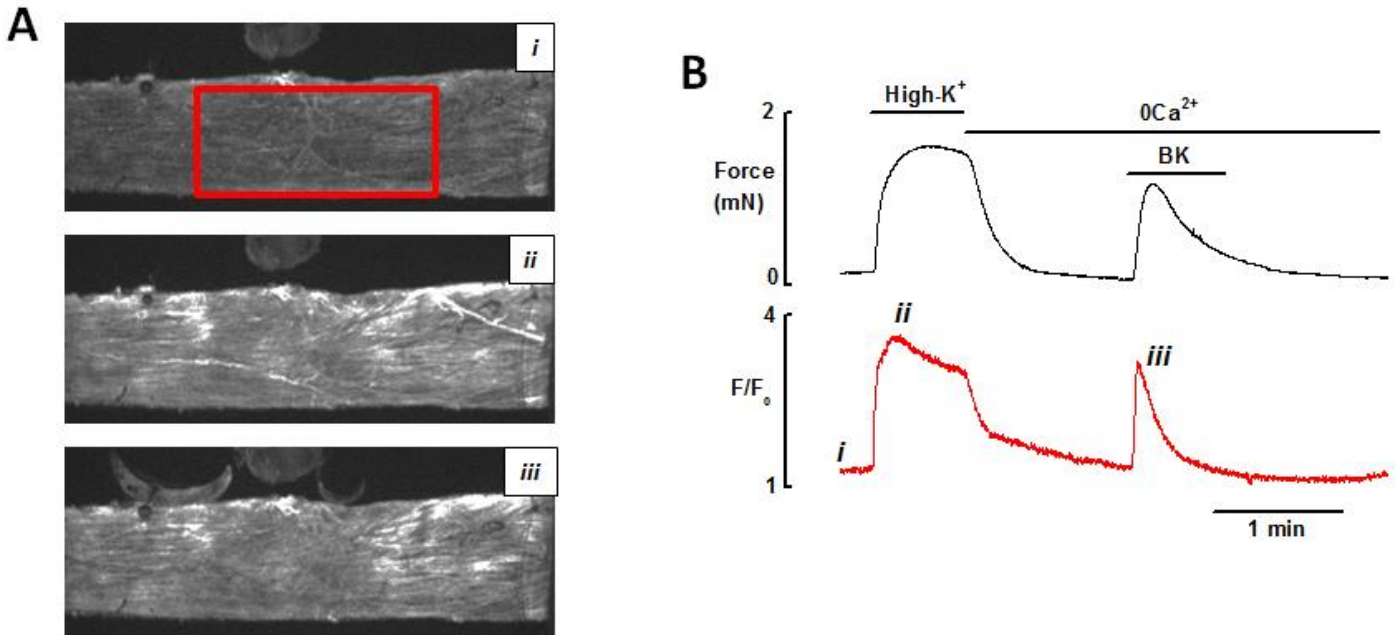
Figure 5.3.2-2. *Effect of 40 seconds application of BK on Ca^{2+} transient and force in Ca^{2+}-free solution. A: Grey scale images showing fluo-4 loaded ureteric smooth muscle of the upper ureter at rest (i), during high-K (ii) and BK stimulation (iii) B: Graph showing Ca^{2+} transient (Bottom trace), measured in region 1 from A and force (Top trace), induced by high-K in Ca^{2+}containing solution and BK in Ca^{2+}-free solution with 2mM EGTA.*
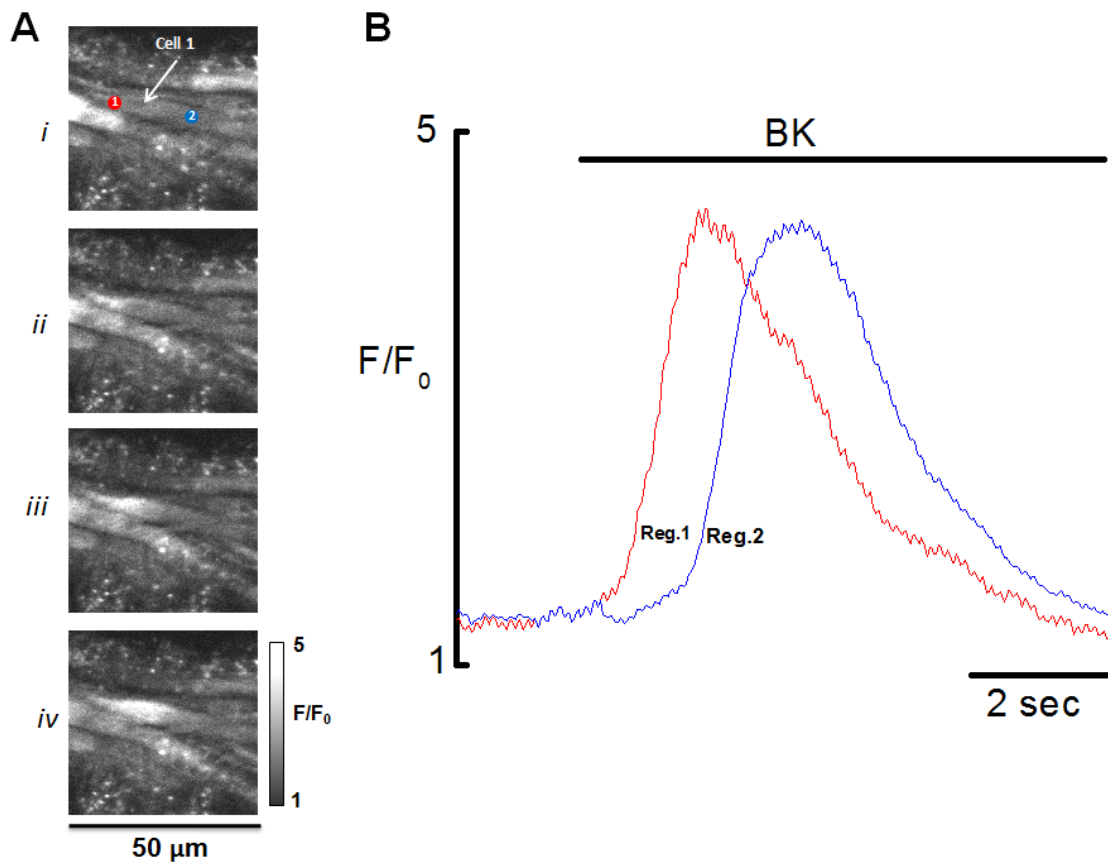
Figure 5.3.2-3. *Temporal and spatial properties of the intracellular Ca^{2+} wave, induced by 40 seconds application of BK (5μM) in the ureteric smooth muscle cell in situ in Ca^{2+}- free solution. A: Stack of images showing Cell 1 at rest (i) and during propagation of the intracellular Ca^{2+} wave, recorded with 0.5 sec interval, induced by BK in Ca^{2+}- free solution (ii-iv); B:Graph showing the superimposed traces of changes of intracellular Ca^{2+} in regions1 and 2, shown in Ai.*

5.3.3 Temporal and spatial properties of BK induced intercellular Ca^{2+} waves.

Simultaneous recording of Ca^{2+} signalling and force showed that each of the BK-induced phasic contractions, superimposing sustained tonic component, was associated with propagating intercellular Ca^{2+} waves. As a rule propagating intercellular Ca^{2+} waves were initiated in one of the region, which started to act as a temporary pacemaker. This pacemaker region in the presence of BK, caused regular spontaneous intercellular Ca^{2+} waves oscillations, which propagated from initiation site in both direction in a regenerative manner and a constant speed. Figure 5.3.3-1 shows an example, where region 1 was acting as a pacemaker, and was initiating the intercellular Ca^{2+} wave, which propagated at a constant speed and amplitude along the ureteric smooth muscle bundles. The Ca^{2+} wave had a fast rising phase, followed by a plateau phase (Figure 5.3.3-1B). These intercellular Ca^{2+} waves looked stereotypic and were shown previously to be associated with the propagating plateau type action potentials (Borysova et al., 2011). The intercellular Ca^{2+} wave was propagating at an average speed of 5.12 ±0.4mm/s (n=11) and was quickly abolished by Nifedipine (Figure 5.3.3-2)
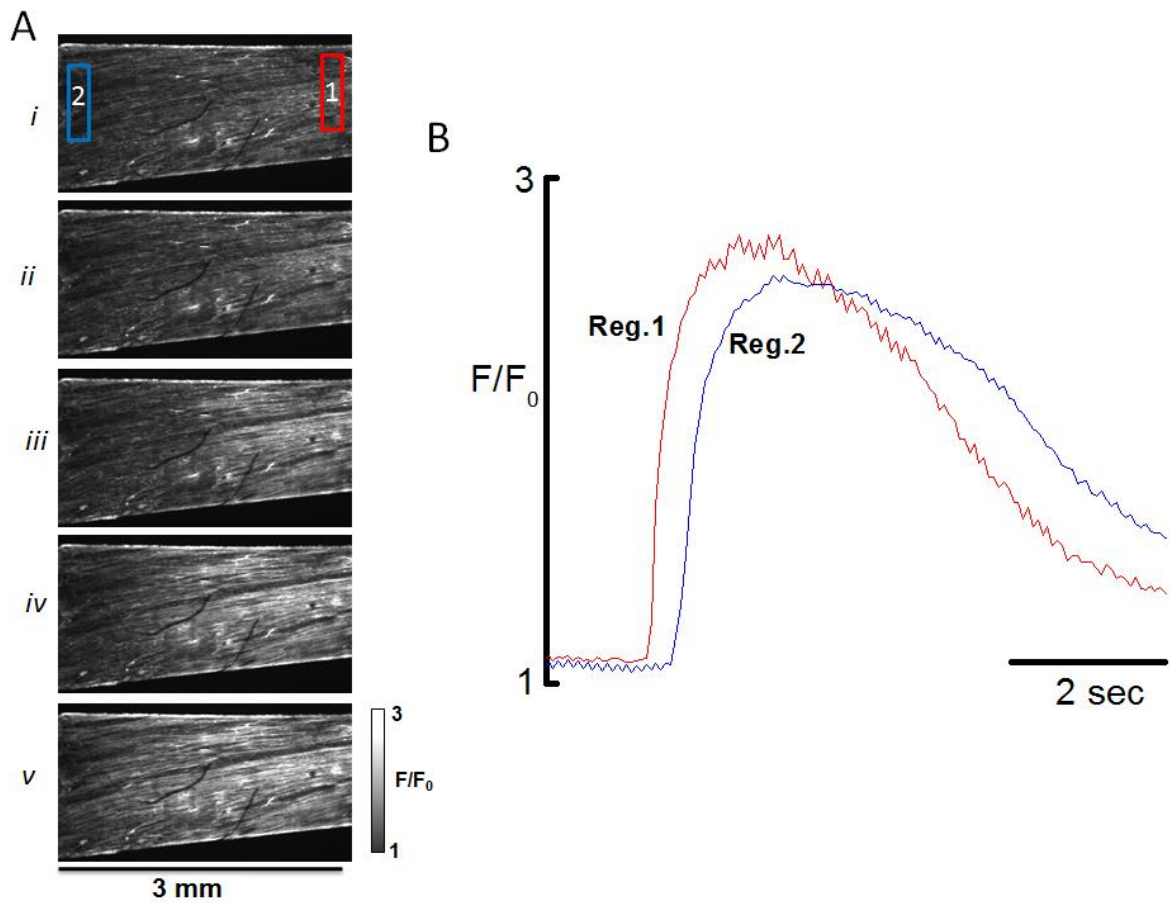
Figure 5.3.3-1. *Temporal and spatial properties of the intercellular Ca²⁺ wave, induced by 5 minutes application of BK (5µM) in the ureteric smooth muscle cell in situ, in normal Krebs solution. A: Stack of images showing the ureteric smooth muscle cells in situ, loaded with fluo-4 at rest (i) and during propagation of the intercellular Ca²⁺wave, recorded with 100msec interval, induced by BK in normal Krebs solution (ii-v); B: Graph showing superimposed traces of the changes of intracellular Ca²⁺in region 1(Red trace) and 2 (Blue trace) shown in Ai.*
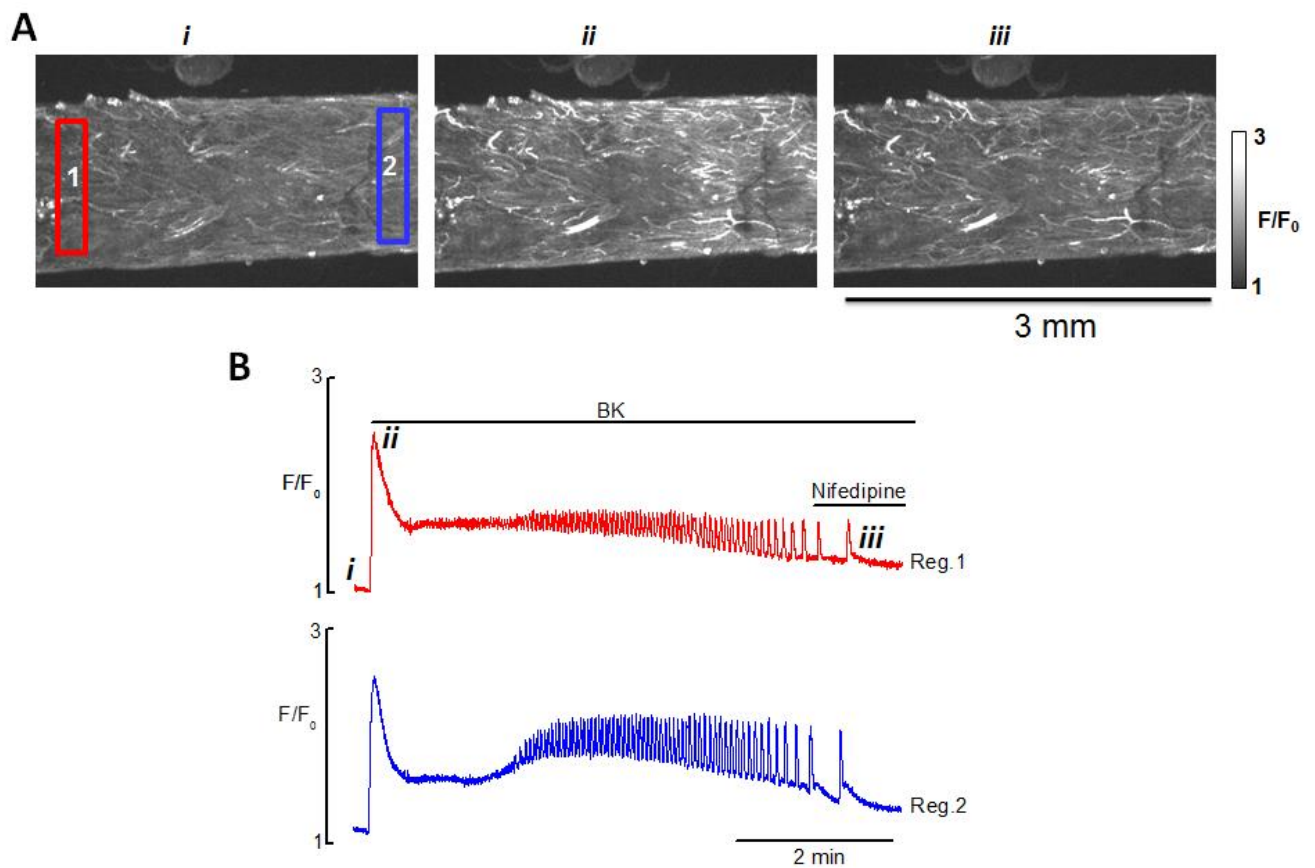
Figure 5.3.3-2. *The effect of 2 minutes application of Nifedipine (10µM) on the propagating intercellular Ca^{2+} waves, induced by 5 minutes application of BK (5µM) in the rat ureteric smooth muscle of the upper ureter. A: Grey scale images showing the ureteric smooth muscle, loaded with fluo-4 at rest (i), during BK simulation in the absence (ii) and the presence (iii) of Nifedipine. B: Graph showing traces of changes of intracellular Ca^{2+} in region 1 (Red trace) and region 2 (Blue trace) shown in Ai.*

5.3.4 Effects of Nifedipine on Ca^{2+} signalling and force induced by BK

The role of VOCCs in the control of the complex Ca^{2+} signalling and force was determined by the use of the L-type Ca^{2+} channel inhibitor (Nifedipine) (10µM), ($n = 3$).

Addition of Nifedipine, quickly and selectively, blocked intercellular Ca^{2+} wave oscillations (Figure 5.3.4), but had no effect on the initial phasic, seen as asynchronous intracellular Ca^{2+} waves, and the sustained tonic component, seen as an elevation of the baseline Ca^{2+} (Figure 5.3.4, Bottom trace), which correlated with the biphasic contractile response (Figure 5.3.4, Top trace). These experiments indicate a key role of the intracellular Ca^{2+} waves in the generation of the initial transient BK-induced phasic contraction and a key role of the propagating intercellular Ca^{2+} waves in the control of the phasic contractions.
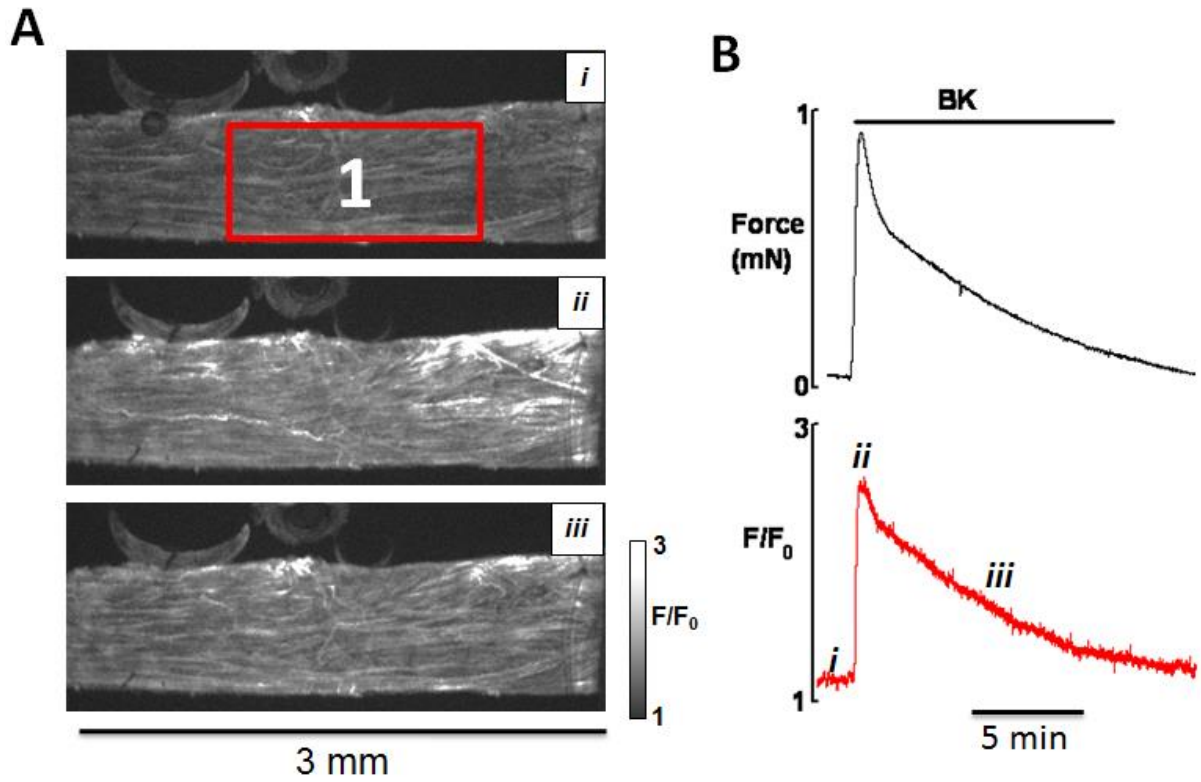
Figure 5.3.4. *Effect of BK (5µM) on Ca²⁺transient and force in the presence of 2 minutes prior application of Nifedipine (10µM). A: Grey scale images showing fluo-4 loaded ureteric smooth muscle of the upper ureter at rest (i), and during BK stimulation at a peak (ii) and plateau phase (iii). B: Graph showing force (Top trace) and Ca²⁺ transient (Bottom trace), measured in region 1 from A.*

5.3.5 Ca^{2+} release/ Ca^{2+} entry coupling induced by BK and CPA in the ureteric SMs

To investigate the possible role of the capacitive Ca^{2+} entry, the effects of a re-addition of external Ca^{2+} to a Ca^{2+}- free solution on the force- Ca^{2+} relationship in the ureteric smooth muscle, following a store depletion either by BK (5µM) or CPA (20µM) in Ca^{2+}- free solution were studied. Ca^{2+} was removed from the external bathing solution containing 2mM EGTA. Re-admittance of Ca^{2+}, after store depletion, induced by BK, resulted in an elevation of the baseline Ca^{2+} and force and initiation of propagating intercellular Ca^{2+} waves (Figure 5.3.5-1B, Bottom trace), associated with the phasic contractions (Figure 5.3.5-1B, Top trace), in the smooth muscle of the upper and the lower ureter. A similar response was seen, when the store was depleted with CPA (Figure 5.3.5-2). Upon re-admittance of Ca^{2+} to Ca^{2+}- free solution, following SR Ca^{2+} depletion by CPA, an elevation of the baseline Ca^{2+} and force superimposed by intercellular Ca^{2+} wave oscillations (Figure 5.3.5-2B, Bottom trace), associated with the phasic contractions (Figure 5.3.5-2B, Top trace) were observed.

