Gestation changes sodium pump isoform expression, leading to changes in ouabain sensitivity, contractility and intracellular calcium in rat uterus

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Abstract

Developmental and tissue-specific differences in isoforms allow Na^+, K^+-ATPase function to be tightly regulated, as they control sensitivity to ions and inhibitors. Uterine contraction relies on the activity of the Na^+, K^+ATPase, which creates ionic gradients that drive excitation-contraction coupling. It is unknown whether Na^+, K^+ATPase isoforms are regulated throughout pregnancy or whether they have a direct role in modulating uterine contractility. We hypothesized that gestation-dependent differential expression of isoforms would affect contractile responses to Na^+, K^+ATPase α subunit inhibition with ouabain. Our aims were therefore: (1) to determine the gestation-dependent expression of mRNA transcripts, protein abundance and tissue distribution of Na^+, K^+ATPase isoforms in myometrium, (2) to investigate the functional effects of differential isoform expression via ouabain sensitivity and, (3) if changes in contractile responses can be explained by changes in intracellular [Ca^{2+}]. Changes in abundance and distribution of the Na^+, K^+ATPase α, β and FXYD1 and 2 isoforms, were studied in rat uterus from non-pregnant, and early, mid-, and term gestation. All α, β subunit isoforms (1,2,3) and FXYD1 were detected but FXYD2 was absent. The α1 and β1 isoforms were unchanged throughout pregnancy, whereas α2 and α3 significant decreased at term while β2 and FXYD1 significantly increased from mid-term onwards. These changes in expression correlated with increased functional sensitivity to ouabain, and parallel changes in intracellular Ca^{2+}, measured with Indo-1. In conclusion, gestation induces specific regulatory changes in expression of Na^+, K^+ATPase isoforms in the uterus which influence contractility and may be related to the physiological requirements for successful pregnancy and delivery.

Introduction

The excitability of human myometrium must be regulated to control contractile activity for successful pregnancy and parturition (Noble et al., 2009, Wray et al., 2003, Wray et al., 2015). While the pivotal role of intracellular [Ca^{2+}] has been well-studied (Floyd and Wray, 2007), other ions including K^+, Na^+ and Cl^- are also known to be important (Khan et al., 2001, Tong et al., 2011, Seda et al., 2007, Jones et al., 2004). Either directly (Na^+, K^+) or indirectly (Ca^{2+}, Cl^-), the concentrations of these ions are dependent upon the Na^+, K^+-ATPase, as it moves $3Na^+$ out of the cell with $2K^+$ entering and contributes to the negative cell membrane potential and the differential concentration gradients of sodium and potassium. The ion distributions across the cell membrane can be linked to the entry or efflux of other ions, including Ca^{2+}, Cl^- and protons, all of which will affect myometrial excitability (Wray et al., 2015). In smooth muscle Moore et al (Moore et al., 1993) showed co-localization of the Na^+, K^+ ATPase and Na-Ca exchanger (NCX), and Blaustein's group; (Blaustein et al., 1992, Golovina et al., 2003, Blaustein, 1993) further showed that intracellular [Na^+] in the sub-sarcolemmal space influences the activity of the NCX and sarcoplasmic reticulum (SR) Ca^{2+} content. As myometrial SR approaches the plasma membrane throughout the cell (Wray and Shmygol, 2007) and NCX contributes about a third to Ca^{2+} efflux (Taggart and Wray, 1997, Matthew et al., 2004), effects of the Na^+, K^+ ATPase on Ca^{2+} availability and contraction will occur. In addition, it has been reported that ouabain, an inhibitor of the Na^+, K^+ ATPase and endogenous cardiotonic steroids (glycosides), target the Na^+, K^+ ATPase and initiate IP_3 Src signalling (Zhang et al., 2008, Aperia et al.). Recently it has been suggested that for this ouabain-dependent signalling, the Na^+, K^+ ATPase resides in the caveolar (lipid raft) domain of the membrane (Yosef et al.). Caveolae are abundant in smooth muscle, including the uterus and thus this could be another way in which the Na^+, K^+ ATPase can influence contraction (Noble et al., 2006, Draeger et al., 2005). In addition, the effect of ouabain on Na^+, K^+ ATPase activity is modified by its isoform composition (see below), as they vary in sensitivity, and this in turn changes Ca signals, due to increasing intracellular [Na^+] deceasing NCX activity, and consequently increasing Ca^{2+} in the myocytes.