It should be noted that the spontaneous phasic activity initiated by the re-admittance of the external Ca^{2+} to Ca^{2+} free solution, following CPA induced Ca^{2+} store depletion, was observed exclusively in the smooth muscle of the upper ureter (n=5).
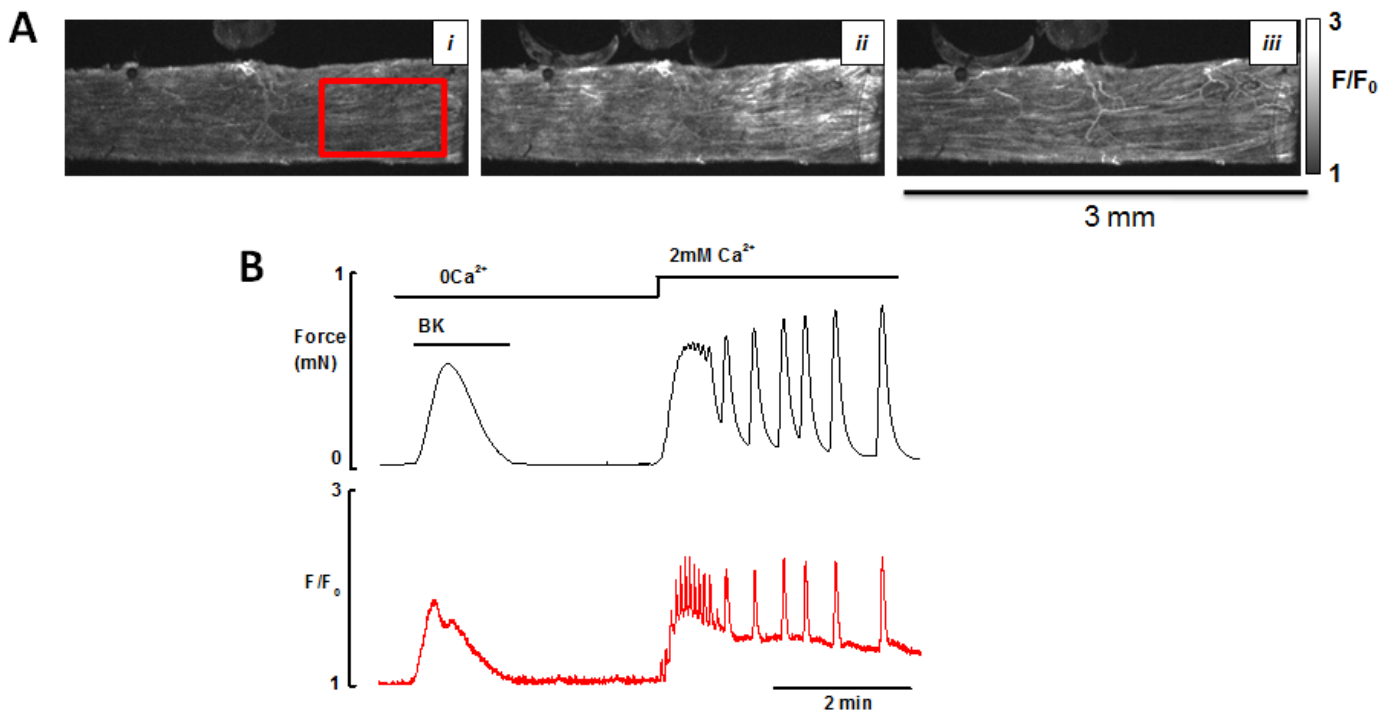
Figure 5.3.5-1. *Effect of the readmission of external Ca^{2+} to Ca^{2+}-free solution, following Ca^{2+} depletion of the SR by 40 seconds application of BK (5µM) in the smooth muscle of the upper ureter. A: Grey scale images showing fluo-4 loaded ureteric smooth muscle of the upper ureter at rest (i), during BK stimulation in Ca^{2+}- free solution (ii) and after readmission of external Ca^{2+} to Ca^{2+} -free solution, following store depletion (iii).B: Ca^{2+} transient (Bottom trace), measured in region 1 from A and force (Top trace), induced by BK in Ca^{2+}-free solution with 2mM EGTA and after readmission of external Ca^{2+} to Ca^{2+}-free solution. Note that the re-admission of external Ca^{2+} to Ca^{2+} -free solution, following SR Ca^{2+} depletion by BK leads to elevation of the baseline Ca^{2+} and force, superimposed by propagating intercellular Ca^{2+} wave*
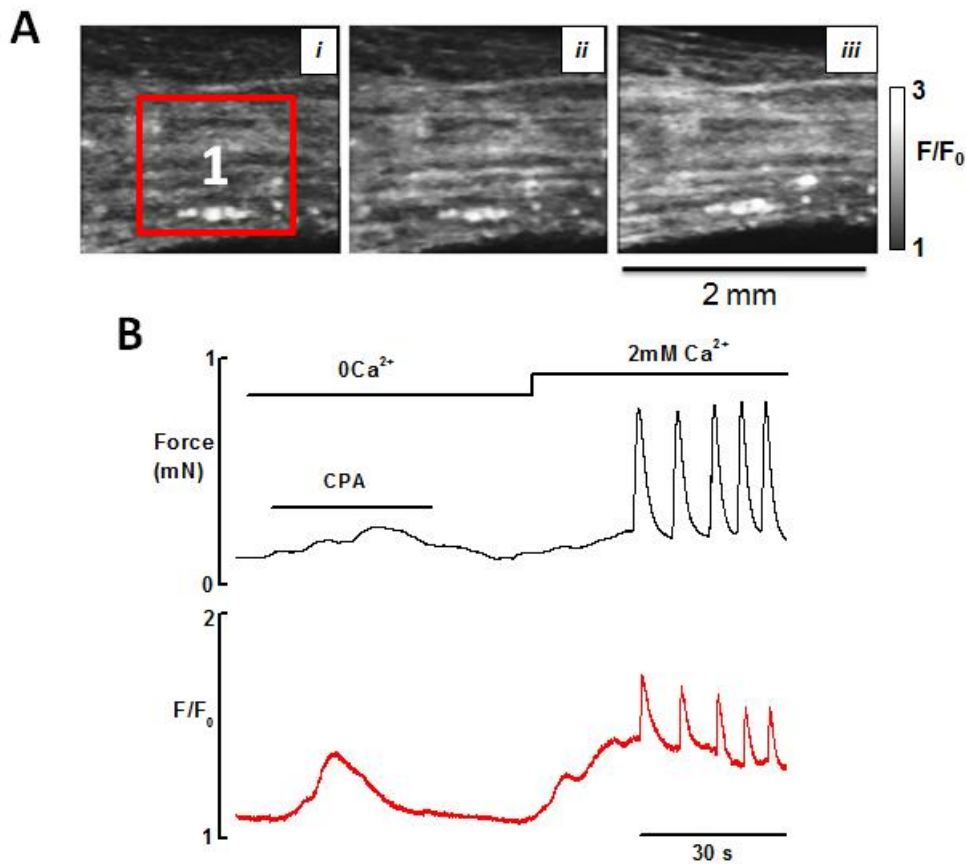
Figure 5.3.5-2. *Effect of the readmission of external Ca^{2+} to Ca^{2+}-free solution, following Ca^{2+} depletion of the SR by 5 minutes application of CPA (20µM) in the smooth muscle of the upper ureter. A: Grey scale images, showing fluo-4 loaded ureteric smooth muscle of the upper ureter at rest (i), during   CPA stimulation in Ca^{2+}- free solution (ii) and after readmission of external Ca^{2+} to Ca^{2+}-free solution, following store depletion (iii).B; Ca^{2+} transient (Bottom red trace), measured in region 1 from A and force (Top black trace) induced by CPA in Ca^{2+}-free solution with 2mM EGTA and after a readmission of external Ca^{2+} to Ca^{2+}-free solution. Note that the re-admission of external Ca^{2+} to Ca^{2+}- free solution, following SR Ca^{2+} depletion by CPA leads to elevation of the baseline Ca^{2+} and force, superimposed by propagating intercellular Ca^{2+} wave oscillations, associated with the phasic contractions.*

5.3.6 Concentration-dependent effects of BK on Ca^{2+} signalling of the smooth muscle of the upper and the lower ureter

In the next series of the experiments, the effects of 0.2, 0, 5 and 5µM BK on Ca^{2+} signalling and force on the smooth muscle of the upper (n=4) and the lower (n=3) ureter was studied. Figure 5.3.6-1 shows typical example of calcium responses of the smooth muscle of the upper ureter evoked by 0.2, 0.5 and 5µM, added cumulatively and measured in two regions of interest shown in figure 5.3.6-1A. Typically, at 0.2 µM, BK induced small elevation of the baseline Ca^{2+}, which could vary between regions (Figure 5.3.6-1B). At 0.5µM, BK further increased the baseline Ca^{2+} and, at this concentration, the smooth muscle of the upper ureter reached a threshold of activation of propagating intercellular Ca^{2+} waves, which were initiated in region 1 (Figure 5.3.6-1A, Red box). The frequency of Ca^{2+} oscillations was dependent on the concentration of BK used and at 0.5 µM, it was in a range of 0.8- 2 cycles/min (n=3). At 5µM the frequency of Ca^{2+} oscillations was increased to 3-8 cycles/min (n=3). On the other hand, the smooth muscle of the lower ureter were significantly less sensitive to BK. There was no significant change in the baseline Ca^{2+} in response to 0.2 µM of BK (Figure 5.3.6-2B). In the presence of 0.5µM BK, the baseline Ca^{2+} was increased, but was not associated with the activation of the spontaneous activity (Figure 5.3.6-2B). Only at the maximal concentration (5µM), BK triggered a spontaneous activity, which appeared as propagating intercellular Ca^{2+} wave oscillations from region 2 to region 1 (Figure 5.3.6-2C). In this sample, region 2 was acting as a pacemaker. The frequency of Ca^{2+} oscillations was in the range of 0.5-0.7 cycles/min (n=3).
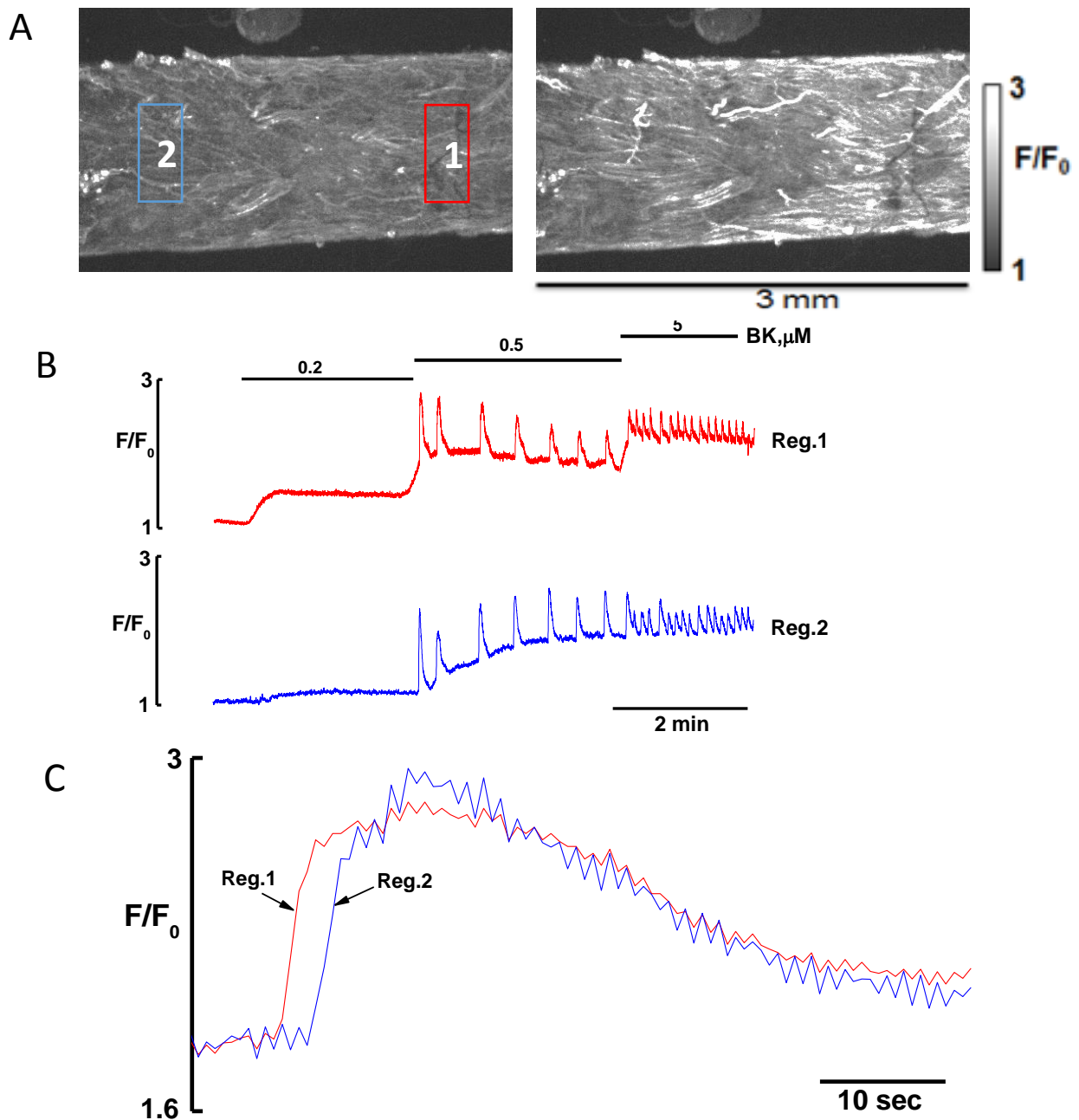
Figure 5.3.6-1. *Ca²⁺ signalling induced by 5 minutes application of different concentrations of BK in the smooth muscle of the upper ureter. A: Grey scale image of Fluo-4 loaded ureteric segment isolated from the upper ureter at rest (i) and during BK stimulation (ii). B: Graph showing changes in Ca²⁺ signals in region 1(Red trace) and region 2 (Blue trace) shown in A. the smooth muscle cells in region 1 are more sensitive to BK and act as a pacemaker. C: Superimposed traces of the propagating intercellular Ca²⁺ wave oscillations from pacemaker region 1(Red trace) to region 2 (Blue trace).*
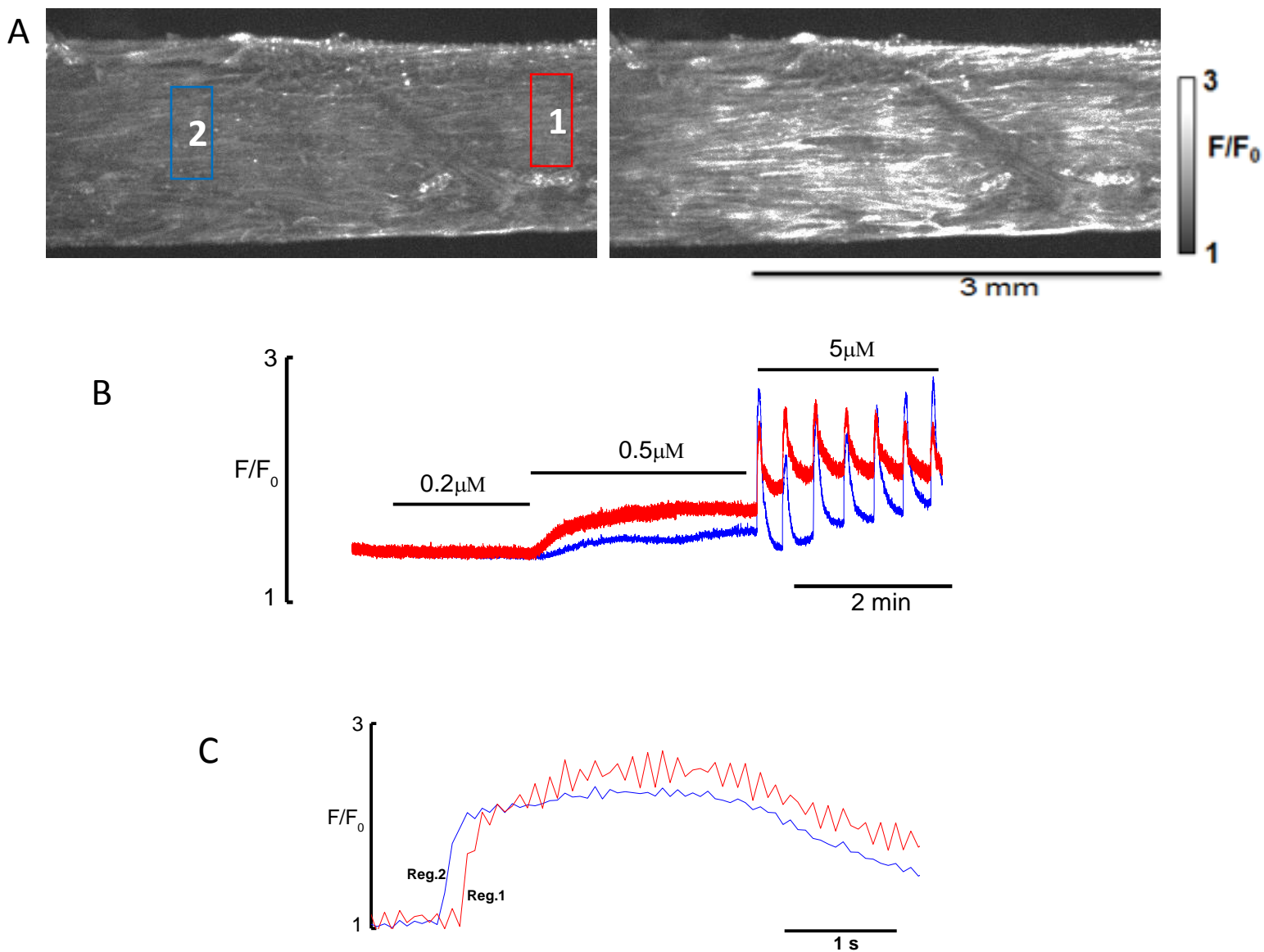
Figure 5.3.6-2. Ca^{2+} *signalling induced by 5 minutes application of different concentrations of BK in the smooth muscle of the lower ureter. A: Grey scale image of Fluo-4 loaded ureteric segment isolated from the lower ureter at rest (i) and during BK stimulation (ii). B: Graph showing the changes in Ca^{2+} signals in region 1 (Red trace) and region 2 (Blue trace) shown in A. Smooth muscle cells in region 2 are more sensitive to BK and act as a pacemaker. C: Superimposed traces of the propagating intercellular Ca^{2+} wave oscillations from pacemaker region 2 (Blue trace) to region 1 (Red trace).*

5.4   Discussion

The results of the present chapter demonstrate that the Ca^{2+} transient, induced by BK, consists of three major components: initial transient phasic component, associated with Ca^{2+} release from the SR, seen as asynchronous intracellular Ca^{2+} waves, and associated with a brief initial phasic contracture. The second component of BK induced Ca^{2+} response is a sustained elevation of the baseline Ca^{2+}, caused mainly by Ca^{2+} influx, via Nifedipine resistant SOCCs/ROCCs, associated with the elevation of the base line tonic contraction. And the third component of the BK induced Ca^{2+} transient are observed as propagating intercellular Ca^{2+} waves, dependent on Ca^{2+} entry, via voltage gated L-type Ca^{2+} channels, associated with the phasic contractions.

The data obtained also indicate that there are dormant pacemaker regions in the main ureter, which can be activated by BK. These dormant pacemaker regions can drive a retrograde peristalsis, which could be potentially damaging for the kidney.

Thus, in the ureteric smooth muscle, B_2 receptors activation is sequentially coupled to Ca^{2+} release, Ca^{2+} entry via the SOCCs/ROCCs and L-type VOCCs.

This conclusion is supported by the following observations: (i) In the presence of Nifedipine, there was a selective inhibition of the phasic intercellular Ca^{2+} wave oscillations, associated with the phasic contractions, which are known to depend on Ca^{2+} entry, via L-type voltage gated Ca^{2+} channels that is responsible for the generation of the plateau type action potential in the rat ureteric smooth muscle (Burdyga and Wray, 1999b); (ii) There was a transient release of Ca^{2+} from the store, associated with the transient phasic contractile response of the ureteric smooth muscle to BK under control conditions, in the presence of Nifidipine and in Ca^{2+}-free solution; (iii) There was an elevation of the baseline Ca^{2+} and force superimposed by intercellular Ca^{2+} wave oscillations, associated with the phasic contractions, induced by

the readmission of external Ca^{2+} to Ca^{2+}- free solution, following Ca^{2+} store depletion, either by BK or CPA.

Collectively, these data indicate that BK produces contraction of the rat ureter smooth muscle, via B_2 receptors, coupled to Ca^{2+} release from the SR, mediated by IP_3R channels, responsible for the generation of the initial phasic contraction, followed by Ca^{2+} entry via the SOCCs/ROCCs that are responsible for the generation of the tonic contraction, and the voltage gated Ca^{2+} channels responsible for the generation of the propagating intercellular Ca^{2+} waves, associated with the phasic contractions.

The results obtained on canine tracheal smooth muscle cells also indicated that bradykinin by acting on B_2 receptors, increases the intracellular Ca^{2+} concentration, which involves the release of Ca^{2+} from the intracellular Ca^{2+} stores, followed by the influx of external Ca^{2+} (Yang et al., 1994).

Based on the data obtained, it is suggested that the spontaneous phasic contractions associated with the propagating intercellular Ca^{2+} waves, mediated by the action potentials induced by BK, are activated by membrane depolarisation, produced by the translocation of the cations across the plasma membrane, caused by Ca^{2+} release/ Ca^{2+} entry coupling mechanism and/or receptor operated channels.

Chapter 6

The importance of Rho-associated kinase-induced Ca^{2+} sensitization as a component of Bradykinin –induced pharmaco-mechanical coupling in the rat ureteric smooth muscle

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6.1   Introduction

It is generally accepted that Ca^{2+} is the primary regulator of the contraction in the smooth

muscle cells (Fukata et al., 2001). The rise of the cytoplasmic free $[Ca^{2+}]$ can be induced by

the electromechanical coupling, through depolarization of the surface membrane, or by the

pharmaco-mechanical coupling, a mechanism independent of the surface membrane

potential, which could involve Ca^{2+} release and/or Ca^{2+} entry, via receptor-operated or store-

operated channels, or a combination of the two. A global rise in the cytoplasmic $[Ca^{2+}]$

activates Ca^{2+}/calmodulin-dependent MLCK, which phosphorylates the 20-kDa regulatory

light chains of myosin (MLC_{20}), thereby triggering cross-bridge cycling and contraction.

Secondary mechanisms that can modulate SMs contractility, independent of $[Ca^{2+}]_i$

have been identified, and the RhoA/Rho-kinase signalling was reported to play a major role

in this process, in different types of the smooth muscles, including the ureter (Fukata et al.,

2001, Shabir et al., 2004).

Rho-kinase directly modulates smooth muscle constriction, via phosphorylation of the

myosin light chain (Fukata et al., 2001).Therefore, inhibition of Rho-kinase might result in

smooth muscle relaxation. It is well established that a decrease or an increase in MLCP

activity, at a constant level of MLCK activity, and at steady-state $[Ca^{2+}]_i$, can sensitize or

desensitize smooth muscle, which results in an increase or a decrease in MLC_{20}

phosphorylation and force, respectively (Somlyo and Somlyo, 2004).

Ca^{2+} sensitization of the smooth muscle produced, following receptor stimulation, involves a

decrease in MLCP activity, mediated through the actions of protein kinase C (PKC) and/or

<center>160</center>

RhoA-associated kinase (Somlyo and Somlyo, 2004). Thus the extent of MLC_{20}

phosphorylation and force results from a balance between MLCK and MLCP activities.

Some early studies, performed on an intact tissue, showed that GPCR agonists can produce a

greater increases in force for a given increase in $[Ca^{2+}]_i$ than KCl, which activates Ca^{2+} entry

via L-type voltage gated Ca^{2+} channels, to induce activation of the contractile response

independently of the plasma membrane receptor activation (Bradley and Morgan, 1987,

Himpens et al., 1988, Himpens and Casteels, 1990).

Mechanisms responsible for Ca^{2+} sensitization of the smooth muscle contraction involve

primarily inhibition of MLCP activity, by ligand occupancy of the receptors that are coupled

to guanine nucleotide exchange factors (GEF), via the heterotrimeric G proteins of the $G_{12/13}$

family (Somlyo and Somlyo, 2004).

MLCP is a heterotrimeric enzyme with a 37 kDa type-1 phosphatase catalytic subunit (PP1c),

a 110 - 130 kDa myosin-targeting subunit (MYPT1) and a 20 kDa subunit of unknown

function (Hartshorne et al., 1998). Rho-kinase phosphorylates MYPT1 at Thr_{697} and/or

Thr_{855}, leading to inhibition of MLCP activity (Somlyo and Somlyo, 2004). There is abundant

evidence showing that the phosphorylation of Thr_{855} catalysed by Rho-kinase occurs in a

variety of smooth muscle tissues, in response to diverse stimuli (Somlyo and Somlyo, 2004,

Kitazawa et al., 2003, Murányi et al., 2005). However, the physiological importance of Thr_{697}

phosphorylation by Rho-kinase remains uncertain. While there are several examples of Thr_{697}

phosphorylation occurring in some types of smooth muscles (Seko et al., 2003, Ito et al.,

2004), there are many examples of T_{855} phosphorylation occurring without Thr_{697}

phosphorylation (Kitazawa et al., 2003, Murányi et al., 2005, Tsai and Jiang, 2006).

The roles of Rho-kinase, in the spontaneous, as well as agonist induced contractions, were

revealed more in tonic muscles (Iizuka et al., 1999, Martinez et al., 2000, Sakurada et al.,

2001). However, recently, it was also shown that Rho-kinase plays an essential role in the

control of the excitation-contraction coupling in the phasic SMs, such as the urinary bladders

of the rabbits, rats, mice, pigs and human (Zhang and DiSanto, 2011), as well as the ureters of

the rabbits (Turna et al., 2007), rats (Borysova et al., 2011, Shabir et al., 2004), sheep (Levent

and Buyukafsar, 2004) and human (Hong et al., 2005).

Furthermore, both ROCK isoforms α and β are expressed in the rat ureteric smooth muscles

(Borysova et al., 2011). In a previous study (Shabir et al., 2004), it was shown that Rho-

kinase plays a key role in the control of the electromechanical coupling in the phasic rat

ureter smooth muscle, via both Ca^{2+}-dependent (modulation of Ca^{2+} influx through L-type

voltage-gated Ca^{2+} channels) and Ca^{2+}-independent (modulation of MLCP activity)

mechanisms. There is a significant contribution of MLCP to MLC_{20} phosphorylation and

force during high-K and CCh stimulation of the rat ureteric smooth muscles (Borysova et al.,

2011, Shabir et al., 2004). Inhibition of Rho-kinase has a dramatic effect on Ca^{2+} –MLC_{20}-

phosphorylation- force relationship in the rat ureteric smooth muscles. It causes an increase in

the activity of MLCP associated with dephosphorylation of MYPT1 at both residues. This

results in a complete dephosphorylation of the MLC_{20} and relaxation of the tonic component

of the high-K^+- or CCh induced contraction, with little or no change in the intracellular Ca^{2+}

(Borysova et al., 2011).

BK, which is one of the G-protein coupled receptor agonist, was shown to have high Rho-

kinase sensitive effects on the contractile response of the chemically skinned smooth muscle

of the rat airways. This observation provided a strong support for a physiological role of Rho-

kinase in BK induced control of the contractile response in at least some tonic smooth

muscles (Shaifta et al., 2015).

Y-27632 is a selective inhibitor of ROCK-1 and ROCK-2, and is a pharmacological tool,

widely used to identify the functional role of Rho-kinase in Ca^{2+} sensitisation, in various

types of smooth muscles (Fu et al., 1998, Amano et al., 1999, Yoshii et al., 1999, Ishizaki et al., 2000, Narumiya et al., 2000, Nakahara et al., 2000, Kishi et al., 2000).

Recently it was shown that in obstructed rabbit ureter, the level of the expression of ROCK 1 and ROCK 2 have been substantially increased and the contractile responses induced by various factors were effectively inhibited by Y-27632 (Turna et al., 2007). Hence, Rho-kinase inhibitors might be considered as potential pharmacological tools for facilitation of kidney stone passage and prevention of renal colic and kidney damage.

The next aim of the present work was to investigate the possible role of Rho-associated kinase-induced Ca^{2+} sensitization as a component of BK –induced pharmaco-mechanical coupling in the rat ureteric SM, by investigating the effects of Y-27632 on the force-Ca^{2+} relationship, induced by BK in the rat ureteric smooth muscle.

6.2   Methods

6.2.1   Tissue preparations

Rats (~200 g) were humanely killed using CO_2 anaesthesia, followed by cervical dislocation, in accordance with UK legislation. The ureters were dissected, carefully cleaned of the connective and the paraureteric adipose tissue, using fine curved scissors, while keeping the sharp edges away from the tissue to avoid physical damage of the ureteric bundles.

6.2.2   Fluo -4 loading

To load the tissue with fluo 4, strips of ureter were placed in a plastic cuvette contained 1 ml of HEPES-buffered Krebs solution, containing 15 µM-fluo 4-AM, dissolved in DMSO premixed with Pluronic F127 (final concentration of 0.01 %).

Loading was performed at room temperature for 3 h with the cuvettes wrapped in a black tape and rotated at 30 rpm. Tissue samples were then removed from the loading medium and placed in normal Krebs solution for at least 30 min to allow cleavage of fluo 4-AM to fluo 4 by intracellular esterases.

6.2.3   Confocal imaging of intact rat ureteric strips

We used fast Nipkow disc-based confocal imaging, attached to a high sensitivity (iXon, Andor) CCD camera, which allow acquisition of the images at 60 fps, and thereby accurate measurement of the temporal and spatial characteristics of Ca^{2+} signaling in individual smooth muscle cells and bundles in intact ureteric strips.

To measure temporal and spatial characteristics of Ca^{2+} signaling in individual smooth muscle cells, a x60 water objective (NA 1.2) was used, while low-power dry objectives (x4, NA 0.08 and x20, NA 0.72) were used to measure the temporal and spatial characteristics of Ca^{2+} signaling in the whole ureteric muscle bundles. Data acquisition was performed using Andor iQ software.

6.2.4 Combined confocal imaging with force measurement

To simultaneously measure Ca^{2+} transients and force, ureters were cut into small segments (5 - 6 mm in length), clipped at both ends, using aluminum foil clips (Laser Services, USA), and attached to a force transducer (FORT 10, WPI) at one end and a stainless steel hook, fixed to the bottom of the experimental chamber at the other end. The force transducer was attached to a 3-d manipulator (Narashige, Japan) allowing movement of the strip in the X-Y-Z directions to position it in the focal plane of the objective and apply an optimal stretch, which did not exceed 40% of the active maximal force induced by high $[K^+]$ depolarization.

6.2.5 Solutions

The standard physiological solution was a HEPES-buffered modified Krebs solution containing (in mM): Nacl, 124; Kcl, 5.4; Cacl$_2$, 2; Mgso$_4$, 1.2, HEPES, 11; and glucose, 11.7 Solutions with increased $[K^+]_o$ were obtained by replacing Na^+ by equimolar K^+. The Ca^{2+}-free solutions contained 2 mM EGTA.

6.2.6 Analysis and statistics

Data analysis was performed using Andor iQ software and Origin 8.6 (Origin Labs). The data were analysed with students (paired or unimpaired) t-test; differences between means were assumed to be significant at $P<0.05$. Data are given as mean $\pm$ s.e.m.; n is the number of samples.

6.3   Results

6.3.1   Effects of Y-27632 on the complex contractile response of the ureteric smooth muscle in normal Krebs solution.

Inhibition of Rho-kinase by Y-27632 (10 µM), in the presence of external Ca^{2+}, strongly reduced all components of BK- induced complex contractile response of the ureteric smooth muscle (n=11, Figure 6.3.1). The amplitude of the initial phasic and tonic components of BK-induced contraction in the presence of Y-27632 were reduced from 85.8±8.7 to 28.7±3.8% and 32.6±7.5 to 8.3±1.2% of the peak high-K response, respectively. The amplitude of the spontaneous phasic contractions, superimposing the tonic contraction was reduced from 124±1.1% to 56±8.3%.

These data indicate that there is a significant effect of Y-27632 on the BK induced complex contractile response of the rat ureteric smooth muscle. A decrease in the amplitude of the initial phasic and tonic contraction, which depend on Ca^{2+} release and Ca^{2+} entry via the ROCCs/SOCCs insensitive to Rho-kinase (Borysova et al., 2011), are most likely caused by Ca^{2+} sensitisation. A decrease in the amplitude of the phasic contractions, associated with the intercellular Ca^{2+} waves, mediated by Ca^{2+} entry, via voltage gated L-type Ca^{2+} channels sensitive to Rho-kinase (Shabir et al., 2004), are likely to involve both Ca^{2+}-dependent and Ca^{2+} independent effects.

In order to separate Ca^{2+} -dependent from Ca^{2+} -independent effects of Rho-kinase, the effects of BK on force- Ca^{2+} relationship induced by BK in the presence of Nifedipine and Ca^{2+}-free solution have been investigated next.
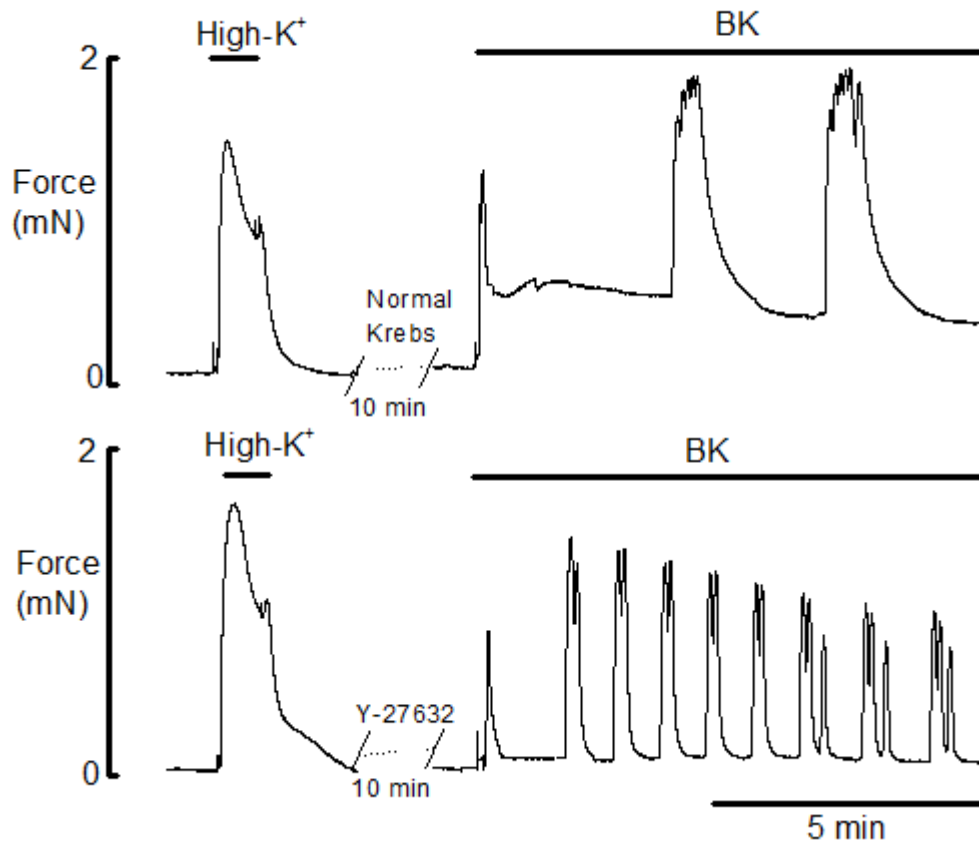
Figure 6.3.1. *Effects of Rho-kinase inhibitor (Y-27632) on BK-induced complex contractile response of the smooth muscle of the upper ureter. Representative traces show typical response of the smooth muscle of the upper ureter to BK (5 µM) under control conditions (Top trace) and in the presence of Y-27632 (10 µM) (Bottom trace). The tissues were pretreated with Y-27632 for 10 minutes. Contractile responses of the ureteric smooth muscle to high-K (40 seconds application) used as a reference is shown on the left*

6.3.2 Effects of Rho-kinase inhibition by Y-27632 on BK-induced force-Ca^{2+} relationship in the presence of Nifedipine

Inhibition of voltage gated L-type Ca^{2+} channels by Nifedipine (10μM) selectively blocked spontaneous contractile activity, but had no effect on the initial phasic and sustained tonic components of Ca^{2+} transient and force (Figure 6.3.2-1A, Left panel).

Pre-treatment of ureteric strips with Y-27632 (10μM) had no effect on the Ca^{2+} transient (Figure 6.3.2-1A, Right panel, Bottom trace), but caused a significant reduction of the amplitude of the initial phasic and strong inhibition of the tonic component of BK induced contraction (Figure 6.3.2-1A, Right panel, Top trace).

This is more clearly seen from the superimposed traces of Ca^{2+} transients (Bottom panel) and force (Top panel) shown in Figure 6.3.2-1B. The amplitude of both phasic and tonic component of BK-induced force in the presence of Y-27632 were reduced from 63.3±4.7 to 29.5±2.7% and 22.3±2.7 to 7.1±0.5% of peak high-K contraction, respectively, (Figure 6.3.2-2, n=16).
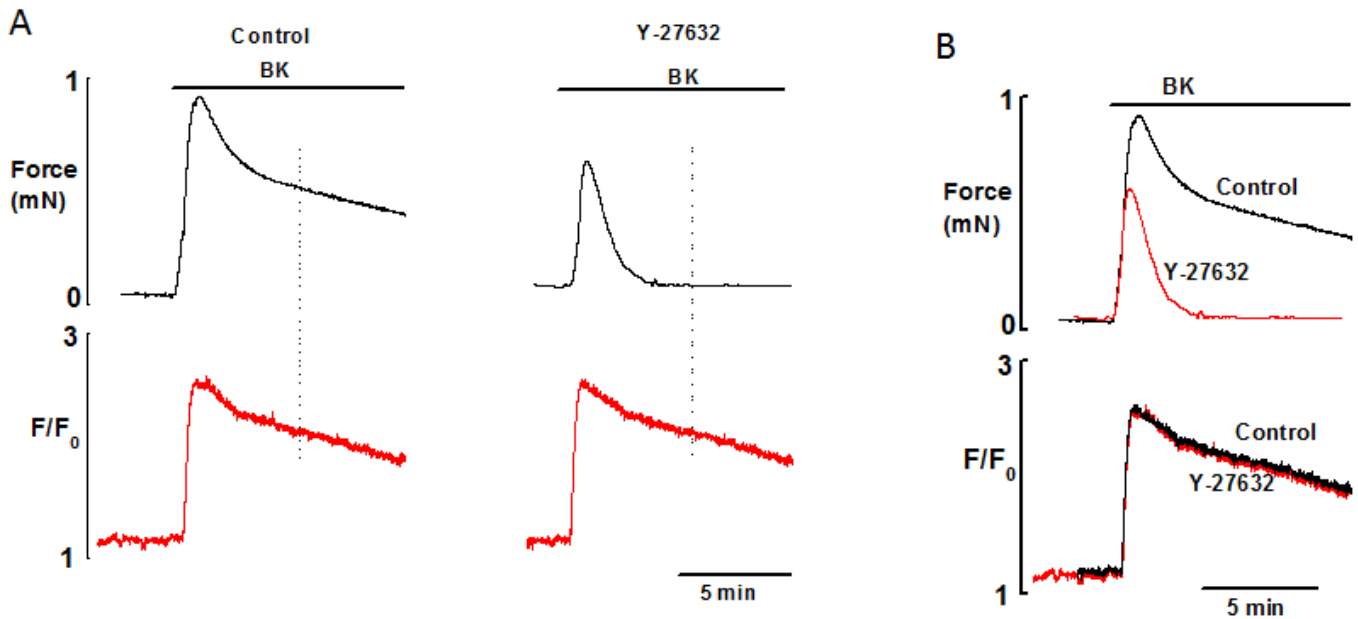
Figure 6.3.2-1. *The effect of Y-27632 (10μM) applied for 10 minutes on force-Ca^{2+} relationship induced by 5 minutes application of BK (5μM) in the ureteric smooth muscle in the presence of 2 minutes prior application of Nifedipine (10μM). A: Ca^{2+}transient (Bottom trace) and force (Top trace) induced by BK in the ureteric smooth muscle, in the presence of Nifedipine, recorded in the absence (Left panel) and the presence of Y-27632 (Right panel); B: Superimposed traces from A. The dotted lines indicates the points of measuring the tonic component of both force and Ca^{2+}transient.*
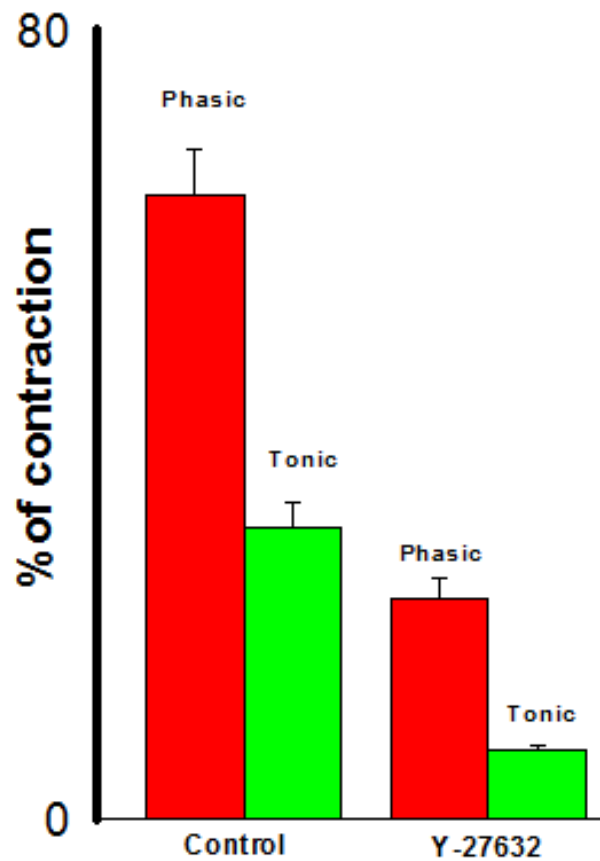
Figure 6.3.2-2. *A bar chart showing the mean amplitudes of the phasic (Red) and tonic (Green) contraction induced by 5 minutes application of BK (5 µM) in the ureteric smooth muscle, recorded in the presence of Nifedipine, in the absence (Control) and the presence of Y-27632 (10 µM). The mean amplitudes of both phasic and tonic contractions were significantly reduced (n=16, P<0.05) in the Y-27632 treated group.*

6.3.3 Effects of Rho-kinase inhibition by Y-27632 on BK-induced Ca^{2+} transient and force in Ca^{2+}-free solution

In Ca^{2+} -free solution, BK induced a transient increase in Ca^{2+}, accompanied by a brief phasic contraction (Figure 6.3.3.1, Left panel). Pre-treatment of ureteric strips, in Ca^{2+}- free solution with Y-27632 (10μM), caused a reduction in the amplitude and the duration of the phasic contraction (Figure 6.3.3-1, Right panel, Top trace), but had little effect on Ca^{2+} transient (Figure 6.3.3-1, Right panel, Bottom trace), which is more clearly seen from the superimposed traces (Figure 6.3.3-2A).