78 The Na^+, K^+ ATPase is a P-type ATPase first described by Skou (Skou, 1957). It
79 is composed of two α subunits, two β subunits and usually a third, FXYD subunit.
80 Each of these subunits has multiple isoforms encoded by distinct genes (Martin-
81 Vasallo et al., 1989, Shull and Lingrel, 1987, Lingrel et al., 1990). The expression of
82 the isoforms is both tissue (Orlowski and Lingrel, 1988b) and species specific (Zahler
83 et al., 1993, Zahler et al., 1996). The α subunit has four isoforms, α1-α4, but α4 is
84 specific to spermatozoa. The α subunit has binding sites for nucleotides, cations and
85 the inhibitory glycosides and is responsible for the Na^+, K^+ ATPase enzymatic activity.
86 Three β isoforms have been identified (β1-β3), which are thought to modulate cation
87 affinity, and the folding and trafficking of the Na^+, K^+ ATPase. To date seven FXYD
88 isoforms have been identified and they lower substrate affinities or Vmax of the Na^+,
89 K^+ ATPase (Geering, 2005).
90 It is now appreciated that each subunit isoform has somewhat different
91 properties, and therefore that the Na^+, K^+ ATPase will have site specific differences in
92 its function, susceptibility to glycosides, and regulation of ion transport. It is
93 considered that all these attributes of the Na^+, K^+ ATPase are varied to best suit the
94 activity of the tissue it is functioning in. Given the uterus relies on the Na^+, K^+ ATPase
95 for its rhythmic contractions but also has a fundamental shift from near quiescence
96 throughout most of pregnancy, to hours of powerful, prolonged contractions during
97 labour, it may be that variations in the isoform composition of the Na^+, K^+ ATPase
98 play a role in supporting these changes in activity (Floyd et al., 2010a). Some insights
99 into what these changes might be can be gathered from the literature in other cells
100 or tissues.
101 The α1 and β1 subunits are ubiquitously present in tissues, and the α1 and β1
102 isozyme combination is considered the basic housekeeping form of the Na^+, K^+
103 ATPase that all cells require (Clausen et al., 2017). The isoforms of the α subunit
104 show differences in K affinity; highest in α1, while α3 has relatively low Na affinity
105 (Blanco, 2005). A relative expression of α3 to α1 several-fold higher in men than in
106 women as judged from RNA levels has also been reported (Gaborit et al., 2010).
107 Interestingly, it has been suggested that the α2 subunit changes in response to
108 activity in skeletal muscle (Kravtsova et al., 2016). This, along with earlier reports
109 suggesting that of all the isoforms, α2 is most concerned with modulating [Ca], and

that in α2 heterozygous mice, skeletal muscles and vascular smooth muscles are hyper-contractile, develop force faster and show greater sensitivity to receptor stimulation than in wild-type animals (Shelly et al., 2004a, He et al., 2001), suggests that changes in the α2 subunit with gestation may be anticipated. Changes in Na^+, K^+ ATPase isoforms with gestation are also pointed to by the work of Tsai and colleagues, reporting in rat uterus that estradiol causes a decrease in α3 expression and a subsequent decrease in contractility (Tsai et al., 2000). As mentioned above, the α subunit isoforms vary in resistance to the effects of ouabain, with rat α1 being particularly resistant. Different concentrations of ouabain can therefore be used as a way to interrogate and functionally separate the α isoforms in a tissue (Monteith and Blaustein, 1998). Ouabain at different concentrations has been reported indirectly to increase Ca^{2+} signalling in myometrium but no measurements of intracellular Ca^{2+} were made, and the very high concentrations of ouabain used were not designed to allow the effects on the different isoforms to be tested (Ausina et al., 1996b).