The mean amplitude of phasic contraction was reduced from 58.5±5.3% to 30.9±4.1% of the peak high-K contraction, (Figure 6.3.3-2B, n=11). Besides, there was a statistically significant reduction in the duration of BK-induced phasic contraction, measured at 50% of the peak amplitude, in the presence Y27632 treated tissue relative to the control responses. The mean duration at 50% of the peak amplitude of the BK –induced phasic contraction, in Ca^{2+} - free solution, for Y-27632 treated tissue was 5.4± 0.7s (n=9) compared to 14.1 ± 0.7s (n=6) under control conditions (Figure 6.3.3-2C).

From the superimposed normalised records of Ca^{2+} transient (Bottom panel) and force (Top panel) shown in Figure 6.3.3-3A and the average data presented in in figure 6.3.3-3B, it can be seen that the half-time of relaxation ($t_{1/2}$) of the phasic contractions of the BK induced contraction in Ca^{2+}- free solution was significantly reduced, while that of the Ca^{2+} transient was unaffected. The mean $t_{1/2}$ of relaxation of the phasic contraction was reduced from 13.8 ± 0.9s in the absence to 4.9± 0.5s in the presence of Y-27632, respectively (Figure 6.3.3-3B). Accordingly, the rate constant of relaxation of the phasic BK-induced phasic contraction ($1/t_{1/2}$) under control conditions (n=6) was 0.07 ± 0.01s. While in the presence of Y-27632 (n=9), it was increased to 0.21 ± 0.03s^{-1} (Figure 6.3.3-4). While that of the phasic BK-induced Ca^{2+} transient had no statistical significant changes. The rate constant of relaxation

was 0.09 ± 0.02 and 0.095 ± 0.03 in the control (n=3) and Y-27632 (n=3) treated group (Figure 6.3.3-4).

These data suggest that a Ca^{2+} -independent increase in the rate of relaxation of force is most likely associated with an increased rate of MLCP activity, and accordingly MLC_{20} dephosphorylation.
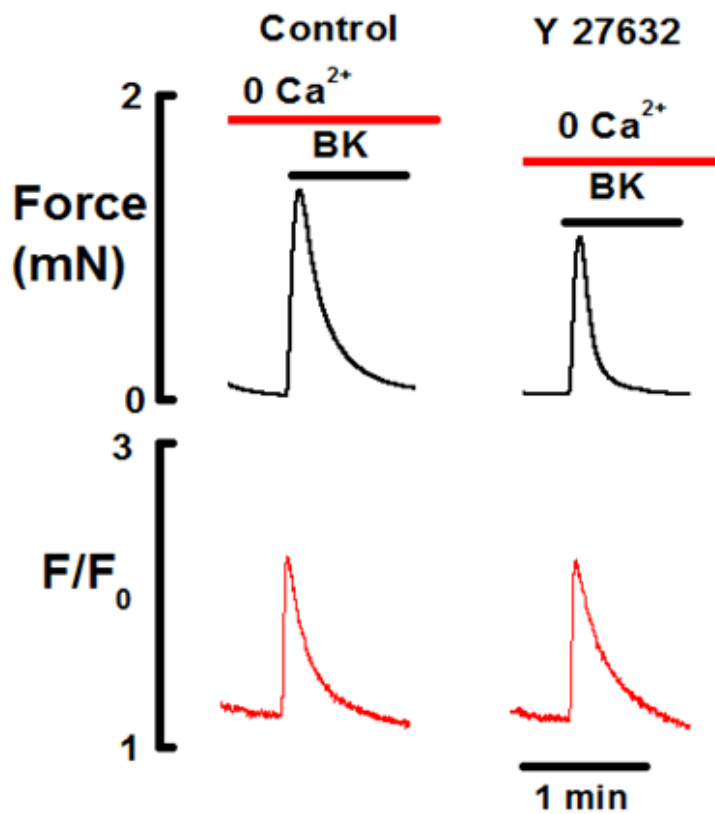
Figure 6.3.3-1. *The effect of Rho-kinase inhibition by Y-27632 (10 µM) on Ca^{2+} transient (Bottom trace) and force (Top trace) induced by BK (5 µM) applied for 40 seconds in Ca^{2+} free solution in the absence (Left panel) and the presence (Right panel) of Y-27632. The tissues were pre-treated with Y-27632 for 10 minutes.*
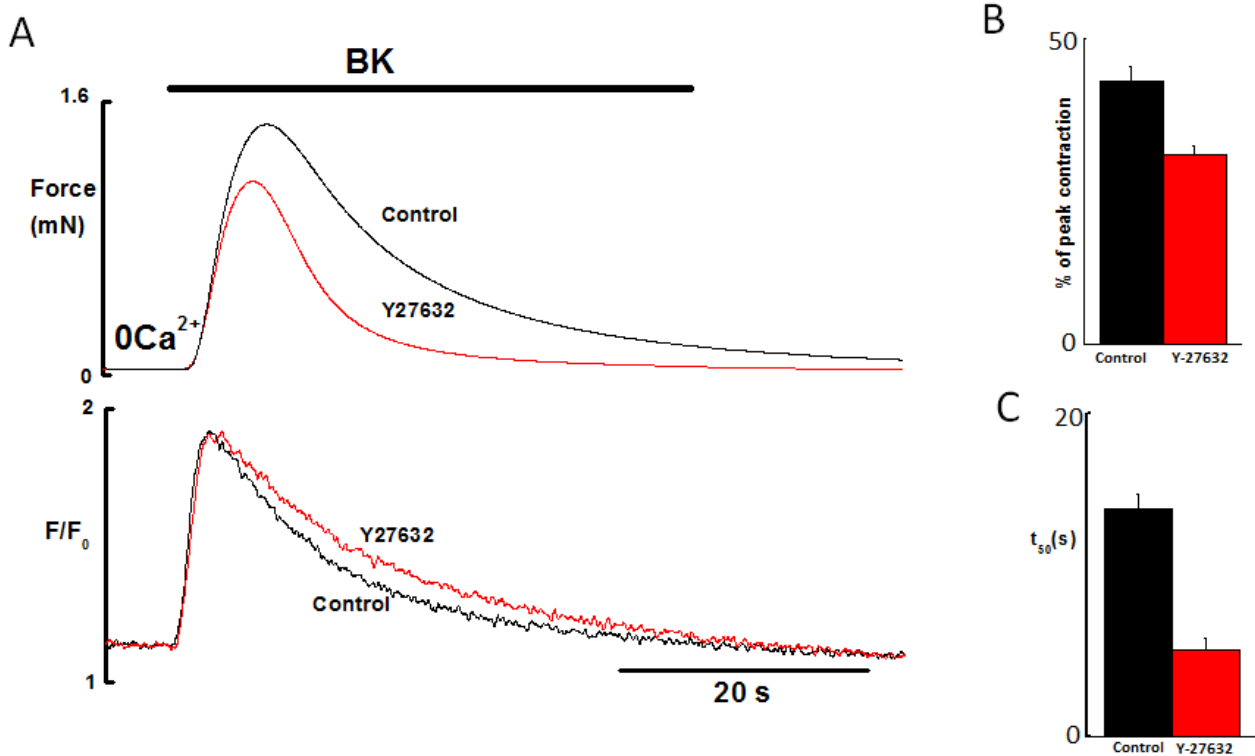
Figure 6.3.3-2. *Effects of Y-27632 (10μM) on kinetics of Ca^{2+} transients and force induced by 40 seconds application of BK (5μM) in Ca^{2+}-free solution. A: Superimposed traces of Ca^{2+} transients (Bottom panel) and force (Top panel) induced by BK in Ca^{2+} -free solution in the absence (Black traces) and the presence (Red traces) of Y-27632. B: The bar chart showing the mean amplitudes of BK-induced phasic contraction in the absence (Black bar) and the presence (Red bar) of Y-27632. There is a significant reduction of the mean amplitudes of BK-induced phasic contraction in the Y-27632 treated group (n=11, P< 0.05) as compare to the control group C: The bar chart showing the effect of Y-27632 on the duration of the BK induced contraction, measured at 50% of the peak amplitude (t_{50}) in Ca^{2+} free solution in the absence (Black column) and the presence (Red column) of Y-27632. The duration of the BK induced contraction at 50% of the peak amplitude significantly reduced in the presence of Y-27632 (n=15, P< 0.05).*
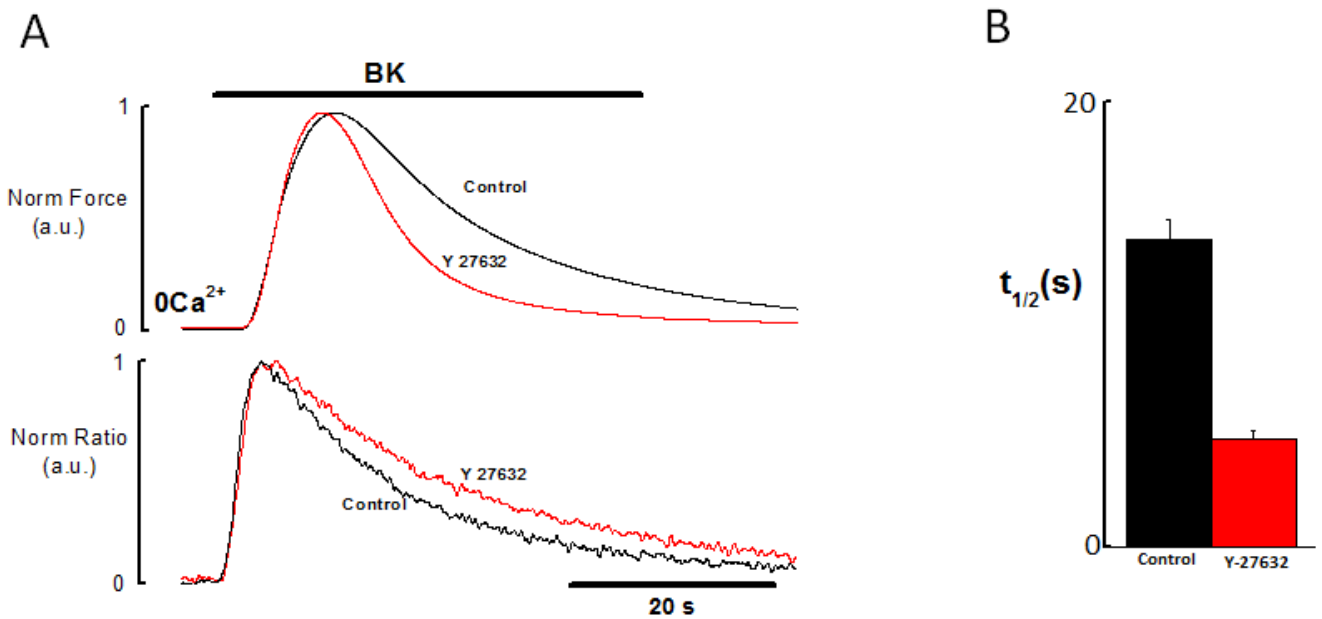
Figure 6.3.3-3. *Effects of Y-27632* (10μM) *on the half-time (t$_{1/2}$) of relaxation of 40 seconds application of BK* (5μM) *induced phasic contraction in Ca^{2+} free solution. A: Normalized superimposed traces of Ca^{2+} transients (Bottom panel) and force (Top panel) induced by BK in Ca^{2+} -free solution in the absence (Black traces) and the presence (Red traces) of Y-27632. B: The bar chart showing the effect of Y-27632 on the half-time (t$_{1/2}$) of relaxation BK induced phasic contraction in Ca^{2+} free solution in the absence (Black bar) and the presence (Red bar) of Y-27632. The half-time (t$_{1/2}$) of relaxation of force demonstrates a significant reduction in Y-27632 group (n=15, P< 0.05) as compare to the control group.*
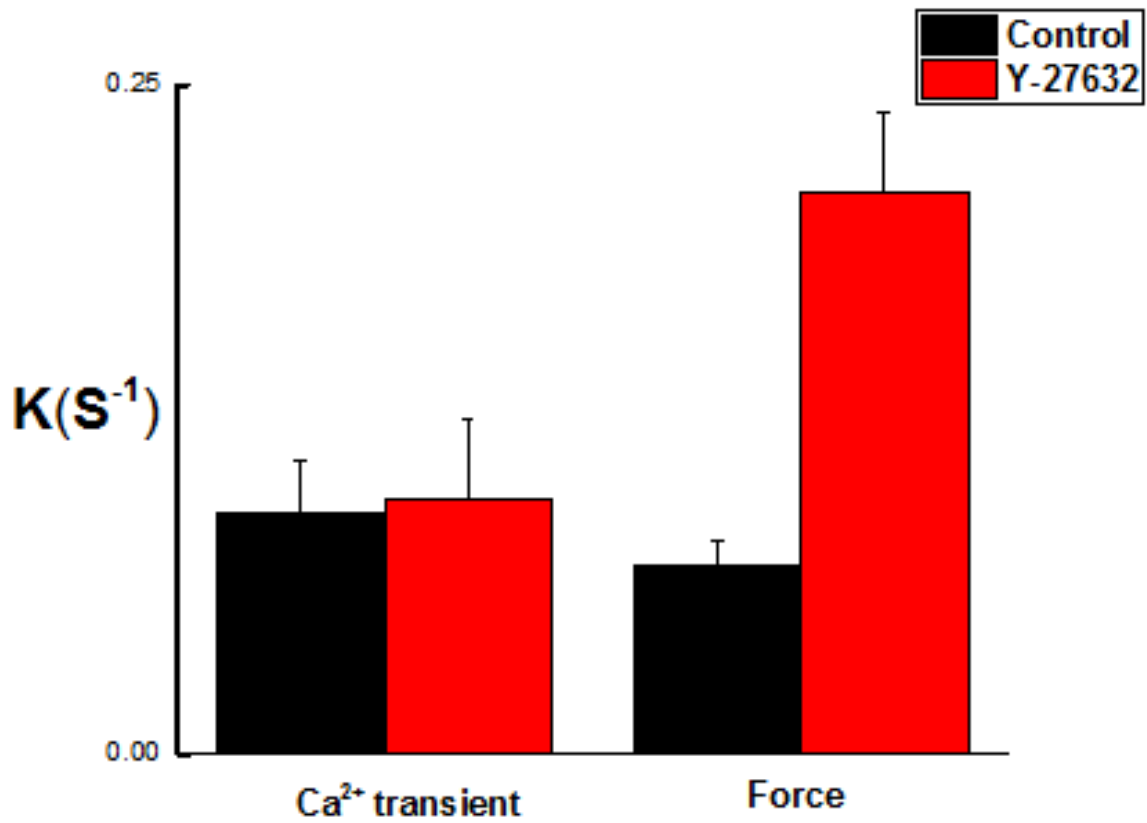
Figure 6.3.3-4. *Effects of Rho-kinase inhibition on the rate constants (k) measured as the reciprocal of $t_{1/2}$ of relaxation of Ca^{2+} transient and force induced by 40 seconds application of BK (5μM) in Ca^{2+} free solution. The rate constant of force shows a significant increase in Y-27632 group (n=15, P< 0.05) as compare to the control group, while that of the Ca^{2+} transient illustrates no statistical significance change (n=3, P> 0.05),*

6.4   Discussion

In previous studies (Shabir et al., 2004, Borysova et al., 2011), it was shown that Rho-kinase plays a key role in the control of the contraction in the rat ureteric smooth muscle, via both Ca^{2+}-dependent (modulation of Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels) and Ca^{2+}-independent mechanisms (modulation of MLCP activity). Use of the live confocal imaging, combined with force, in the present study, clearly showed that in the presence of Nifedipine or Ca^{2+}-free solution, Ca^{2+} signals induced by BK, under these conditions, were totally immune to Rho-kinase inhibitor (Y-27632). This was in marked contrast to its effect on force. The amplitude and the duration of the initial phasic contraction, both in the presence of Nifedipine or Ca^{2+}-free solution, were markedly reduced and the tonic contraction was almost completely blocked.

This data suggests that there is a substantial inhibition of the *in situ* MLCP activity by BK in the rat ureteric smooth muscle, via Rho-kinase activation, although the mechanism of its activation was not investigated. Inhibition of Rho-kinase has a dramatic effect on the balance of MLCK and MLCP, so that an increase in the activity of MLCP results in antagonizing the contractile response induced by BK.

The data obtained indicate that Rho-kinase inhibition leads to premature relaxation of the BK induced phasic contraction and shift of its peak to a higher level of intracellular Ca^{2+}, as well as a complete inhibition of the tonic contraction induced by BK in the presence of Nifedipine. Recently it was shown that in an obstructed rabbit ureter, the level of the expression of ROCK 1 and ROCK 2 have been substantially increased, and the contractile responses induced by various factors were effectively inhibited by Y-27632 (Turna et al., 2007). BK is an inflammatory factor and contribute to pain caused by ureteric obstruction. One could suggest that Rho-kinase inhibitors might be considered as potential pharmacological

tools for facilitation of kidney stone passage and prevention of renal colic pain and kidney damage.

Chapter 7

General discussion

Chapter 7

General discussion

7.1 Discussion

From the clinical perspective, pharmacological research on the ureteric contractility has been prompted by renal colic. There is a great interest in the pharmacologic modulation of ureteral contractility during stone passage, as the pain associated with acute ureteral colic has been described as one of the most intense experienced by humans.

Recent research has focused on the mechanisms involved in pain relief, how to facilitate stone passage, and to protect the kidney from injury induced by acute obstruction. Consequently, numerous anatomical, neurophysiological and hormonal studies have attempted to identify the behaviour of the ureter under normal and pathological conditions. Recently, it was shown that in the obstructed rabbit ureter, the level of the expression of ROCK 1 and ROCK 2 have been substantially increased and the contractile responses induced by various factors were effectively inhibited by the selective blocker of Rho-kinase (Y-27632) (Turna et al., 2007). Hence, Rho-kinase inhibitors might be considered as potential pharmacological tools for the facilitation of kidney stone passage and prevention of renal pain and kidney damage.

BK is an inflammatory agent and could be involved in causing ureteric contraction during stone passage. To date, little is known about the distribution of bradykinin receptors and it's relation to ureteral contractility. The present study has characterised the expression and the distribution of B_2 receptors, as well as the complex Ca^{2+} signalling and the contractile response induced by BK in the smooth muscle of the rat ureter.

The data obtained present evidence for the involvement of both Ca^{2+} dependent and Ca^{2+} - independent mechanisms.

7.1.1 Expression and distribution of B_2 receptors in the rat ureter

The results of the present investigation provide morphological and functional evidence, which indicate that the anatomic distribution of B_2 receptors, in the rat ureter, corresponded to regions of high sensitivity of the ureteric smooth muscle to BK, more specifically the proximal upper ureter. Ureteral segments taken from the proximal ureter showed a larger rise of the base line Ca^{2+} and force, as well the frequency of the propagating intercellular Ca^{2+} waves, associated with the phasic contractions, than the middle and the lower areas of the ureter and corresponded to increased levels of B_2 receptor expression.

The frequency of the spontaneous propagating intercellular Ca^{2+} waves, associated with the phasic contractions, superimposing the baseline Ca^{2+} and the tonic component of the contractile response induced by BK, were significantly higher in the proximal segments of the rat ureter and could appear as continuous burst of phasic contractions, mediated by propagating intercellular Ca^{2+} wave oscillations.

Alterations in the contractility patterns also occurred. The number of contractions within the "signature" for each segment was greater in the most proximal segments. Thus, a higher level of expression of B_2 receptors in the proximal ureter corresponded to areas of increased sensitivity of the rat ureteric smooth muscle to BK and higher frequencies of the phasic oscillations. This was in contrast to the contractile responses of the rat ureter to high-K and CCh, which were even along the length of the ureter.

The data obtained in this study demonstrate that BK effects on the ureteric smooth muscle are due to the activation of B_2 receptors, since they were blocked by the selective bradykinin B_2 receptor antagonist (HOE- 141) (Hock et al., 1991, Wirth et al., 1991).

7.1.2 Mechanisms of action of BK on ureteric contractility.

A. Ca^{2+}-dependent effects

The present study shows that BK causes a complex contractile response on the rat ureteric smooth muscle, which consists of an initial transient phasic component, associated with Ca^{2+} release from the SR, followed by sustained tonic component, associated with Nifedipine resistant Ca^{2+} entry, via store/ receptor-operated channels and spontaneous phasic contractions, associated with Nifedipine sensitive propagating intercellular Ca^{2+} waves.

(I) Ca^{2+} release from the store

Previous studies have shown that in the rat ureteric smooth muscle, Ca^{2+} release from the intracellular Ca^{2+} store is mediated mainly by IP_3 receptor channels (Burdyga et al., 1998, Boittin et al., 2000). The mechanism by which agonists mobilize intracellular Ca^{2+} in most types of smooth muscles, including the rat ureter (Burdyga et al., 1998) involves the activation of the phosphatidylinositol-specific phospholipase C, resulting in an increase in the cellular levels of inositol 1,4,5-trisphosphate and diacylglycerol. While inositol 1, 4, 5-trisphosphate releases intracellular Ca^{2+} from the sarcoplasmic reticulum, diacylglycerol directly activates protein kinase C (Farmer and Burch, 1992).

B_2 receptor is a member of G-protein coupled receptor that activates PLC (Hess et al., 1994). In several tissues, the action of BK on B_2 receptors involves the activation of PLC, leading to an increase in IP_3 concentration, and subsequently the intracellular Ca^{2+} (Den Hertog et al., 1988, Hess et al., 1994, Hall, 1992, Field et al., 1994). The IP_3 receptors cause Ca^{2+} release from the store (Patel et al., 1999).

In the present study, it was shown that the initial transient phasic contraction, exhibited by BK, was associated with Ca^{2+} signal, which appeared as asynchronous intracellular Ca^{2+} waves. These Ca^{2+} waves were resistant to Nifedipine and SKF 96365, as well as the removal of external Ca^{2+}.

CCh has been reported to cause a release of Ca^{2+} from the SR through IP_3 production in the rat ureteric smooth muscle (Burdyga et al., 1998, Boittin et al., 2000). Abolition of BK – induced contraction after CCh application in Ca^{2+} -free conditions indicates that both agonists release Ca^{2+} from the same intracellular Ca^{2+} store and cause it's complete Ca^{2+} depletion, which is coupled to Ca^{2+} entry via store operated Ca^{2+} channels.

(II) Ca^{2+} release/ Ca^{2+} entry coupling

It is well established that in some types of smooth muscles, Ca^{2+} depletion of the intracellular Ca^{2+} store can induce the entry of Ca^{2+} via store operated Ca^{2+} channels. This phenomenon is known as the 'capacitive Ca^{2+} entry', in which the influx of extracellular Ca^{2+}, through SOCCs is coupled to Ca^{2+} release from the store. This phenomenon was documented in the vascular (Casteels and Droogmans, 1981, Marthan, 2004) and the visceral (McCarron et al., 2002) smooth muscles, where IP_3-induced Ca^{2+} release from the store resulted in SOCE, which contributes to the tonic contraction (McCarron et al., 2002). IP_3R inhibition by 2-Aminoethoxydiphenyl borate (2-APB) completely diminished both, the phasic and the tonic components, indicating a link between IP_3-induced Ca^{2+} releases and SOCE.

Previous studies also demonstrated that SERCA inhibitors such as Cyclopiazonic acid (CPA) and Thapsigargin caused depolarisation-independent activation of SOCE (McFadzean and Gibson, 2002). This Ca^{2+} entry can refill the stores and directly activate SMs contraction. In addition, Ca^{2+} entry via SOCCs can depolarise smooth muscle and activate additional Ca^{2+} entry via voltage gated Ca^{2+} channels (Albert and Large, 2003).

In the present study, it was shown that there was an elevation of the baseline Ca^{2+} and force, superimposed by intercellular Ca^{2+} wave oscillations associated with phasic contractions induced by readmission of external Ca^{2+} to Ca^{2+}- free solution, following Ca^{2+} store depletion either by BK or CPA, which were blocked by the non-selective blocker of store/receptor operated channels (SKF 96365) (Miwa et al., 2000, Weirich et al., 2004).

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In addition, SKF 96365 significantly reduced Nifedipine resistant tonic component of BK-induced contractile response in the rat ureteric smooth muscle, along with the spontaneous superimposed phasic contraction, without affecting the initial phasic component associated with Ca^{2+} release from the store.

These physiological and pharmacological data indicate that the tonic component of BK induced ureteric contraction could be partly produced by Ca^{2+} entry via store operated channels. It is suggested that Ca^{2+} entry via store operated channels induced by BK, following SR depletion, leads to depolarization of the cell membrane to a threshold of the activation of the action potentials, associated with the propagating intercellular Ca^{2+} waves, accompanied by the phasic contractions. It is also known that agonists can activate Ca^{2+} entry via receptor operated channels in different types of smooth muscles, such as the smooth muscles of the guinea pig and the rabbit small intestine (McFadzean and Gibson, 2002), ear artery (Suzuki, 1985), and portal vein (Xiong et al., 1991), the rat vas deferens (Friel, 1988), gastric (Kim et al., 1998) and tracheal myocyte (McFadzean and Gibson, 2002), as well as the smooth muscle of the human's saphenous vein (Loirand and Pacaud, 1995). Also, it has become known that both ROCCs and SOCCs have very similar physical properties, potentially being members of the Transient Receptor Potential channel (TRPC) family (Yue et al., 2001, Warnat et al., 1999). It cannot be excluded, these channels could also contribute to BK-induced stimulant action on the rat ureteric smooth muscle, which should be investigated in the future experiments.

(III) Ca^{2+} entry via voltage gated Ca^{2+} channels.

In the current investigation, the potent inhibition of the BK-induced intercellular Ca^{2+} waves, associated with the phasic contractions by the selective blocker of the L-type VOCCs (Nifidipine) indicates the importance of Ca^{2+} entry via VOCCs in the control of the propagating phasic contractions, responsible for ureteric peristalsis.

Use of confocal imaging revealed that BK produce pacemaker regions, which initiate spontaneous intercellular Ca^{2+} waves, propagating in both directions, causing peristalsis and anti-peristalsis of the rat ureter.

Previously, it was shown that these intercellular Ca^{2+} waves are associated with the generation of the plateau type action potentials (Borysova et al., 2011). The action potential in the main ureter is normally triggered by the invading pacemaker potentials, initiated by the pacemaker cells, located in renal pelvis (Hashitani et al., 2017, Lang et al., 2010). However in the presence of the agonists, pacemaker regions in the main ureter could be activated, which could override the pacemaker activity.

In the ureteric smooth muscle, Ca^{2+} transient is produced mainly by the entry of Ca^{2+} into the cell from the extracellular space, L-type Ca^{2+} VOCCs. These channels are responsible for the generation of both components of AP. The AP, inward Ca^{2+}current, Ca^{2+} transient and the twitch contraction are all fully blocked by the selective L-type Ca^{2+} channel blocker (Nifedipine) (Lang, 1989, Floyd et al., 2008).

The data obtained on the ureteric smooth muscle are in good agreement with the data obtained on some other types of smooth muscles such as the guinea pig ileum, urinary bladder, as well as the rat uterus and vas deferens, where the stimulant action of BK was mediated mainly by activating of Ca^{2+} influx through L-Type VOCC (Calixto, 1995).

B. Ca^{2+}-independent effects

BK receptors are G-protein coupled receptors (AbdAlla et al., 1996). Activation of G-protein is one of the known mechanisms of increase Ca^{2+} sensitivity of the SMs (Nishimura et al., 1988, Kitazawa et al., 1989). Agonist activating GPCR ($G_{\alpha q}$) can stimulate RhoA (Somlyo and Somlyo, 2003a). Activated RhoA causes activation of ROCK by pathway known as the RhoA/ROCK pathway. Activated ROCK causes an increase in the force of the SMs via inhibition of MLCP (Kitazawa et al., 1991a, Kitazawa et al., 1991b).

In a previous studies (Shabir et al., 2004, Borysova et al., 2011), it was shown that Rho-kinase plays a key role in the control of the rat ureteric smooth muscles contraction , via both Ca^{2+}-dependent (modulation of Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels) and Ca^{2+}-independent mechanisms (modulation of MLCP activity).

Use of live confocal imaging combined with force in the present study, clearly showed that in the presence of Nifedipine or Ca^{2+} -free solution, Ca^{2+} signals induced by BK under these conditions were totally immune to Rho-kinase inhibitor (Y-27632). This was in marked contrast to its effect on force. The amplitude and the duration of the initial phasic contraction both in the presence of Nifedipine or Ca^{2+} -free solution were markedly reduced and the tonic contraction was almost completely blocked. The data suggest that there is a substantial inhibition of the *in situ* MLCP activity by BK in the rat ureteric smooth muscle, via Rho-kinase activation, although the mechanism of its activation was not investigated.

Inhibition of Rho-kinase has a dramatic effect on the balance of MLCK and MLCP, so that an increase in the activity of MLCP results in antagonizing contractile response induced by BK. The data obtained indicate that Rho-kinase inhibition leads to premature relaxation of the BK induced phasic contraction and shifts of its peak to a higher level of intracellular Ca^{2+}, as well as almost complete inhibition of the tonic contraction, induced by BK in the presence of Nifedipine.

The result obtained with BK agree with the data obtained by Borysova et al., (2011), which showed that in the presence of external Ca^{2+}, Y-27632 pre-treatment decreased the amplitude of the phasic component of the Ca^{2+} transient and force, abolished the tonic component of the CCh-induced contraction, without affecting steady-state membrane potential or $[Ca^{2+}]_i$. Inhibition of Rho-kinase by H-1152 or Y-27632 was shown to reduce MLC_{20} phosphorylation, the amplitude of force, but had no effects on Ca^{2+} transients induced by CCh in the presence of Nifedipine or Ca^{2+} -free solution (Borysova et al., 2011).

It is understood that ROCK-dependent MLCP phosphorylation, and thus deactivation, results in an increase in force (Kureishi et al., 1997). Therefore, ROCK inhibition by Y-27632 results in reduced MLCP phosphorylation and increased phosphatase activity. ROCK inhibition largely influences the ratio of MLCP and MLCK activity. Reduced levels of activated ROCK results in the dephosphorylation of MYPT1 at the specific sites (Borysova et al., 2011) causing higher level of MLCP activity. Resultantly, the myosin regulatory light chain undergoes faster dephosphorylation, which is responsible for the more rapid rate of relaxation of BK induced contraction in Ca^{2+} - free solution in the presence of Y-27632. Recently, it was shown that in an obstructed rabbit ureter, the level of the expression of ROCK1 and ROCK2 have been substantially increased, and the contractile responses induced by various factors were effectively inhibited by Y-27632 (Turna et al., 2007).

It is well established that BK links with the pathophysiological process of inflammation and damage resulting in pain sensation in different tissues. In keeping with this, Rho-kinase inhibitors could be a useful agent that might be used to allow relieving of the renal colic and prevent kidney injury.

7.2   Summary and conclusions

B_2 receptor activation by BK is coupled to Ca^{2+} release from the store, which appear as asynchronous Ca^{2+} waves, and is associated with the initial phasic contraction, followed by Nifedipine resistant and SKF 96365 sensitive Ca^{2+} entry, via SOCCs/ROCCs, associated with tonic contraction and Nifedipine sensitive L-type VGCCs, responsible for the generation of the propagating intercellular Ca^{2+} waves, associated with the burst of phasic contractions. In addition, BK activates Rho-kinase, which decreases activity of MLCP, causing Ca^{2+} sensitisation of force in the rat ureteric smooth muscle. Dephosphorylation of MLC_{20} by MLCP causes relaxation, a process inhibited by Rho-kinase activation. Figure 7.2 summarises the possible mechanisms of Ca^{2+} mobilization and Rho-kinase dependent Ca^{2+} sensitization induced by BK in the rat ureteric smooth muscles.
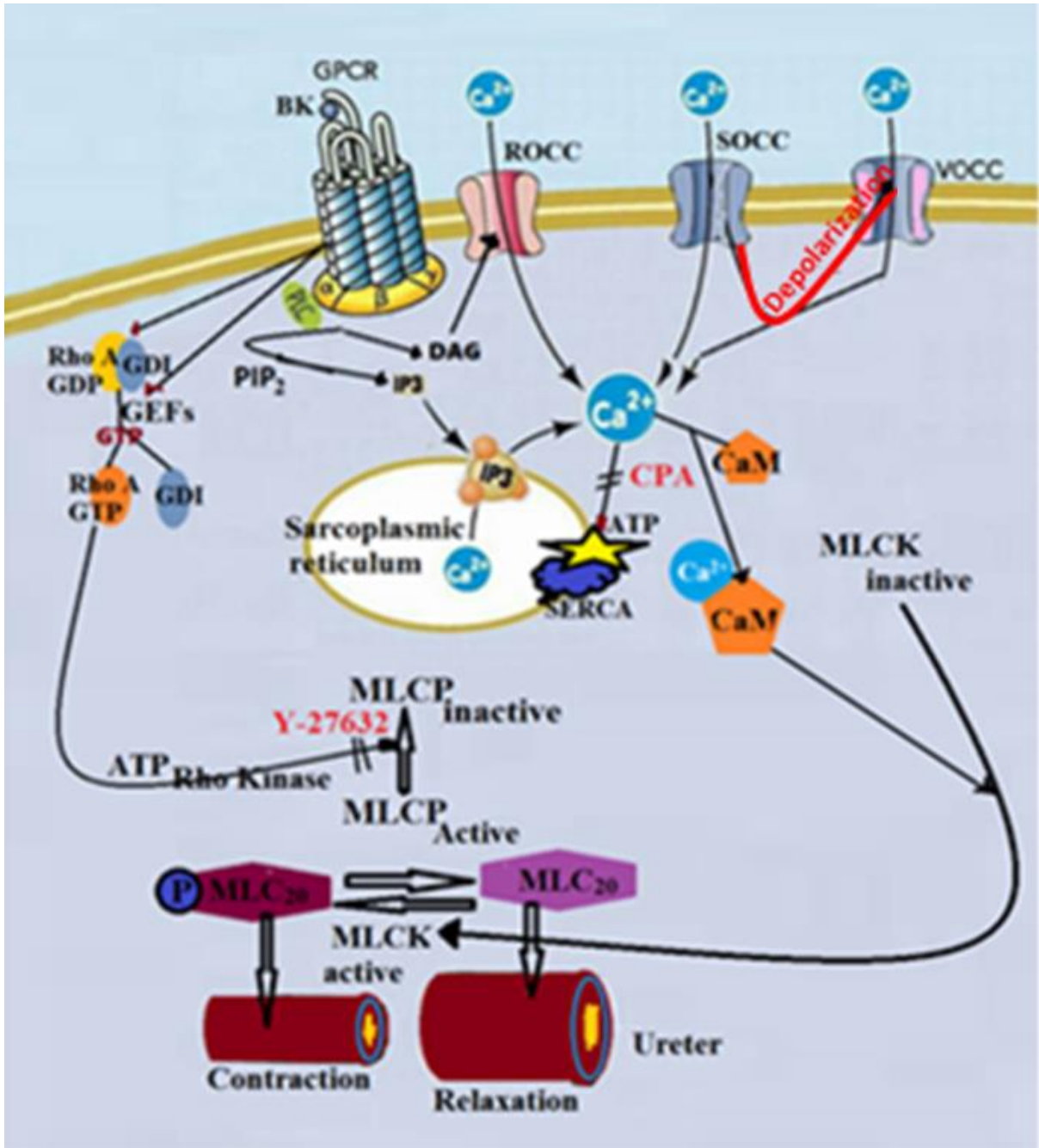
Figure 7.2 *A Schematic diagram showing the possible mechanisms of Ca^{2+} mobilization and Rho-kinase dependent calcium sensitization induced by BK in the rat ureteric smooth muscles.*

7.3 Future work

In order to get better insights into the mechanisms of action of BK on the ureteric smooth muscles, the effects of BK on MLC_{20} and MYPT1 phosphorylation in the absence and the presence of Y-27632 should be investigated. These data will offer a more direct examination related to Ca^{2+}-independent effects on Ca^{2+} sensitivity of the ureteric smooth muscles. Moreover, use of the electrophysiological method, combined with Ca^{2+} imaging would allow study the relationship between different components of BK induced Ca^{2+} signaling and the membrane potential. Also, when the selective SOCCs blockers become available, it might be a benefit to use them in the future work.

Chapter 8

References

Chapter 8

References

AARSEN, P. N. & VAN CASPEL-DE BRUYN, M. 1970. Effect of changes in ionic environment on the action of bradykinin on the guinea-pig taenia coli. *European Journal of Pharmacology,* 12**,** 348-358.

ABDALLA, S., GODOVAC-ZIMMERMANN, J., BRAUN, A., ROSCHER, A. A., MULLER-ESTERL, W. & QUITTERER, U. 1996. Structure of the bradykinin B2 receptors' amino terminus. *Biochemistry,* 35**,** 7514-9.

ABEL, P. D. 1994. Atlas of urosurgical anatomy. F. Hinman Jr. 310 × 235 mm. Pp. 553. Illustrated. 1993. Philadelphia, Pennsylvania: W. B. Saunders. £104. *British Journal of Surgery,* 81**,** 628.

ADRIENNE, E. M., EARL, R. S., SUNIL, B., RENA, O., CHINH, B., PATRICIA, Z., JILL, F., RICHARD, W. A. & KURT, J. 1991. Expression Cloning of a Rat B 2 Bradykinin Receptor. National Academy of Sciences of the United States of America.

AICKIN, C. C., BRADING, A. F. & BURDYGA, T. V. 1984. Evidence for sodium-calcium exchange in the guinea-pig ureter. *The Journal of Physiology,* 347**,** 411-430.

AKTORIES, K. & JUST, I. 1995. Monoglucosylation of low-molecular-mass GTP-binding Rho proteins by clostridial cytotoxins. *Trends Cell Biol,* 5**,** 441-3.

ALBERT, A. P. & LARGE, W. A. 2003. Store-operated Ca2+-permeable non-selective cation channels in smooth muscle cells. *Cell Calcium,* 33**,** 345-356.

ALLEN, B. G. & WALSH, M. P. 1994. The biochemical basis of the regulation of smooth-muscle contraction. *Trends in Biochemical Sciences,* 19**,** 362-368.

AMANO, M., CHIHARA, K., NAKAMURA, N., KANEKO, T., MATSUURA, Y. & KAIBUCHI, K. 1999. The COOH terminus of Rho-kinase negatively regulates rho-kinase activity. *J Biol Chem,* 274**,** 32418-24.

AMEDEE, T., BENHAM, C. D., BOLTON, T. B., BYRNE, N. G. & LARGE, W. A. 1990. Potassium, chloride and non-selective cation conductances opened by noradrenaline in rabbit ear artery cells. *J Physiol,* 423**,** 551-68.

AMEDEE, T. & LARGE, W. A. 1989. Microelectrode study on the ionic mechanisms which contribute to the noradrenaline-induced depolarization in isolated cells of the rabbit portal vein. *Br J Pharmacol,* 97**,** 1331-7.

ANDERSSON, K.-E. & ARNER, A. 2004. Urinary Bladder Contraction and Relaxation: Physiology and Pathophysiology. *Physiological Reviews,* 84**,** 935.

ANDERSSON, K. E. 1992. Clinical pharmacology of potassium channel openers. *Pharmacol Toxicol,* 70**,** 244-54.

ANDERSSON, K. E. & FORMAN, A. 1978. Effects of prostaglandins on the smooth muscle of the urinary tract. *Acta Pharmacol Toxicol (Copenh),* 43 Suppl 2**,** 90-5.

ANDRE, M., RAHIMI, S., SCHOLLMEYER, P. & PAVENSTADT, H. 1998. Bradykinin stimulates the intracellular calcium activity in human mesothelial cells. *Nephrol Dial Transplant,* 13**,** 2047-52.

ARKIN, I. T., ADAMS, P. D., BRUNGER, A. T., SMITH, S. O. & ENGELMAN, D. M. 1997. Structural perspectives of phospholamban, a helical transmembrane pentamer. *Annu Rev Biophys Biomol Struct,* 26**,** 157-79.

AULLO, P., GIRY, M., POPOFF, M. R., BOQUET, KOCKS, P. & OLSNES, S. 1993. A chimeric toxin to study the role of the 21 kDa GTP binding protein rho in the control of actin microfilament assembly. *EMBO Journal,* 12**,** 921-931.

BADER, M. 2011. *Kinins: History and outlook*, Walter de Gruyter GmbH and Co. KG.

BAO, L., RAPIN, A. M., HOLMSTRAND, E. C. & COX, D. H. 2002. Elimination of the BK(Ca) Channel's High-Affinity Ca(2+) Sensitivity. *The Journal of General Physiology,* 120**,** 173-189.

BARROW, S. E., DOLLERY, C. T., HEAVEY, D. J., HICKLING, N. E., RITTER, J. M. & VIAL, J. 1986. Effect of vasoactive peptides on prostacyclin synthesis in man. *Br J Pharmacol,* 87**,** 243-7.

BASCANDS, J. L., MARIN-CASTAÑO, M. E., SCHANSTRA, J. P., PRADDAUDE, F., PESQUERO, J. B., ADER, J. L. & GIROLAMI, J. P. 1998. Differential induction of functional B1-bradykinin receptors along the rat nephron in endotoxin induced inflammation. *Kidney International,* 54**,** 1888-1898.

BENEDITO, S., PRIETO, D., RIVERA, L., COSTA, G. & GARCIA-SACRISTAN, A. 1991. Mechanisms implicated in the histamine response of the sheep ureterovesical junction. *J Urol,* 146**,** 184-7.

BENHAM, C. D., BOLTON, T. B. & LANG, R. J. 1985. Acetylcholine activates an inward current in single mammalian smooth muscle cells. *Nature,* 316**,** 345-7.

BENHAM, C. D., BOLTON, T. B., LANG, R. J. & TAKEWAKI, T. 1986. Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *J Physiol,* 371**,** 45-67.

BERRIDGE, M. J. 1984. Inositol trisphosphate and diacylglycerol as second messengers. *Biochemical Journal,* 220**,** 345-360.

BERTACCINI, G., ZAPPIA, L., BEZZI, E. & POTENZONI, D. 1983. Histamine receptors in the human ureter. *Pharmacol Res Commun,* 15**,** 157-66.

BHOOLA, K. D., FIGUEROA, C. D. & WORTHY, K. 1992. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacological Reviews,* 44**,** 1-80.

BJORK, L. & NYLEN, O. 1972. Cineradiographic investigations of contraction in the normal upper urinary tract in man. *Acta Radiol Diagn (Stockh),* 12**,** 25-33.

BOITTIN, F.-X., COUSSIN, F., MOREL, J.-L., HALET, G., MACREZ, N. & MIRONNEAU, J. 2000. Ca^{2+} signals mediated by Ins(1,4,5)P3-gated channels in rat ureteric myocytes. *Biochemical Journal,* 349**,** 323.