The β subunits of the Na^+, K^+ ATPase are considered to modulate its functions as well as acting as chaperones to ensure maturation, expression at tight junctions and contributing to cellular adhesion and polarity (Geering, 2008). The different β isoforms differ in their degree of post-translational modification, especially glycosylation. The myometrium undergoes repeated transient hypoxic episodes as the strength of contractions is sufficient to compress blood vessels (Harrison et al., 1994, Alotaibi et al., 2015). Thus it is of interest that the β1 subunit responds to oxidative stress by glutathionylation (Rasmussen et al., 2010), while β2 with the strongest effects on Na^+, K^+ ATPase kinetics, (reducing the apparent K affinity and raising external Na affinity (Larsen et al., 2014), may be anticipated to increase with myometrial activity. FXYD isoforms and changes in them have not been studied for functional effects in any smooth muscle, as far as we are aware.

Thus, different subunit isoforms convey distinct properties to the Na^+, K^+ ATPase, from kinetic properties, membrane localization and trafficking, sensitivity to endogenous and applied Na^+, K^+ ATPase inhibitors, which will also have secondary effects, such as on Ca fluxes (Horisberger and Kharoubi-Hess, 2002, He et al., 2001, Blanco and Mercer, 1998). As all of these aspects are likely to impact on excitation contraction by varying ionic gradients and potentials, the question arises as to which

142 isoforms are expressed in the uterus and whether this expression is varied during
143 gestation, and if it can have any functional effect on uterine contractility. There is
144 little literature concerning the α and β isoform or FYXD expression in the
145 myometrium (Floyd et al., 2003, Esplin et al., 2003b) and no information about
146 changes throughout pregnancy.
147 We have used a variety of techniques to test the hypothesis that there would
148 be isoform-specific changes with gestation in the myometrium and that these would
149 affect the contractile and Ca responses of the myometrium to Na^+, K^+ ATPase
150 inhibition with ouabain. Therefore, the aims of this work were: (1) to determine the
151 mRNA transcripts and quantitative protein expressions and tissue distribution of Na^+,
152 K^+ ATPase isoforms from rat myometrium, (2) to determine the effects of gestation
153 on their expression and distribution, (3) to investigate the functional effects of
154 differential isoform expression by testing ouabain sensitivity and (4) if changes in
155 contractile responses can be explained by changes in intracellular Ca^{2+}
156 concentration.
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Materials and Methods

Tissue

All studies were performed on female Sprague-Dawley rats (Charles River, Kent UK). Uterine horns from Non-pregnant rats (n=4), early-stage pregnant rats (10/11 days n=4), mid-stage pregnant rats (16/17 days n=4) and late-stage pregnant rats (21/22 days n=4) were cleaned of adipose tissue and either used fresh for contractility studies, snap frozen in liquid nitrogen for western blot analysis or preserved in RNAlater for RT-PCR studies. Tissues were fixed in neutral buffered formalin (NBF) for 24 hours prior to paraffin embedding and preparation of custom designed tissue microarrays (TMAs) (Floyd et al., 2010a).

SDS PAGE and western Blotting

Tissues were homogenized in the presence of protease inhibitor cocktail (Sigma, UK) and centrifuged at 10,000g for 10 minutes at 4°c to remove insoluble material. Protein concentration was determined using the BioRad Dc protein assay as directed by the manufacturer (BioRad, UK).