BOLTON, T. B., PRESTWICH, S. A., ZHOLOS, A. V. & GORDIENKO, D. V. 1999. Excitation-contraction coupling in gastrointestinal and other smooth muscles. *Annu Rev Physiol,* 61**,** 85-115.

BORYSOVA, L., SHABIR, S., WALSH, M. P. & BURDYGA, T. 2011. The importance of Rho-associated kinase-induced Ca(2+) sensitization as a component of electromechanical and pharmacomechanical coupling in rat ureteric smooth muscle. *Cell calcium,* 50**,** 393-405.

BORYSOVA, L., WRAY, S., EISNER, D. A. & BURDYGA, T. 2013. How calcium signals in myocytes and pericytes are integrated across in situ microvascular networks and control microvascular tone(). *Cell Calcium,* 54**,** 163-174.

BRADLEY, A. B. & MORGAN, K. G. 1987. Alterations in cytoplasmic calcium sensitivity during porcine coronary artery contractions as detected by aequorin. *The Journal of Physiology,* 385**,** 437-448.

BURDYGA, T., SHMYGOL, A., EISNER, D. A. & WRAY, S. 2003. A new technique for simultaneous and in situ measurements of Ca^{2+} signals in arteriolar smooth muscle and endothelial cells. *Cell Calcium,* 34**,** 27-33.

BURDYGA, T. & WRAY, S. 1997. Simultaneous measurements of electrical activity, intracellular [Ca2+] and force in intact smooth muscle. *Pflugers Archiv European Journal of Physiology,* 435**,** 182-184.

BURDYGA, T. & WRAY, S. 2005. Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. *Nature,* 436**,** 559-62.

BURDYGA, T. V., TAGGART, M. J., CRICHTON, C., SMITH, G. L. & WRAY, S. 1998. The mechanism of Ca^{2+} release from the SR of permeabilised guinea-pig and rat ureteric smooth muscle. *Biochim Biophys Acta,* 1402**,** 109-14.

BURDYGA, T. V., TAGGART, M. J. & WRAY, S. 1995. Major difference between rat and guinea-pig ureter in the ability of agonists and caffeine to release Ca^{2+} and influence force. *J Physiol,* 489 (Pt 2)**,** 327-35.

BURDYGA, T. V. & WRAY, S. 1999a. The effect of cyclopiazonic acid on excitation-contraction coupling in guinea-pig ureteric smooth muscle: role of the sarcoplasmic reticulum. *The Journal Of Physiology,* 517 (Pt 3)**,** 855-865.

BURDYGA, T. V. & WRAY, S. 1999b. The relationship between the action potential, intracellular calcium and force in intact phasic, guinea-pig uretic smooth muscle. *The Journal of Physiology,* 520**,** 867-883.

BURDYGA, T. V. & WRAY, S. 2002. On the mechanisms whereby temperature affects excitation-contraction coupling in smooth muscle. *JOURNAL OF GENERAL PHYSIOLOGY,* 119**,** 93-104.

BURNSTOCK, G. 2006. Purinergic P2 receptors as targets for novel analgesics. *Pharmacol Ther,* 110**,** 433-54.

BURNSTOCK, G. & PROSSER, C. L. 1960. Responses of smooth muscles to quick stretch: relation of stretch to conduction. *The American journal of physiology,* 198**,** 921-925.

BURNSTOCK, G. & WILLIAMS, M. 2000. P2 purinergic receptors: modulation of cell function and therapeutic potential. *J Pharmacol Exp Ther,* 295**,** 862-9.

BUSTAMANTE, S., ORENSANZ, L. M., BARAHONA, M. V., GARCIA-SACRISTAN, A. & HERNANDEZ, M. 2001. NK2 tachykinin receptors mediate contraction of the pig intravesical ureter: tachykinin-induced enhancement of non-adrenergic non-cholinergic excitatory neurotransmission. *Neurourol Urodyn,* 20**,** 297-308.

BYRNE, N. G. & LARGE, W. A. 1988. Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein. *J Physiol,* 404**,** 557-73.

CALIXTO, J. B. 1995. Multiple mechanisms of bradykinin-induced contraction in rat and guinea pig smooth muscles in vitro. *European Journal of Pharmacology,* 281**,** 279-288.

CANDA, A. E., TURNA, B., CINAR, G. M. & NAZLI, O. 2007. Physiology and pharmacology of the human ureter: basis for current and future treatments. *Urol Int,* 78**,** 289-98.

CASTEELS, R. & DROOGMANS, G. 1981. Exchange characteristics of the noradrenaline-sensitive calcium store in vascular smooth muscle cells or rabbit ear artery. *The Journal Of Physiology,* 317**,** 263-279.

CATACUTAN-LABAY, P. & BOYARSKY, S. 1966a. Bradykinin: Effect on Ureteral Peristalsis. *Science,* 151**,** 78.

CATACUTAN-LABAY, P. & BOYARSKY, S. 1966b. Bradykinin: effect on ureteral peristalsis. *Science,* 151**,** 78-9.

CATTERALL, W. A. 2011. Voltage-gated calcium channels. *Cold Spring Harbor Perspectives In Biology,* 3**,** a003947-a003947.

CHOI, S.-Y., KIM, Y.-H., LEE, Y.-K. & KIM, K.-T. 2001. Chlorpromazine inhibits store-operated calcium entry and subsequent noradrenaline secretion in PC12 cells. *British Journal of Pharmacology,* 132**,** 411-418.

CHOPRA, B., BARRICK, S. R., MEYERS, S., BECKEL, J. M., ZEIDEL, M. L., FORD, A. P. D. W., DE GROAT, W. C. & BIRDER, L. A. 2005. Expression and function of bradykinin B1 and B2 receptors in normal and inflamed rat urinary bladder urothelium. *The Journal Of Physiology,* 562**,** 859-871.

COLEMAN, H. A. & PARKINGTON, H. C. 1988. Induction of prolonged excitability in myometrium of pregnant guinea-pigs by prostaglandin F2 alpha. *J Physiol,* 399**,** 33-47.

CONSTANTINOU, C. E., GRANATO JR, J. J. & GOVAN, D. E. 1974. Dynamics of the upper urinary tract. *Urologia internationalis,* 29**,** 249-264.

CONSTANTINOU, C. E. & YAMAGUCHI, O. 1981. Multiple-coupled pacemaker system in renal pelvis of the unicalyceal kidney. *Am J Physiol,* 241**,** R412-8.

COOPER, D. M. F. 1983. Receptor-Mediated Stimulation and Inhibition of Adenylate Cyclase. *In:* ARNOST KLEINZELLER AND, B. R. M. (ed.) *Current Topics in Membranes and Transport.* Academic Press.

COXHEAD, P. D. 2000. Intracellular calcium oscillations.

CRAIG, R. & WOODHEAD, J. L. 2006. Structure and function of myosin filaments. *Current Opinion in Structural Biology,* 16**,** 204-212.

CRAVEN, M., SERGEANT, G. P., HOLLYWOOD, M. A., MCHALE, N. G. & THORNBURY, K. D. 2004. Modulation of spontaneous Ca(2+)-activated Cl(−) currents in the rabbit corpus cavernosum by the nitric oxide–cGMP pathway. *The Journal of Physiology,* 556**,** 495-506.

CRIDDLE, D. N., MEIRELES, A. V. P., MACÊDO, L. B., LEAL-CARDOSO, J. H., SCARPARO, H. C. & JAFFAR, M. 2002. Comparative inhibitory effects of niflumic acid and novel synthetic derivatives on the rat isolated stomach fundus. *Journal of Pharmacy and Pharmacology,* 54**,** 283-288.

DEN HERTOG, A., NELEMANS, A. & VAN DEN AKKER, J. 1988. The multiple action of bradykinin on smooth muscle of guinea-pig taenia caeci. *Eur J Pharmacol,* 151**,** 357-63.

DILLON, S. T., RUBIN, E. J., YAKUBOVICH, M., POTHOULAKIS, C., LAMONT, J. T., FEIG, L. A. & GILBERT, R. J. 1995. Involvement of Ras-related Rho proteins in the mechanisms of action of Clostridium difficile toxin A and toxin B. *Infect Immun,* 63**,** 1421-6.

DIXON, J. S. & GOSLING, J. A. 1990. Ultrastructure of smooth muscle cells in the urinary system. *In:* MOTTA, P. M. (ed.) *Ultrastructure of Smooth Muscle.* Boston, MA: Springer US.

DJURHUUS, J. C. 1977. Dynamics of upper urinary tract. III. The activity of renal pelvis during pressure variations. *Investigative urology,* 14**,** 475-477.

DOBRYDNEVA, Y., WILLIAMS, R. L. & BLACKMORE, P. F. 1999. trans-Resveratrol inhibits calcium influx in thrombin-stimulated human platelets. *Br J Pharmacol,* 128**,** 149-57.

DODEL, R. C., HAFNER, D. & BORCHARD, U. 1996. Characterization of histamine receptors in the ureter of the dog. *Eur J Pharmacol,* 318**,** 395-402.

DOMINGUEZ, R. & HOLMES, K. C. 2011. Actin Structure and Function. *Annual review of biophysics,* 40**,** 169-186.

DRAKE, R. L., VOGL, W. & MITCHELL, A. W. M. 2015. *Gray's Anatomy for students. [electronic book],* Philadelphia, PA : Churchill Livingstone/Elsevier, 2015.

3rd ed.

DRAY, A. & PERKINS, M. 1993. Bradykinin and inflammatory pain. *Trends in Neurosciences,* 16**,** 99-104.

DUCHENE, J., LECOMTE, F., AHMED, S., CAYLA, C., PESQUERO, J., BADER, M., PERRETTI, M. & AHLUWALIA, A. 2007. A novel inflammatory pathway involved in leukocyte recruitment: role for the kinin B1 receptor and the chemokine CXCL5. *J Immunol,* 179**,** 4849-56.

EGGERICKX, D., RASPE, E., BERTRAND, D., VASSART, G. & PARMENTIER, M. 1992. Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B2 receptor gene. *Biochemical and Biophysical Research Communications,* 187**,** 1306-1313.

ELLIOTT, D. F. 1970. The Discovery and Characterization of Bradykinin. *In:* ERDÖS, E. G. & WILDE, A. F. (eds.) *Bradykinin, Kallidin and Kallikrein.* Berlin, Heidelberg: Springer Berlin Heidelberg.

ETO, M., OHMORI, T., SUZUKI, M., FURUYA, K. & MORITA, F. 1995. A Novel Protein Phosphatase-1 Inhibitory Protein Potentiated by Protein Kinase C. Isolation from Porcine Aorta Media and Characterization. *The Journal of Biochemistry,* 118**,** 1104-1107.

ETO, M., SENBA, S., MORITA, F. & YAZAWA, M. 1997. Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: Its specific localization in smooth muscle. *FEBS Letters,* 410**,** 356-360.

EXINTARIS, B. & LANG, R. J. 1999. K+ channel blocker modulation of the refractory period in spontaneously active guinea-pig ureters. *Urological Research,* 27**,** 319-327.

FARMER, S. G. & BURCH, R. M. 1992. Biochemical and Molecular Pharmacology of Kinin Receptors. *Annual Review of Pharmacology & Toxicology,* 32**,** 511.

FEENEY, M. M. & ROSENBLUM, N. D. 2014. Urinary tract pacemaker cells: current knowledge and insights from nonrenal pacemaker cells provide a basis for future discovery. *Pediatr Nephrol,* 29**,** 629-35.

FERREIRA, S. H., NG, K. K. & VANE, J. R. 1973. The continuous bioassay of the release and disappearance of histamine in the circulation. *British Journal of Pharmacology,* 49**,** 543-553.

FIELD, J. L., BUTT, S. K., MORTON, I. K. & HALL, J. M. 1994. Bradykinin B2 receptors and coupling mechanisms in the smooth muscle of the guinea-pig taenia caeci. *British Journal of Pharmacology,* 113**,** 607-613.

FIGINI, M., RICCIARDOLO, F. L., JAVDAN, P., NIJKAMP, F. P., EMANUELI, C., PRADELLES, P., FOLKERTS, G. & GEPPETTI, P. 1996. Evidence that epithelium-derived relaxing factor released by bradykinin in the guinea pig trachea is nitric oxide. *Am J Respir Crit Care Med,* 153**,** 918-23.

FILENKO, A. M., DANILOVA, V. M. & SOBIESZEK, A. 1997. Smooth muscle myosin light chain kinase, supramolecular organization, modulation of activity, and related conformational changes. *Biophysical Journal,* 73**,** 1593-1606.

FINBERG, J. P. & PEART, W. S. 1970. Function of smooth muscle of the rat renal pelvis--response of the isolated pelvis muscle to angiotensin and some other substances. *Br J Pharmacol,* 39**,** 373-81.

FIXMAN, E. D., TOLLOCZKO, B. & LAUZON, A.-M. 2005. Airway smooth muscle: The contractile phenotype. *In:* HAMID, Q. (ed.) *Physiological Basis of Respiratory Disease.* USA: BC Decker.

FLOYD, R. V., BORISOVA, L., BAKRAN, A., HART, C. A., WRAY, S. & BURDYGA, T. V. 2008. Morphology, calcium signaling and mechanical activity in human ureter. *J Urol,* 180**,** 398-405.

FRIEL, D. D. 1988. An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens. *J Physiol,* 401**,** 361-80.

FROSSARD, N., STRETTON, C. D. & BARNES, P. J. 1990. Modulation of bradykinin responses in airway smooth muscle by epithelial enzymes. *Agents Actions,* 31**,** 204-9.

FU, X., GONG, M. C., JIA, T., SOMLYO, A. V. & SOMLYO, A. P. 1998. The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, GTPgammaS-, and phorbol ester-induced Ca2+-sensitization of smooth muscle. *FEBS Lett,* 440**,** 183-7.

FUKATA, Y., MUTSUKI, A. & K, K. 2001. Rho-Rho Kinase pathway in smooth muscle contraction and cytoskeletal reorganisation of non-muscle cells. *Trends Pharmacol Sci***,** 22:32-9.

GEISZT, M., KAPUS, A., NEMET, K., FARKAS, L. & LIGETI, E. 1997. Regulation of capacitative Ca2+ influx in human neutrophil granulocytes. Alterations in chronic granulomatous disease. *J Biol Chem,* 272**,** 26471-8.

GESTRELIUS, S. & BORGSTRÖM, P. 1986. A dynamic model of smooth muscle contraction. *Biophysical Journal,* 50**,** 157-169.

GOLIAS, C., CHARALABOPOULOS, A., STAGIKAS, D., CHARALABOPOULOS, K. & BATISTATOU, A. 2007. The kinin system - bradykinin: biological effects and clinical implications. Multiple role of the kinin system - bradykinin. *Hippokratia,* 11**,** 124-128.

GOSLING, J. A. & DIXON, J. S. 1974. Species variation in the location of upper urinary tract pacemaker cells. *Invest Urol,* 11**,** 418-23.

HAEBERLE, J. R. 1999. Thin-filament linked regulation of smooth muscle myosin. *J Muscle Res Cell Motil,* 20**,** 363-70.

HAGIWARA, Y., KOJIMA, M., KURAISHI, T., HAYASHI, I., OH-ISHI, S. & MIYATA, T. 1995. Identification of rat urinary kinin as bradykinin. *Life Sciences,* 57**,** 997-1002.

HALL, J. M. 1992. Bradykinin receptors: pharmacological properties and biological roles. *Pharmacol Ther,* 56**,** 131-90.

HALL, J. M. 1997a. Bradykinin receptors. *Gen Pharmacol,* 28**,** 1-6.

HALL, J. M. 1997b. Bradykinin receptors. *General Pharmacology: The Vascular System,* 28**,** 1-6.

HANNAPPEL, J. 1981. Effects of Adrenergic Activators and Inhibitors on the Urinary Tract. *In:* SZEKERES, L. (ed.) *Adrenergic Activators and Inhibitors.* Springer Berlin Heidelberg.

HARTSHORNE, D. J. 1998. Myosin phosphatase: subunits and interactions. *Acta Physiol Scand,* 164**,** 483-93.

HARTSHORNE, D. J., ITO, M. & ERDODI, F. 1998. Myosin light chain phosphatase: subunit composition, interactions and regulation. *J Muscle Res Cell Motil,* 19**,** 325-41.

HASHITANI, H., NODA, H., MITSUI, R., NGUYEN, M. J., LANG, R. J., HIGASHI, R., OHTA, K. & NAKAMURA, K. I. 2017. Interstitial cell modulation of pyeloureteric peristalsis in the mouse

renal pelvis examined using FIBSEM tomography and calcium indicators. *Pflugers Archiv European Journal of Physiology*, 1-17.

HAUSER, D. S., MEVISSEN, M., WEISS, R., PORTIER, C. J., SCHOLTYSIK, G., STUDER, U. E. & DANUSER, H. 2002. Effects of ketanserin and DOI on spontaneous and 5-HT-evoked peristalsis of the pig ureter in vivo. *Br J Pharmacol,* 135**,** 1026-32.

HERNANDEZ, M., BARAHONA, M. V., RECIO, P., RIVERA, L., BENEDITO, S., MARTINEZ, A. C., GARCIA-SACRISTAN, A., ORENSANZ, L. M. & PRIETO, D. 2004. Heterogeneity of neuronal and smooth muscle receptors involved in the VIP- and PACAP-induced relaxations of the pig intravesical ureter. *Br J Pharmacol,* 141**,** 123-31.

HERNANDEZ, M., BARAHONA, M. V., SIMONSEN, U., RECIO, P., RIVERA, L., MARTINEZ, A. C., GARCIA-SACRISTAN, A., ORENSANZ, L. M. & PRIETO, D. 2003. Characterization of the 5-hydroxytryptamine receptors mediating contraction in the pig isolated intravesical ureter. *Br J Pharmacol,* 138**,** 137-44.

HESS, J. F., BORKOWSKI, J. A., STONESIFER, G. Y., MACNEIL, T., STRADER, C. D. & RANSOM, R. W. 1994. Cloning and pharmacological characterization of bradykinin receptors. *Braz J Med Biol Res,* 27**,** 1725-31.

HICKS, R. M. 1965. THE FINE STRUCTURE OF THE TRANSITIONAL EPITHELIUM OF RAT URETER. *The Journal of Cell Biology,* 26**,** 25-48.

HIGASHIHARA, M. & IKEBE, M. 1988. Increase in the actin-activated Mg2+-ATPase activity of smooth muscle myosin by increasing myosin concentration. *Biochemical and Biophysical Research Communications,* 154**,** 928-933.

HILL-EUBANKS, D. C., WERNER, M. E., HEPPNER, T. J. & NELSON, M. T. 2011. Calcium Signaling in Smooth Muscle. *Cold Spring Harbor Perspectives in Biology,* 3**,** a004549.

HIMPENS, B. & CASTEELS, R. 1990. Different effects of depolarization and muscarinic stimulation on the Ca2+/force relationship during the contraction-relaxation cycle in the guinea pig ileum. *Pflügers Archiv,* 416**,** 28-35.

HIMPENS, B., MATTHIJS, G., SOMLYO, A. V., BUTLER, T. M. & SOMLYO, A. P. 1988. Cytoplasmic free calcium, myosin light chain phosphorylation, and force in phasic and tonic smooth muscle. *J Gen Physiol,* 92**,** 713-29.

HIRANO, K., PHAN, B. C. & HARTSHORNE, D. J. 1997. Interactions of the subunits of smooth muscle myosin phosphatase. *J Biol Chem,* 272**,** 3683-8.

HOCK, F. J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H. J., WIEMER, G., HENKE, S., BREIPOHL, G., KONIG, W., KNOLLE, J. & ET AL. 1991. Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro studies. *Br J Pharmacol,* 102**,** 769-73.

HOFMANN, F., BUSCH, C., PREPENS, U., JUST, I. & AKTORIES, K. 1997. Localization of the glucosyltransferase activity of Clostridium difficile toxin B to the N-terminal part of the holotoxin. *J Biol Chem,* 272**,** 11074-8.

HONG, F., HALDEMAN, B. D., JACKSON, D., CARTER, M., BAKER, J. E. & CREMO, C. R. 2011. Biochemistry of smooth muscle myosin light chain kinase. *Archives of Biochemistry and Biophysics,* 510**,** 135-146.

HONG, S. K., KWAK, C., CHANG JEONG, B., KIM, B. S. & KIM, H. H. 2005. Involvement of Rho-kinase in the contractile mechanism of human ureteral smooth muscle. *Neurourol Urodyn,* 24**,** 136-41.

HORMAN, S., MOREL, N., VERTOMMEN, D., HUSSAIN, N., NEUMANN, D., BEAULOYE, C., NAJJAR, N. E., FORCET, C., VIOLLET, B., WALSH, M. P., HUE, L. & RIDER, M. H. 2008. AMP-activated Protein Kinase Phosphorylates and Desensitizes Smooth Muscle Myosin Light Chain Kinase. *Journal of Biological Chemistry,* 283**,** 18505-18512.

HOROWITZ, A., MENICE, C. B., LAPORTE, R. & MORGAN, K. G. 1996. Mechanisms of smooth muscle contraction. *Physiol Rev,* 76**,** 967-1003.

HOTH, M. & PENNER, R. 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature,* 355**,** 353-6.

HYMEL, L., INUI, M., FLEISCHER, S. & SCHINDLER, H. 1988. Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum forms Ca2+-activated oligomeric Ca2+ channels in planar bilayers. *Proc Natl Acad Sci U S A,* 85**,** 441-5.

IGNARRO, L. J., BYRNS, R. E., BUGA, G. M. & WOOD, K. S. 1987. Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am J Physiol,* 253**,** H1074-82.

IIZUKA, K., YOSHII, A., SAMIZO, K., TSUKAGOSHI, H., ISHIZUKA, T., DOBASHI, K., NAKAZAWA, T. & MORI, M. 1999. A major role for the Rho-associated coiled coil forming protein kinase in G-protein-mediated Ca2+ sensitization through inhibition of myosin phosphatase in rabbit trachea. *British Journal of Pharmacology,* 128**,** 925-933.

IKEBE, M., HARTSHORNE, D. J. & ELZINGA, M. 1986. Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J Biol Chem,* 261**,** 36-9.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. 1989. Ionic currents in single smooth muscle cells from the ureter of the guinea-pig. *Journal of Physiology,* 411**,** 131-159.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. 1990. Characteristics of transient outward currents in single smooth muscle cells from the ureter of the guinea-pig. *Journal of Physiology,* 427**,** 301-324.

INOUE, R. & ISENBERG, G. 1990. Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. *J Physiol,* 424**,** 73-92.

ISELIN, C. E., ALM, P., SCHAAD, N. C., LARSSON, B., GRABER, P. & ANDERSSON, K. E. 1997. Localization of nitric oxide synthase and haemoxygenase, and functional effects of nitric oxide and carbon monoxide in the pig and human intravesical ureter. *Neurourol Urodyn,* 16**,** 209-27.

ISELIN, C. E., NY, L., LARSSON, B., SCHAAD, N. C., ALM, P., GRABER, P., MOREL, D. R. & ANDERSSON, K. E. 1998. The nitric oxide synthase/nitric oxide and heme oxygenase/carbon monoxide pathways in the human ureter. *Eur Urol,* 33**,** 214-21.

ISHIZAKI, T., UEHATA, M., TAMECHIKA, I., KEEL, J., NONOMURA, K., MAEKAWA, M. & NARUMIYA, S. 2000. Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol,* 57**,** 976-83.

ISLAM, M. S. M. D. 2012. *Calcium signaling. [electronic book],* Dordrecht ; Springer, c2012.

ITO, M., NAKANO, T., ERDODI, F. & HARTSHORNE, D. J. 2004. Myosin phosphatase: structure, regulation and function. *Mol Cell Biochem,* 259**,** 197-209.

JERDE, T. J., MELLON, W. S., FISCHER, S. M., LIEBERT, M., BJORLING, D. E. & NAKADA, S. Y. 2004. Suppression of 15-Hydroxyprostaglandin Dehydrogenase Messenger RNA Concentration, Protein Expression, and Enzymatic Activity during Human Ureteral Obstruction. *Journal of Pharmacology and Experimental Therapeutics,* 309**,** 398.

JUDE, J. A., WYLAM, M. E., WALSETH, T. F. & KANNAN, M. S. 2008. Calcium Signaling in Airway Smooth Muscle. *Proceedings of the American Thoracic Society,* 5**,** 15-22.

KAMM, K. E. & STULL, J. T. 1985. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol,* 25**,** 593-620.

KAPLAN, A. P., JOSEPH, K., SHIBAYAMA, Y., NAKAZAWA, Y., GHEBREHIWET, B., REDDIGARI, S. & SILVERBERG, M. 1998. Bradykinin formation - Plasma and tissue pathways and cellular interactions. *CLINICAL REVIEWS IN ALLERGY & IMMUNOLOGY,* 16**,** 403-429.

KAPLAN, A. P., JOSEPH, K., SHIBAYAMA, Y., REDDIGARI, S., GHEBREHIWET, B. & SILVERBERG, M. 1997. The intrinsic coagulation/kinin-forming cascade: assembly in plasma and cell surfaces in inflammation. *Adv Immunol,* 66**,** 225-72.

KAPLAN, A. P., JOSEPH, K. & SILVERBERG, M. 2002. Pathways for bradykinin formation and inflammatory disease. *JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY,* 109**,** 195-209.

KAPLON, D. M. & NAKADA, S. Y. 2011. Ureteral Physiology and Pharmacology. *In:* CHAPPLE, R. C. & STEERS, D. W. (eds.) *Practical Urology: Essential Principles and Practice: Essential Principles and Practice.* London: Springer London.

KHAKH, B. S., BURNSTOCK, G., KENNEDY, C., KING, B. F., NORTH, R. A., SEGUELA, P., VOIGT, M. & HUMPHREY, P. P. 2001. International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol Rev,* 53**,** 107-18.

KIERNAN, J. A. 1999. Strategies for preventing detachment of sections from glass slides. Microscopy Today.

KIM, S. J., AHN, S. C., SO, I. & KIM, K. W. 1995. Role of calmodulin in the activation of carbachol-activated cationic current in guinea-pig gastric antral myocytes. *Pflugers Arch,* 430**,** 757-62.

KIM, S. J., KOH, E.-M., KANG, T. M., KIM, Y. C., SO, I., ISENBERG, G. & KIM, K. W. 1998. Ca2+ influx through carbachol-activated non-selective cation channels in guinea-pig gastric myocytes. *The Journal of Physiology,* 513**,** 749-760.

KISHI, H., BAO, J. & KOHAMA, K. 2000. Inhibitory effects of ML-9, wortmannin, and Y-27632 on the chemotaxis of vascular smooth muscle cells in response to platelet-derived growth factor-BB. *J Biochem,* 128**,** 719-22.

KITAZAWA, T., ETO, M., WOODSOME, T. P. & BRAUTIGAN, D. L. 2000. Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J Biol Chem,* 275**,** 9897-900.

KITAZAWA, T., ETO, M., WOODSOME, T. P. & KHALEQUZZAMAN, M. 2003. Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca2+ sensitization in rabbit smooth muscle. *JOURNAL OF PHYSIOLOGY-LONDON,* 546**,** 879-889.

KITAZAWA, T., GAYLINN, B. D., DENNEY, G. H. & SOMLYO, A. P. 1991a. G-protein-mediated Ca2+ sensitization of smooth muscle contraction through myosin light chain phosphorylation. *Journal of Biological Chemistry,* 266**,** 1708-1715.

KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A. V. & SOMLYO, A. P. 1989. Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca2+. *J Biol Chem,* 264**,** 5339-42.

KITAZAWA, T., MASUO, M. & SOMLYO, A. P. 1991b. G Protein-Mediated Inhibition of Myosin Light-Chain Phosphatase in Vascular Smooth Muscle. National Academy of Sciences of the United States of America.

KLEMM, M. F., EXINTARIS, B. & LANG, R. J. 1999. Identification of the cells underlying pacemaker activity in the guinea-pig upper urinary tract. *J Physiol,* 519 Pt 3**,** 867-84.

KOBAYASHI, M. 1965. Effects of Na and Ca on the generation and conduction of excitation in the ureter. *American Journal of Physiology--Legacy Content,* 208**,** 715-719.

KOBAYASHI, S., KITAZAWA, T., SOMLYO, A. V. & SOMLYO, A. P. 1989. Cytosolic heparin inhibits muscarinic and alpha-adrenergic Ca2+ release in smooth muscle. Physiological role of inositol 1,4,5-trisphosphate in pharmacomechanical coupling. *J Biol Chem,* 264**,** 17997-8004.

KONDO, A. 1970. The contraction of the ureter. I. Observations in normal human and dog ureters. *Nagoya Journal Of Medical Science,* 32**,** 387-394.

KOTLIKOFF, M. I. & WANG, Y. X. 1998. Calcium release and calcium-activated chloride channels in airway smooth muscle cells. *Am J Respir Crit Care Med,* 158**,** S109-14.

KUREISHI, Y., KOBAYASHI, S., AMANO, M., KIMURA, K., KANAIDE, H., NAKANO, T., KAIBUCHI, K. & ITO, M. 1997. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem,* 272**,** 12257-60.

KURIYAMA, H., KITAMURA, K. & NABATA, H. 1995. Pharmacological and physiological significance of ion channels and factors that modulate them in vascular tissues. *Pharmacological Reviews,* 47**,** 387.

LANG, R. J. 1989. Identification of the major membrane currents in freshly dispersed single smooth muscle cells of guinea-pig ureter. *Journal of Physiology,* 412**,** 375-395.

LANG, R. J. 1990. The whole-cell Ca2+ channel current in single smooth muscle cells of the guinea-pig ureter. *The Journal of Physiology,* 423**,** 453-473.

LANG, R. J., DAVIDSON, M. E. & EXINTARIS, B. 2002. Pyeloureteral motility and ureteral peristalsis: essential role of sensory nerves and endogenous prostaglandins. *Exp Physiol,* 87**,** 129-46.

LANG, R. J., EXINTARIS, B., TEELE, M. E., HARVEY, J. & KLEMM, M. F. 1998a. Electrical basis of peristalsis in the mammalian upper urinary tract. *Clinical And Experimental Pharmacology & Physiology,* 25**,** 310-321.

LANG, R. J., EXINTARIS, B., TEELE, M. E., HARVEY, J. & KLEMM, M. F. 1998b. Electrical basis of peristalsis in the mammalian upper urinary tract. *Clin Exp Pharmacol Physiol,* 25**,** 310-21.

LANG, R. J., HASHITANI, H., TONTA, M. A., BOURKE, J. L., PARKINGTON, H. C. & SUZUKI, H. 2010. Spontaneous electrical and Ca2+ signals in the mouse renal pelvis that drive pyeloureteric peristalsis. *Clinical & Experimental Pharmacology & Physiology,* 37**,** 509-515.

LANG, R. J., TONTA, M. A., ZOLTKOWSKI, B. Z., MEEKER, W. F., WENDT, I. & PARKINGTON, H. C. 2006. Pyeloureteric peristalsis: role of atypical smooth muscle cells and interstitial cells of Cajal-like cells as pacemakers. *The Journal of Physiology,* 576**,** 695-705.

LEE, H. K., BAYGUINOV, O. & SANDERS, K. M. 1993. Role of nonselective cation current in muscarinic responses of canine colonic muscle. *Am J Physiol,* 265**,** C1463-71.

LENNON, G. M., BOURKE, J., RYAN, P. C. & FITZPATRICK, J. M. 1993. Pharmacological options for the treatment of acute ureteric colic. An in vitro experimental study. *Br J Urol,* 71**,** 401-7.

LEVENT, A. & BUYUKAFSAR, K. 2004. Expression of Rho-kinase (ROCK-1 and ROCK-2) and its substantial role in the contractile activity of the sheep ureter. *Br J Pharmacol,* 143**,** 431-7.

LIOU, J., KIM, M. L., HEO, W. D., JONES, J. T., MYERS, J. W., FERRELL, J. E., JR. & MEYER, T. 2005. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol,* 15**,** 1235-41.

LIU, Y. J. & GYLFE, E. 1997. Store-operated Ca2+ entry in insulin-releasing pancreatic beta-cells. *Cell Calcium,* 22**,** 277-86.

LOIRAND, G. & PACAUD, P. 1995. Mechanism of the ATP-induced rise in cytosolic Ca2+ in freshly isolated smooth muscle cells from human saphenous vein. *Pflugers Arch,* 430**,** 429-36.

LOWRY, P. S., JERDE, T. J., BJORLING, D. E., MASKEL, J. L. & NAKADA, S. Y. 2005. Obstruction alters the effect of prostaglandin E2 on ureteral contractility. *J Endourol,* 19**,** 183-7.

LUCIUS, C., ARNER, A., STEUSLOFF, A., TROSCHKA, M., HOFMANN, F., AKTORIES, K. & PFITZER, G. 1998. Clostridium difficile toxin B inhibits carbachol-induced force and myosin light chain phosphorylation in guinea-pig smooth muscle: role of Rho proteins. *The Journal of Physiology,* 506**,** 83-93.

MA, J. X., WANG, D. Z., WARD, D. C., CHEN, L., DESSAI, T., CHAO, J. & CHAO, L. 1994. Structure and chromosomal localization of the gene (BDKRB2) encoding human bradykinin B2 receptor. *Genomics,* 23**,** 362-9.

MAGGI, C. A. & GIULIANI, S. 1995. A pharmacological analysis of calcium channels involved in phasic and tonic responses of the guinea-pig ureter to high potassium. *J Auton Pharmacol,* 15**,** 55-64.

MAGGI, C. A., GIULIANI, S. & SANTICIOLI, P. 1994. Multiple mechanisms in the smooth muscle relaxant action of calcitonin gene-related peptide (CGRP) in the guinea-pig ureter. *Naunyn Schmiedebergs Arch Pharmacol,* 350**,** 537-47.

MAGGI, C. A., GIULIANI, S. & SANTICIOLI, P. 1995a. CGRP inhibition of electromechanical coupling in the guinea-pig isolated renal pelvis. *Naunyn-Schmiedeberg's Archives of Pharmacology,* 352**,** 529-537.

MAGGI, C. A., GIULIANI, S. & SANTICIOLI, P. 1995b. Effect of the Ca(2+)-ATPase inhibitor, cyclopiazonic acid, on electromechanical coupling in the guinea-pig ureter. *British Journal of Pharmacology,* 114**,** 127-137.

MAGURA, I. S., KUCHER, V. V. & BOIKO, N. Y. 2004. Voltage-operated potassium channels and mechanisms controlling their activity. *Neurophysiology,* 36**,** 285-292.

MAHABEER, R. & BHOOLA, K. D. 2000. Kallikrein and kinin receptor genes. *PHARMACOLOGY & THERAPEUTICS,* 88**,** 77-89.

MALIN, J. M., DEANE, R. F. & BOYARSKY, S. 1970. CHARACTERISATION OF ADRENERGIC RECEPTORS IN HUMAN URETER1. *British Journal of Urology,* 42**,** 171.

MALMQVIST, U. & ARNER, A. 1990. Isoform distribution and tissue contents of contractile and cytoskeletal proteins in hypertrophied smooth muscle from rat portal vein. *Circulation Research,* 66**,** 832.

MANDLE, R. J., COLMAN, R. W. & KAPLAN, A. P. 1976. Identification of prekallikrein and high-molecular-weight kininogen as a complex in human plasma. *Proceedings of the National Academy of Sciences of the United States of America,* 73**,** 4179-4183.

MANNING, D. C. & SNYDER, S. H. 1989. Bradykinin receptors localized by quantitative autoradiography in kidney, ureter, and bladder. *Am J Physiol,* 256**,** F909-15.

MARIN-GREZ, M., BÖNNER, G. & GROSS, F. 1980. Ureteral contractions induced by rat urine in vitro: Probable involvement of renal kallikrein. *Cellular and Molecular Life Sciences (CMLS),* 36**,** 865.

MARSTON, S. B. & SMITH, C. W. 1984. Purification and properties of Ca2+-regulated thin filaments and F-actin from sheep aorta smooth muscle. *J Muscle Res Cell Motil,* 5**,** 559-75.

MARSTON, S. B. & TAYLOR, E. W. 1980. Comparison of the myosin and actomyosin ATPase mechanisms of the four types of vertebrate muscles. *J Mol Biol,* 139**,** 573-600.

MARTHAN, R. 2004. Store-operated calcium entry and intracellular calcium release channels in airway smooth muscle. *American Journal Of Physiology. Lung Cellular And Molecular Physiology,* 286**,** L907-L908.

MARTINEZ, M. C., RANDRIAMBOAVONJY, V., OHLMANN, P., KOMAS, N., DUARTE, J., SCHNEIDER, F., STOCLET, J. C. & ANDRIANTSITOHAINA, R. 2000. Involvement of protein kinase C, tyrosine kinases, and Rho kinase in Ca(2+) handling of human small arteries. *Am J Physiol Heart Circ Physiol,* 279**,** H1228-38.

MASTRANGELO, D., BAERTSCHI, A. J., ROATTI, A., AMHERDT, M. & ISELIN, C. E. 2003. Nitric oxide production within rat urothelial cells. *J Urol,* 170**,** 1409-14.

MASTRANGELO, D., WISARD, M., ROHNER, S., LEISINGER, H. & ISELIN, C. E. 2000. Diclofenac and NS-398, a selective cyclooxygenase-2 inhibitor, decrease agonist-induced contractions of the pig isolated ureter. *Urol Res,* 28**,** 376-82.

MCCARRON, J. G., CRAIG, J. W., BRADLEY, K. N. & MUIR, T. C. 2002. Agonist-induced phasic and tonic responses in smooth muscle are mediated by InsP3. *JOURNAL OF CELL SCIENCE,* 115**,** 2207-2218.

MCFADZEAN, I. & GIBSON, A. 2002. The developing relationship between receptor-operated and store-operated calcium channels in smooth muscle. *British Journal of Pharmacology,* 135**,** 1-13.

MCINTYRE, P., PHILLIPS, E., SKIDMORE, E., BROWN, M. & WEBB, M. 1993. Cloned murine bradykinin receptor exhibits a mixed B1 and B2 pharmacological selectivity. *Mol Pharmacol,* 44**,** 346-55.

MELDOLESI, J. & POZZAN, T. 1998. The endoplasmic reticulum Ca2+ store: a view from the lumen. *Trends In Biochemical Sciences,* 23**,** 10-14.

MERRITT, J. E., ARMSTRONG, W. P., BENHAM, C. D., HALLAM, T. J., JACOB, R., JAXA-CHAMIEC, A., LEIGH, B. K., MCCARTHY, S. A., MOORES, K. E. & RINK, T. J. 1990. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem J,* 271**,** 515-22.

MICHEL, L. Y. M., VERKAART, S., KOOPMAN, W. J. H., WILLEMS, P. H. G. M., HOENDEROP, J. G. J. & BINDELS, R. J. M. 2014. Function and Regulation of the Na(+)-Ca(2+) Exchanger NCX3 Splice Variants in Brain and Skeletal Muscle. *The Journal of Biological Chemistry,* 289**,** 11293-11303.

MITCHELL, G. A. G. 1935. The Innervation of the Kidney, Ureter, Testicle and Epididymis. *Journal of Anatomy,* 70**,** 10-32.15.

MIWA, S., IWAMURO, Y., ZHANG, X. F., OKAMOTO, Y., FURUTANI, H. & MASAKI, T. 2000. Pharmacological properties of calcium entry channels in A7r5 cells activated by endothelin-1. *Journal of Cardiovascular Pharmacology,* 36**,** S107-S109.

MOLINARO, G., GERVAIS, N. & ADAM, A. 2002. Biochemical basis of angioedema associated with recombinant tissue plasminogen activator treatment: an in vitro experimental approach. *Stroke,* 33**,** 1712-6.

MOMBOULI, J. V., ILLIANO, S., NAGAO, T., SCOTT-BURDEN, T. & VANHOUTTE, P. M. 1992. Potentiation of endothelium-dependent relaxations to bradykinin by angiotensin I converting enzyme inhibitors in canine coronary artery involves both endothelium-derived relaxing and hyperpolarizing factors. *Circ Res,* 71**,** 137-44.

MONSEES, T. K., BLÖCHER, S., HEIDORN, F., WINKLER, A., SIEMS, W.-E., MÜLLER-ESTERL, W., HAYATPOUR, J., MISKA, W. & SCHILL, W.-B. 2002. Expression and location of the bradykinin B2 receptor in rat testis. *Biology Of Reproduction,* 67**,** 1832-1839.

MOREAU, M. E., GARBACKI, N., MOLINARO, G., BROWN, N. J., MARCEAU, F. & ADAM, A. 2005. The Kallikrein-Kinin System: Current and Future Pharmacological Targets. *Journal of Pharmacological Sciences,* 99**,** 6-38.

MORI, K., SAKAMOTO, W. & NAGASAWA, S. 1981. Studies on human high molecular weight (HMW) kininogen. III. Cleavage of HMW kininogen by the action of human salivary kallikrein. *J Biochem,* 90**,** 503-9.

MORITA, T., ANDO, M., KIHARA, K. & OSHIMA, H. 1994. Function and distribution of autonomic receptors in canine ureteral smooth muscle. *Neurourol Urodyn,* 13**,** 315-21.

MOTTA, G., ROJKJAER, R., HASAN, A. A., CINES, D. B. & SCHMAIER, A. H. 1998. High molecular weight kininogen regulates prekallikrein assembly and activation on endothelial cells: a novel mechanism for contact activation. *Blood,* 91**,** 516-28.

MURPHEY, L. J., HACHEY, D. L., OATES, J. A., MORROW, J. D. & BROWN, N. J. 2000. Metabolism of Bradykinin In Vivo in Humans: Identification of BK1-5 as a Stable Plasma Peptide Metabolite. *Journal of Pharmacology and Experimental Therapeutics,* 294**,** 263-269.

MURÁNYI, A., DERKACH, D., ERDŐDI, F., KISS, A., ITO, M. & HARTSHORNE, D. J. 2005. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: Inhibitory effects and occurrence in A7r5 cells. *FEBS Letters,* 579**,** 6611-6615.

NAKAHARA, T., MORIUCHI, H., YUNOKI, M., SAKAMATO, K. & ISHII, K. 2000. Y-27632 potentiates relaxant effects of beta 2-adrenoceptor agonists in bovine tracheal smooth muscle. *Eur J Pharmacol,* 389**,** 103-6.

NAKAZAWA, K. & MATSUKI, N. 1987. Adenosine triphosphate-activated inward current in isolated smooth muscle cells from rat vas deferens. *Pflugers Arch,* 409**,** 644-6.

NARUMIYA, S., ISHIZAKI, T. & UEHATA, M. 2000. Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol,* 325**,** 273-84.

NGAI, P. K. & WALSH, M. P. 1985. Properties of caldesmon isolated from chicken gizzard. *Biochemical Journal,* 230**,** 695-707.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. 1988. Norepinephrine and GTP-gamma-S increase myofilament Ca2+ sensitivity in alpha-toxin permeabilized arterial smooth muscle. *Biochem Biophys Res Commun,* 157**,** 677-83.

NISHIZUKA, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature,* 308**,** 693-698.

NOBLE, D., BORYSOVA, L., WRAY, S. & BURDYGA, T. 2014. Store-operated Ca(2+) entry and depolarization explain the anomalous behaviour of myometrial SR: Effects of SERCA inhibition on electrical activity, Ca(2+) and force. *Cell Calcium,* 56**,** 188-194.