Extracted total proteins were separated by SDS-PAGE on 12% gels for analysis of α and β subunits and 14% gels for FXYD- determination with 50µg protein loaded per lane against 5µl SeeBlue® Plus2 Pre-Stained Protein Standard (Invitrogen Ltd, UK) using previously described methods (Floyd et al., 2008). Primary antibodies were as follows: α620 1:100, α1 specific (a generous gift from Michael Kaplan), McHERED 1:2000, α 2 specific (a generous gift from Alicia McDonough, University of Southern California (Muller-Ehmsen et al., 2001), MA3-915 1:1500, α 3 specific (Affinity Bioreagents), SpET β1 1:1500, β 1 specific and SpET β2 1:1500, β 2 specific (both were generous gifts from Pablo Martín-Vasallo, Universidad de La Laguna, Spain)(Gonzalez-Martinez et al., 1994) and RNTβ3 1:1500, β 3 specific (a generous gift from Dr Kathleen Sweadner, Harvard University). Positive and negative controls were included on each of the four repeat-blots for each subunit to ensure a consistent, reproducible signal was generated.

RT-PCR

Total RNA was extracted from frozen rat tissues and quantified by spectrophotometry at 260nM. Reverse transcription was performed on equal amounts of template from each tissue group and template was assessed for integrity using intron spanning β actin primers. Amplification of cDNA templates was performed with species and isoform specific primer pairs using published primer sequences for α1-3 and β1-3 (MacPhee et al., 2000, Betts et al., 1997) Na^+, K^+ ATPase, FXYD1 (Arystarkhova et al., 2007) and FXYD2a-c (Jones et al., 2001) and transcripts were run on 1% agarose for analysis and sequencing.

Immunohistochemistry

Tissue microarrays were constructed from formalin fixed, paraffin embedded rat tissues as described previously (Floyd et al., 2010a). Briefly, sections were de-waxed in xylene and transferred through graded alcohols before antigen retrieval in boiling 10mM citrate buffer pH6. Endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol before slides were washed in TBS/0.05% Tween 20 (TBST) and blocked in 5% BSA/TBST for 1 hour at room temperature. Slides were washed with TBST and incubated with the following primary antibodies diluted in blocking solution overnight at 4°c: α6F neat supernatant, α1 specific (Developmental Studies Hybridoma Bank (Arystarkhova and Sweadner, 1996), HERED 1:300, α2 specific and TED 1:300, α3 specific (both generous gifts from Thomas Pressley, Texas Tech University) (Pressley, 1992), SpET β1 1:200, β1 specific and SpET β2 1:200, β2 specific (both generous gifts from Pablo Martín-Vasallo, Universidad de La Laguna, Spain (Gonzalez-Martinez et al., 1994), RNTβ3 1:200, β3 specific (also Dr Kathleen Sweadner, Harvard University 3 (Arystarkhova and Sweadner, 1997), γa/b a and b specific, 1:200, (a generous gift from Steven Karlish, Biological Chemistry, Weizmann Institute of Science, Israel (Kuster et al., 2000) and Plm 1:100, phospholemman specific (also Steven Karlish, Weizmann Institute) (Crambert et al., 2002). Antibody-antigen complexes were visualised with peroxidase-conjugated EnVision™ polymer and DAB substrate (from DAKO, UK). Sections were counterstained in haematoxylin and mounted in DPX.

Measurement of Force and Calcium

Myometrial tissue strips (3 x 1mm) were dissected and loaded for 3 hours at room temperature with a solution containing the ratiometric fluorophore Indo-1 as described previously (Babiychuk et al., 2004). After the tissues had been incubated with the calcium indicator, strips were rinsed in physiological saline and transferred to a small (200µL) bath on the stage of an inverted microscope. The loaded tissues were excited with light at 340 nm and photomultipliers used to record the emitted light at 400 and 500 nm, and the ratio of these two emissions used to indicate changes in intracellular Ca. Tissues were fitted with aluminium clips, with one end attached to a force transducer (Grass FT03) and perfused with physiological saline at 32°C, to maximise Indo-1 signalling and remain close to physiological parameters.