NOOR, A., NAJMI, M. H. & BUKHTIAR, S. 2011. Effect of Montelukast on bradykinin-induced contraction of isolated tracheal smooth muscle of guinea pig. *Indian Journal of Pharmacology,* 43**,** 445-449.

NOTLEY, R. G. 1978. Ureteral morphology: anatomic and clinical considerations. *Urology,* 12**,** 8-14.

O'KANE, K. P., WEBB, D. J., COLLIER, J. G. & VALLANCE, P. J. 1994. Local L-NG-monomethyl-arginine attenuates the vasodilator action of bradykinin in the human forearm. *Br J Clin Pharmacol,* 38**,** 311-5.

O'RAHILLY, R. 1983. *Basic human anatomy: a regional study of human structure*, Philadelphia: Saunders, 1983.

ONO, S. 2010. Dynamic Regulation of Sarcomeric Actin Filaments in Striated Muscle. *Cytoskeleton (Hoboken, N.j.),* 67**,** 677-692.

PACAUD, P. & BOLTON, T. B. 1991. Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. *J Physiol,* 441**,** 477-99.

PARADISO, A. M., CHENG, E. H. C. & BOUCHER, R. C. 1991. Effects of bradykinin on intracellular calcium regulation in human ciliated airway epithelium. *American Journal of Physiology: Lung Cellular & Molecular Physiology,* 5**,** L63.

PARK, Y. C., TOMIYAMA, Y., HAYAKAWA, K., AKAHANE, M., AJISAWA, Y., MIYATAKE, R., KIWAMOTO, H., SUGIYAMA, T. & KURITA, T. 2000. Existence of a beta3-adrenoceptro and its functional role in the human ureter. *J Urol,* 164**,** 1364-70.

PARKASH, J. & ASOTRA, K. 2012. Calcium oscillations and waves in cells. *Adv Exp Med Biol,* 740**,** 521-9.

PARKINGTON, H. C. & COLEMAN, H. A. 1990. The Role of Membrane Potential in the Control of Uterine Motility. *In:* CARSTEN, M. E. & MILLER, J. D. (eds.) *Uterine Function: Molecular and Cellular Aspects.* Boston, MA: Springer US.

PATEL, S., JOSEPH, S. K. & THOMAS, A. P. 1999. Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium,* 25**,** 247-64.

PELAIA, G., RENDA, T., GALLELLI, L., VATRELLA, A., BUSCETI, M. T., AGATI, S., CAPUTI, M., CAZZOLA, M., MASELLI, R. & MARSICO, S. A. 2008. Molecular mechanisms underlying airway smooth muscle contraction and proliferation: Implications for asthma. *Respiratory Medicine,* 102**,** 1173-1181.

POTJER, R. M., KIMOTO, Y. & CONSTANTINOU, C. E. 1992. Topological Localization of the Frequency and Amplitude Characteristics of the Whole and Segmented Renal Pelvis. *Urologia Internationalis,* 48**,** 278-283.

PRITCHARD, K. & MOODY, C. J. 1986. Caldesmon: a calmodulin-binding actin-regulatory protein. *Cell Calcium,* 7**,** 309-27.

RAEYMAEKERS, L., EGGERMONT, J. A., WUYTACK, F. & CASTEELS, R. 1990. Effects of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca2+ pump of bovine pulmonary artery. *Cell Calcium,* 11**,** 261-8.

REUTER, H. 1979. Properties of Two Inward Membrane Currents in the Heart. *Annual Review of Physiology,* 41**,** 413.

RIBEIRO, A. S., FERNANDES, V. S., MARTINEZ, M. P., LOPEZ-OLIVA, M. E., BARAHONA, M. V., RECIO, P., MARTINEZ, A. C., BLAHA, I., ORENSANZ, L. M., BUSTAMANTE, S., GARCIA-SACRISTAN, A., PRIETO, D. & HERNANDEZ, M. 2016. Pre- and post-junctional bradykinin B2 receptors regulate smooth muscle tension to the pig intravesical ureter. *Neurourol Urodyn,* 35**,** 115-21.

RICCIARDOLO, F. L. M., LOVETT, M., BUNNETT, N. W., HALLIDAY, D. A., NADEL, J. A., KANEKO, T. & GEPPETTI, P. 1998. Bradykinin increases intracellular calcium levels in a human bronchial epithelial cell line via the B2 receptor subtype. *Inflammation Research,* 47**,** 231-235.

RICHARD, J. L., MARGRET, E. D. & BETTY, E. 2002. Pyeloureteral motility and ureteral peristalsis: essential role of sensory nerves and endogenous prostaglandins. *Experimental Physiology,* 87**,** 129.

ROCHA, E. S. M., BERALDO, W. T. & ROSENFELD, G. 1949. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Am J Physiol,* 156**,** 261-73.

RONG, W. & BURNSTOCK, G. 2004. Activation of ureter nociceptors by exogenous and endogenous ATP in guinea pig. *Neuropharmacology,* 47**,** 1093-101.

SAKAMOTO, K., SURI, D. & RAJASEKARAN, M. 2006. Characterization of muscarinic receptor subtypes in human ureter. *J Endourol,* 20**,** 939-42.

SAKURADA, S., OKAMOTO, H., TAKUWA, N., SUGIMOTO, N. & TAKUWA, Y. 2001. Rho activation in excitatory agonist-stimulated vascular smooth muscle. *Am J Physiol Cell Physiol,* 281**,** C571-8.

SANTICIOLI, P. & MAGGI, C. A. 1998. Myogenic and neurogenic factors in the control of pyeloureteral motility and ureteral peristalsis. *Pharmacological Reviews,* 50**,** 683-722.

SASAKI, Y., SUZUKI, M. & HIDAKA, H. 2002. The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. *Pharmacol Ther,* 93**,** 225-32.

SCHMIDT-NIELSEN, B., CHURCHILL, M. & REINKING, L. N. 1980. Occurrence of renal pelvic refluxes during rising urine flow rate in rats and hamsters. *Kidney Int,* 18**,** 419-31.

SCHMIDT–NIELSEN, B. 1987. The renal pelvis. *Kidney International,* 31**,** 621-628.

SCOFIELD, M. A., LIU, F., ABEL, P. W. & JEFFRIES, W. B. 1995. Quantification of steady state expression of mRNA for alpha-1 adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. *J Pharmacol Exp Ther,* 275**,** 1035-42.

SEKO, T., ITO, M., KUREISHI, Y., OKAMOTO, R., MORIKI, N., ONISHI, K., ISAKA, N., HARTSHORNE, D. J. & NAKANO, T. 2003. Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res,* 92**,** 411-8.

SHABIR, S., BORISOVA, L., WRAY, S. & BURDYGA, T. 2004. Rho-kinase inhibition and electromechanical coupling in rat and guinea-pig ureter smooth muscle: Ca2+-dependent and -independent mechanisms. *J Physiol,* 560**,** 839-55.

SHAIFTA, Y., IRECHUKWU, N., PRIETO-LLORET, J., MACKAY, C. E., MARCHON, K. A., WARD, J. P. T. & KNOCK, G. A. 2015. Divergent modulation of Rho-kinase and Ca2+ influx pathways by Src family kinases and focal adhesion kinase in airway smooth muscle. *British Journal of Pharmacology,* 172**,** 5265-5280.

SHMIGOL, A. V., EISNER, D. A. & WRAY, S. 2001. Simultaneous measurements of changes in sarcoplasmic reticulum and cytosolic [Ca(2+)] in rat uterine smooth muscle cells. *The Journal of Physiology,* 531**,** 707-713.

SHMYGOL, A. & WRAY, S. 2005. Modulation of agonist-induced Ca2+ release by SR Ca2+ load: direct SR and cytosolic Ca2+ measurements in rat uterine myocytes. *Cell Calcium,* 37**,** 215-23.

SHUBA, M. F. 1977. The mechanism of the excitatory action of catecholamines and histamine on the smooth muscle of guinea-pig ureter. *The Journal Of Physiology,* 264**,** 853-864.

SHUKLA, A. R., NGUYEN, T., ZHENG, Y., ZDERIC, S. A., DISANTO, M., WEIN, A. J. & CHACKO, S. 2004. Over expression of smooth muscle specific caldesmon by transfection and intermittent agonist induced contraction alters cellular morphology and restores differentiated smooth muscle phenotype. *J Urol,* 171**,** 1949-54.

SIMS, S. M. 1992. Cholinergic activation of a non-selective cation current in canine gastric smooth muscle is associated with contraction. *J Physiol,* 449**,** 377-98.

SMEDLER, E. & UHLÉN, P. 2014. Frequency decoding of calcium oscillations. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1840**,** 964-969.

SMITH, R. D., BORISOVA, L., WRAY, S. & BURDYGA, T. 2002. Characterisation of the ionic currents in freshly isolated rat ureter smooth muscle cells: evidence for species-dependent currents. *PFLUGERS ARCHIV-EUROPEAN JOURNAL OF PHYSIOLOGY,* 445**,** 444-453.

SMYTH, J. T., DEHAVEN, W. I., BIRD, G. S. & PUTNEY, J. W., JR. 2008. Ca2+-store-dependent and -independent reversal of Stim1 localization and function. *J Cell Sci,* 121**,** 762-72.

SMYTH, J. T., HWANG, S.-Y., TOMITA, T., DEHAVEN, W. I., MERCER, J. C. & PUTNEY, J. W. 2010. Activation and regulation of store-operated calcium entry. *Journal of Cellular and Molecular Medicine,* 14**,** 2337-2349.

SOMLYO, A. P. & SOMLYO, A. V. 1994. Signal transduction and regulation in smooth muscle. *Nature,* 372**,** 231-6.

SOMLYO, A. P. & SOMLYO, A. V. 2000. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol,* 522 Pt 2**,** 177-85.

SOMLYO, A. P. & SOMLYO, A. V. 2003a. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiological Reviews,* 83**,** 1325-1358.

SOMLYO, A. P. & SOMLYO, A. V. 2003b. Ca<sup>2+</sup> Sensitivity of Smooth Muscle and Nonmuscle Myosin II: Modulated by G Proteins, Kinases, and Myosin Phosphatase. *Physiological Reviews,* 83**,** 1325.

SOMLYO, A. P. & SOMLYO, A. V. 2004. Signal transduction through the RhoA/Rho-kinase pathway in smooth muscle. *J Muscle Res Cell Motil,* 25**,** 613-5.

STASIEWICZ, J., SZAŁAJ, W. & GABRYEŁEWICZ, A. 1977. MODIFYING EFFECT OF BRADYKININ ON MOTOR ACTIVITY IN THE GUINEA-PIG GALL BLADDER. *Clinical & Experimental Pharmacology & Physiology,* 4**,** 561.

STREB, H., IRVINE, R. F., BERRIDGE, M. J. & SCHULZ, I. 1983. Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature,* 306**,** 67-9.

STULL, J. T., TANSEY, M. G., TANG, D.-C., WORD, R. A. & KAMM, K. E. 1993. Phosphorylation of myosin light chain kinase: a cellular mechanism for Ca2+ desensitization. *Molecular and Cellular Biochemistry,* 127**,** 229-237.

STÜHMER, W., RUPPERSBERG, J. P., SCHRÖTER, K. H., SAKMANN, B., STOCKER, M., GIESE, K. P., PERSCHKE, A., BAUMANN, A. & PONGS, O. 1989. Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *The EMBO journal,* 8**,** 3235-3244.

SUI, J. L. & KAO, C. Y. 1997a. Properties of inward calcium current in guinea pig ureteral myocytes. *Am J Physiol,* 272**,** C543-9.

SUI, J. L. & KAO, C. Y. 1997b. Roles of Ca2+ and Na+ in the inward current and action potentials of guinea pig ureteral myocytes. *Am J Physiol,* 272**,** C535-42.

SUZUKI, H. 1985. Electrical responses of smooth muscle cells of the rabbit ear artery to adenosine triphosphate. *J Physiol,* 359**,** 401-15.

SZTEYN, K., GOMEZ, R., BERG, K. A. & JESKE, N. A. 2015. Divergence in Endothelin-1- and Bradykinin-Activated Store-Operated Calcium Entry in Afferent Sensory Neurons. *ASN NEURO,* 7**,** 1759091415578714.

TACHIBANA, S., TAKEUCHI, M. & UEHARA, Y. 1985. The architecture of the musculature of the guinea-pig ureter as examined by scanning electron microscopy. *J Urol,* 134**,** 582-6.

TINDALL, A. R. 1972. Preliminary observations on the mechanical and electrical activity of the rat ureter. *The Journal of physiology,* 223**,** 633.

TODA, N., BIAN, K., AKIBA, T. & OKAMURA, T. 1987. Heterogeneity in mechanisms of bradykinin action in canine isolated blood vessels. *European Journal of Pharmacology,* 135**,** 321-329.

TORO, L., WALLNER, M., MEERA, P. & TANAKA, Y. 1998. Maxi-KCa, a unique member of the voltage-gated K channel superfamily. *News in Physiological Sciences,* 13**,** 112-117.

TRAPANI, A., MATSUKI, N., ABEL, P. W. & HERMSMEYER, K. 1981. Norepinephrine produces tension through electromechanical coupling in rabbit ear artery. *Eur J Pharmacol,* 72**,** 87-91.

TSAI, M. H. & JIANG, M. J. 2006. Rho-kinase-mediated regulation of receptor-agonist-stimulated smooth muscle contraction. *Pflugers Arch,* 453**,** 223-32.

TSIEN, R. W., LIPSCOMBE, D., MADISON, D. V., BLEY, K. R. & FOX, A. P. 1988. Multiple types of neuronal calcium channels and their selective modulation. *Trends In Neurosciences,* 11**,** 431-438.

TSUCHIYA, T. & TAKEI, N. 1990. Pressure responses and conduction of peristaltic wave in guinea-pig ureter. *The Japanese journal of physiology,* 40**,** 139-149.

TURNA, B., CINAR, M. G., CANDA, A. E., ORHAN, E. C., TIFTIK, N. R., NAZLI, O. & BUYUKAFSAR, K. 2007. Role of Rho-kinase in contractions of ureters from rabbits with unilateral ureteric obstruction. *BJU International,* 100**,** 1166-1171.

UEHARA, Y. & BURNSTOCK, G. 1970. Demonstration of" gap junctions" between smooth muscle cells. *The Journal of cell biology,* 44**,** 215.

UEHATA, M. & ISHIZAKI, T. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature,* 389**,** 990.

VAN BREEMEN, C. & SAIDA, K. 1989. Cellular mechanisms regulating [Ca2+]i smooth muscle. *Annu Rev Physiol,* 51**,** 315-29.

VAN ELDIK, L. J. & WATTERSON, D. M. 1998. 1 - Calmodulin and Calcium Signal Transduction: An Introduction. *Calmodulin and Signal Transduction.* San Diego: Academic Press.

VARNAI, P., TOTH, B., TOTH, D. J., HUNYADY, L. & BALLA, T. 2007. Visualization and manipulation of plasma membrane-endoplasmic reticulum contact sites indicates the presence of additional molecular components within the STIM1-Orai1 Complex. *J Biol Chem,* 282**,** 29678-90.

VEREECKEN, R. L., HENDRICKX, H. & CASTEELS, R. 1975. The influence of calcium on the electrical and mechanical activity of the guinea pig ureter. *Urol Res,* 3**,** 149-53.

VETTER, S. W. & LECLERC, E. 2003. Novel aspects of calmodulin target recognition and activation. *Eur J Biochem,* 270**,** 404-14.

VICENTE-MANZANARES, M., MA, X., ADELSTEIN, R. S. & HORWITZ, A. R. 2009. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature reviews. Molecular cell biology,* 10**,** 778-790.

VOGALIS, F. & SANDERS, K. M. 1990. Cholinergic stimulation activates a non-selective cation current in canine pyloric circular muscle cells. *J Physiol,* 429**,** 223-36.

WANG, H., KOHNO, T., AMAYA, F., BRENNER, G. J., ITO, N., ALLCHORNE, A., JI, R.-R & WOOLF, C. J. 2005. Bradykinin Produces Pain Hypersensitivity by Potentiating Spinal Cord Glutamatergic Synaptic Transmission. *The Journal of Neuroscience,* 25**,** 7986.

WANG, Q. & LARGE, W. A. 1991. Noradrenaline-evoked cation conductance recorded with the nystatin whole-cell method in rabbit portal vein cells. *J Physiol,* 435**,** 21-39.

WANG, Y.-X. & KOTLIKOFF, M. I. 1997. Inactivation of calcium-activated chloride channels in smooth muscle by calcium/calmodulin-dependent protein kinase. *Proceedings of the National Academy of Sciences,* 94**,** 14918-14923.

WARNAT, J., PHILIPP, S., ZIMMER, S., FLOCKERZI, V. & CAVALIE, A. 1999. Phenotype of a recombinant store-operated channel: highly selective permeation of Ca2+. *J Physiol,* 518 (Pt 3)**,** 631-8.

WASHIZU, Y. 1966. Grouped discharges in ureter muscle. *Comparative Biochemistry and Physiology,* 19**,** 713-728.

WEI, J. W. & WEI, M. M. 1992. Characterization of some biochemical properties of bradykinin-induced cAMP and cGMP formation in guinea pig ileum. *Chin J Physiol,* 35**,** 303-16.

WEIRICH, J., DUMONT, L. & FLECKENSTEIN-GRÜN, G. 2004. Contribution of store-operated Ca2+ entry to pHo-dependent changes in vascular tone of porcine coronary smooth muscle. *Cell Calcium,* 35**,** 9-20.

WEISS, R. M. 2002. Physiology and pharmacology of the renal pelvis and ureter. *Campbell's urology,* 1**,** 94-128.

WILDE, C., GENTH, H., AKTORIES, K. & JUST, I. 2000. Recognition of RhoA by Clostridium botulinum C3 exoenzyme. *J Biol Chem,* 275**,** 16478-83.

WILLIAMS, D. A. & FAY, F. S. 1986. Calcium transients and resting levels in isolated smooth muscle cells as monitored with quin 2. *Am J Physiol,* 250**,** C779-91.

WILLIS, L. R., LUDENS, J. H., HOOK, J. B. & WILLIAMSON, H. E. 1969. Mechanism of natriuretic action of bradykinin. *American Journal of Physiology -- Legacy Content,* 217**,** 1.

WINDER, S. J., SUTHERLAND, C. & WALSH, M. P. 1991. Biochemical and functional characterization of smooth muscle calponin. *Adv Exp Med Biol,* 304**,** 37-51.

WIRTH, K., HOCK, F. J., ALBUS, U., LINZ, W., ALPERMANN, H. G., ANAGNOSTOPOULOS, H., HENKE, S., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B. A. 1991. Hoe 140 a new potent and long acting bradykinin-antagonist: in vivo studies. *British Journal of Pharmacology,* 102**,** 774-777.

WOOD, D. & GREENWELL, T. 2010. Surgical anatomy of the kidney and ureters. *Surgery (Oxford),* 28**,** 314-316.

WRAY, S. & BURDYGA, T. 2010. Sarcoplasmic reticulum function in smooth muscle. *Physiol Rev,* 90**,** 113-78.

XIONG, Z. L., KITAMURA, K. & KURIYAMA, H. 1991. ATP activates cationic currents and modulates the calcium current through GTP-binding protein in rabbit portal vein. *J Physiol,* 440**,** 143-65.

YAGI, S., BECKER, P. L. & FAY, F. S. 1988. Relationship between force and Ca2+ concentration in smooth muscle as revealed by measurements on single cells. *Proc Natl Acad Sci U S A,* 85**,** 4109-13.

YAMAGUCHI, O. A. & CONSTANTINOU, C. E. 1989. Renal calyceal and pelvic contraction rhythms. *Am J Physiol,* 257**,** R788-95.

YANG, C. M., HSIA, H. C., HSIEH, J. T., ONG, R. & LUO, S. F. 1994. Bradykinin-stimulated calcium mobilization in cultured canine tracheal smooth muscle cells. *Cell Calcium,* 16**,** 59-70.

YOSHII, A., IIZUKA, K., DOBASHI, K., HORIE, T., HARADA, T., NAKAZAWA, T. & MORI, M. 1999. Relaxation of contracted rabbit tracheal and human bronchial smooth muscle by Y-27632 through inhibition of Ca2+ sensitization. *Am J Respir Cell Mol Biol,* 20**,** 1190-200.

YUE, L., PENG, J. B., HEDIGER, M. A. & CLAPHAM, D. E. 2001. CaT1 manifests the pore properties of the calcium-release-activated calcium channel. *Nature,* 410**,** 705-9.

ZHANG, X. & DISANTO, M. E. 2011. Rho-Kinase, a Common Final Path of Various Contractile Bladder and Ureter Stimuli. *In:* ANDERSSON, K.-E. & MICHEL, M. C. (eds.) *Urinary Tract.* Berlin, Heidelberg: Springer Berlin Heidelberg.

ZHANG, X. F., IWAMURO, Y., ENOKI, T., OKAZAWA, M., LEE, K., KOMURO, T., MINOWA, T., OKAMOTO, Y., HASEGAWA, H., FURUTANI, H., MIWA, S. & MASAKI, T. 1999. Pharmacological characterization of Ca2+ entry channels in endothelin-1-induced contraction of rat aorta using LOE 908 and SK&F 96365. *Br J Pharmacol,* 127**,** 1388-98.

ZHAO, Y., QIU, Q., MAHDI, F., SHARIAT-MADAR, Z., ROJKJAER, R. & SCHMAIER, A. H. 2001. Assembly and activation of HK-PK complex on endothelial cells results in bradykinin liberation and NO formation. *Am J Physiol Heart Circ Physiol,* 280**,** H1821-9.