Chemicals

All chemicals were purchased from Sigma (Dorset, UK). Antibodies were from DSHB (α6F) or otherwise kind gifts from Dr A. McDonough (McHERED), Dr T. Pressley (HERED and TED), Dr P. Martín-Vasallo (SpETβ1 and SpETβ2), Dr K. Sweadner (RNTβ3), Dr S. J. Karlish (γ a/b and plm). Protein quantification reagents were from Bio-Rad, detection materials were purchased from Dako, Perbio Science (UK) or Amersham Biosciences (UK). Primers were synthesised by Proligo (France) all PCR substrates were purchased from Promega, Qiagen or Ambion (UK). Indo-1 and Pleuronic® F-127 were from Molecular Probes (Oregon, USA). Ouabain was prepared in a stock solution of 10mM in distilled H_2O and further diluted in physiological saline for use at 50µM, 75µM and 100µM.

Data Presentation and Statistical Analysis

Western blot data was quantified using ImageJ software where data are expressed as a percentage of control β actin gels in terms of relative band intensity. Immunohistochemical analysis is referred to in terms of localisation and distribution with inter-tissue variability expressed in terms of density as TMA samples allow direct comparison to be made. Images were quantitatively evaluated and scored using previously published methods based on the spectral deconvolution method for DAB and hematoxylin, using IHC Profiler open source plugin for ImageJ. Five regions

of interest were analysed per image for areas delineated as smooth muscle, stroma and epithelia at 40X magnification (Varghese et al., 2014).

Force/calcium data are given as mean and standard error of the mean (SEM), where '*n*' represents the number of samples, each taken from a different animal. Significance was tested, using ANOVA and where appropriate, Tukey's multiple comparison post-hoc test where P values < 0.05 were accepted as significant. Data are expressed as percent changes in integral, (area under the curve, arbitrary units) for contractions and Ca changes and compared to preceding ten minutes' control responses.

Results

Expression of mRNA transcripts encoding Na^+, K^+ ATPase α and β and FXYD2 isoforms in rat myometrium

The expression of multiple isoforms of the Na^+, K^+ ATPase and FXYD1 and FXYD2 was studied in non-pregnant rat uterus and at different stages of pregnancy (days 10, 16, 21). The typical expression pattern for each tissue group is shown in Figure 1. Amplicons of 336bp encoding the α1 isoform, were consistently generated from each of the four groups of cDNA. The 335bp transcripts encoding the α2 subunit were detected in all samples, similarly, detection of the 336bp α3 isoform transcript was reliably reproducible in all groups. These data correlate with quantitative protein expression data shown in Figure 2. Amplicons corresponding to the β1, β2 and β3 genes (378bp, 441bp, and 384bp respectively), were generated with 100% frequency in all cDNA preparations from all groups studied. Amplicons corresponding to FXYD1 were detected in all samples. Detection of amplicons of 234bp and 217bp corresponding to the FXYD2a and 2b mRNA were consistently detected in samples from all groups with equal frequency. No evidence of FXYD2c mRNA was detected in any of the samples (bottom of Figure 1). All repeat analysis on subsequent cDNA preparations produced identical results. Beta actin was consistently detected in all preparations from all gestational time points. The positive mRNA transcripts provided the basis for undertaking the investigation of protein expression and quantification.

Expression of Na^+, K^+ ATPase α isoform proteins in rat uteri

Identification of changes in isoform expression during pregnancy through western blotting, allowed quantitative comparison to be made between protein samples. Figures are expressed as a percentage of the 45kDa β actin control in units of relative intensity. The rat uterus (n=4) displayed a pattern of distinct regulatory changes in Na^+, K^+ ATPase isoform expression throughout gestation as compared to non-pregnant animals of the same age. The 112kDa α1 isoform (Figure 2) was detected at all stages of gestation and also in the non-pregnant uterus. Expression in the uterus did not significantly change throughout the course of pregnancy, showing similar expression levels to control samples of kidney and brain. As gestation progressed, the expression of the 112kDa α2 isoform decreased significantly by approximately 62% at term (22 days), after an initial significant 45% increase in intensity on the 10th day of pregnancy when compared to the non-pregnant uteri. Expression of α3 protein was significantly increased from extremely low non-pregnant values, at day 10 and 16 of gestation, before significantly decreasing (by 90%) in density towards term. There also appears to be no renal α3 but dense expression of the isoform in the brain which agrees with published literature (Feraille et al., 1995, McDonough et al., 1994).

Expression of Na^+, K^+ ATPase β isoforms and FXYD2 proteins in rat uteri

The β1 isoform was detected by western blot at two different molecular weights that correspond to the variable state of glycosylation seen in most tissues that express this protein. The 56kDa protein (Figure 2) shows identical expression patterns to that of the 35kDa unglycosylated form in all stages of pregnancy. The non-pregnant rat uterus and all stages of pregnancy show maintained levels of β1 protein. Expression of the β1 subunit was also present in both kidney and brain which agrees with previous studies (Feraille et al., 1995, Martin-Vasallo et al., 1997, Martin-Vasallo et al., 2000). The 56kDa β1 subunit is normally found in brain and skeletal muscle, not renal tissues so the pattern of expression seen in the control tissues is supported by current literature. Western blots show that the β2 isoform is more abundantly expressed at 16 days of gestation and at term in the rat uterus. Our studies show that the 56kDa β3 isoform is consistently expressed in the rat uterus

both in the virgin and pregnant states, with comparatively lower levels seen in kidney and brain as expected (Arystarkhova and Sweadner, 1997).

Detection of FXYD2 using a pan-γ specific antibody reveals a characteristic doublet on SDS PAGE gels at 6kDa (Figure 3.3) (Kuster et al., 2000). This data shows that γa/b is not detected in rat uterus at any stage of gestation or in adult rat brain by Western blot but is abundant in the kidney, while FXYD1 is ubiquitously expressed and rises significantly towards term.

Distribution of Na^+, K^+ ATPase α isoforms in rat uterus

The localisation of isoforms of the Na, K-ATPase was studied in fixed tissues from non-pregnant, day 10, 16 and 21 of pregnancy (Figure 3). Quantitative evaluation scores are included in Supplementary Figure S1 for smooth muscle (A) and epithelial cell layers (B). Expression of the α1 isoform was determined using a well characterised specific monoclonal antibody α6F which binds to an N-terminal region of the protein between residues 27-55 (DSHB literature). Expression of α1 protein was most dense in the basolateral membranes of the secretory epithelia lining the endometrium (Figure 3, panels A, D, G and J and Figure S1A and S1B). Restricted pockets of sparse α1 expression were observed in the smooth muscle layers but was absent from within the endometrium.

Widespread expression of α2 protein was seen in all uterine samples analysed (Figure 3, B, E, H and K and Figure S1A and S1B). Distinct localisation was apparent in the longitudinal and circular smooth muscles of all samples, with little cross-reactivity in the connective tissues of the myometrium and endometrium. Furthermore, epithelia lining secretory glands within the endometrium showed particularly strong expression of α2 in basolateral membranes (Panel B). Of particular interest is the distinct decrease in the intensity of immunostaining seen in the smooth muscle of samples taken from rats at term gestation (Panel K). This data correlates well with the significant decrease in α2 protein (Figure 2) at this late stage of pregnancy (21 days). Immunohistochemical analysis of uteri from all groups of animals demonstrated very diffuse expression of α3 protein across all anatomical regions (Figure 3, panels C, F, I, and L and Figure S1A and S1B). Expression of the α3 isoform in smooth muscle was sparse across both the longitudinal and circular

regions with little variation seen between the 5 groups, apart from being particularly faint on day 21, (Panel L). Expression of α3 in the luminal and secretory epithelia was low with no polarisation in expression as seen in the α1 and α2 localisation. No expression was observed in the connective tissue of the endometrium and myometrium.

Distribution of Na^+, K^+ ATPase β isoforms in rat uterus

The distribution of β isoforms of the Na^+, K^+ ATPase showed much less variability than that seen in the α subunit isoforms in the same tissues. What becomes immediately apparent is the widespread expression of both β1 and β2 isoforms in most of the tissue groups analysed (Figure 4). The most abundant expression is localised in the smooth muscle bundles, this is clearly most prominent in the non-pregnant samples (Figure 4, panels A and B and Figure S1A and S1B). This is primarily due to the non-pregnant smooth muscle myocytes being relatively compact compared to the pregnant samples which show a marked increase in cell surface area and therefore give the appearance of more diffuse staining. Furthermore, the prevalent expression of β1 protein at all stages of gestation relates well to our previous western blot and mRNA analysis showing ubiquitous expression in all samples (Figures 1 and 2). In contrast, the immunohistochemical analysis does not detect any specific decrease in β2 expression in non-pregnant and 10 day pregnant tissues (Panels B and E).

The abundant nature of the immunostaining of the β3 isoform suggests that this may be the predominant β subunit present in uterine tissue as the binding specificity of this antibody in rat tissue is well documented (Arystarkhova and Sweadner, 1997, Arteaga et al., 2004). Biochemical data also confirms that this isoform is present in all uterine samples at uniform levels (Figure 2).

Distribution of Na^+, K^+ ATPase FXYD1 and FXYD2 in rat uterus

Immunohistochemical analysis of the FXYD1 and 2 proteins was also performed on the rat tissue microarrays (Figure 5 and Figure S1A and S1B). Expression of FXYD1 was found to be widespread in all samples tested with no specific variation in abundance or localisation. Conversely, very sparse

immunolocalisation corresponding to FXYD2 expression was seen in the smooth muscle bundles and secretory epithelia of all samples (Figure 5 and Figure S1A and S1B). No further expression of FXYD2 protein was seen in any other regions of these samples (Figure 5 Panels B, D F and H). Again, this is in good correspondence with the western data (Figure 2) which failed to detect protein in all stages of gestation.

Effects of Ouabain on contractility and intracellular Ca in NP, 10, 16 and 21 day pregnant rats

Contractile responses were analysed by measuring the percentage change in the integral of contractions compared to a similar control period where tissues were perfused with physiological saline alone. The ouabain concentrations were chosen to differentially inhibit the α subunits (α 1 more resistant than α 2 which in turn is more resistant than α 3) (Jewell and Lingrel, 1991) and contractile responses were investigated at the four stages of gestation. The lowest concentration at which any significant response was seen in both non-pregnant and term pregnant tissues was 50μM (data from lower concentrations not shown).

The most marked and consistent effect on contractility was on frequency, calculated as number of contractions in a 10-minute period. All samples, at all gestational stages, showed an increase in the frequency of contractions when exposed to 50, 75 or 100μM ouabain.

Figure 6 shows representative force recordings from NP, 10, 16 and 21 day pregnant rats for each of the three concentrations of ouabain studied, and the bottom panel gives the mean concentration for each dose (*n* = 4). It can be seen that the effects on contractility are dependent on the concentration of ouabain, with the largest difference occurring between 50 and 75 μM. The contractions of the uterus remained rhythmic and not tonic at all stages of gestation and with each ouabain concentration. The data also showed that the contractile activity in non-pregnant and term pregnant myometrium were consistently less affected by ouabain at each concentration, than the days 10 and 16 samples. As shown in the mean data the responses at day 16 were statistically larger than those at other gestations. These data are consistent with the protein data showing α subunits 2 and 3 being significantly more highly expressed at days 10 and 16 compared to non-pregnant and

term myometrium, with these functional differences being more apparent at the lower ouabain concentrations.

Figure 7 shows typical traces from simultaneously recorded contractions and intracellular Ca (from Indo-1 fluorescence) in non-pregnant rats. It can be seen that even at the highest ouabain concentrations there was no increase in basal Ca levels, consistent with there being no increase in basal force in these traces. The increases in active force were mirrored in the calcium transients in all experiments (n= 3).

Discussion

The aims of this study were to determine the expression and distribution of the α and β isoforms of the Na^+, K^+ ATPase and the regulatory FXYD proteins in rat myometrium at different stages of pregnancy, in order to better understand how the Na^+, K^+ ATPase may affect uterine function. These data are the first to examine the distribution of both α and β Na^+, K^+ ATPase isoforms in uterine tissue from non-pregnant rats and throughout gestation. It is now appreciated that the Na^+, K^+ ATPase activity is modified by the expression of different isoforms of its molecular subunits, and so a determination of their expression and isozyme combination in the myometrium will increase our understanding of how the Na^+, K^+ ATPase will contribute to excitation-contraction coupling during different stages of pregnancy. We show using biochemical, immunohistochemical and mRNA analyses that all three isoforms of both α and β subunits are expressed, along with FXYD1 in the myometrium. Interestingly three of these isoforms, α2, α3, and β2 change their expression with gestational changes suggesting that they are functionally regulated. The housekeeping isoforms α1 and β1 (Beguin et al., 2002), and β3 remained consistently expressed throughout all stages of pregnancy and in the non-pregnant myometrium. Detection of the β3 isoform was strong in all samples, in agreement with previous data suggesting that this isoform is widespread in its distribution (Arystarkhova and Sweadner, 1997); it appeared to be the dominant β isoform of the myometrium. We also found that changes in sensitivity to ouabain occurred as a result of pregnancy and in a dose-dependent manner. These changes are likely related to the aforementioned changes in isoform expression with gestation, as the

subunits vary in their sensitivity (Jewell and Lingrel, 1991). We also examined how inhibition of the Na^+, K^+ ATPase with low, medium and high ouabain concentrations affected Ca within the cell and found changes in the Ca transients, especially their frequency, underlay the force changes, but found little or no change in basal Ca or change from rhythmic to tonic contraction, as had been suggested from studies in other tissues (Hartford et al., 2004, Dostanic-Larson et al., 2006, Dostanic-Larson et al., 2005, Saini-Chohan et al., 2010).

Our study used a range of techniques to help build the fullest picture of the Na^+, K^+ ATPase isoforms in myometrium; mRNA transcripts, western blotting, immunohistochemistry, contractility assays and measurement of intracellular Ca. These different approaches provided a consistent pattern of data increasing confidence in the conclusions draw from the study. Western blotting from tissue homogenates will of course detect cells other than uterine myocytes, but it is estimated that the smooth muscle cells contribute >95% of the cellular content of the uterus (Wynn, 1977). In addition, western blotting is not as sensitive as immunohistochemistry. Our data suggests that all the gene products for α and β subunits in mRNA are transcribed into protein and detectable by immunohistochemistry, but in non-pregnant samples western blots failed to detect α3 and β2. In addition, western blots showed all three β isoforms but the predominance of β3 was a clear finding on all the tissue sections, using immunohistochemistry. Similarly, the findings concerning Na^+, K^+ ATPase inhibition were consistent when force alone was measured and when intracellular Ca was simultaneously with force; the frequency of contractions, and Ca transients, increased. These consistent outcomes allowed us to minimize the number of rats required, in keeping with ARRIVE guidelines, although, while clearly able to detect the major changes occurring in contractions, the reduced statistical power may have led to us missing some smaller effects as being significant, due to the biological variations affecting force.

The impetus for this investigation came from previous studies showing a difference in α isoform expression between non-pregnant and pregnant human myometrium (Floyd et al., 2010b). These data pointed to a dynamic regulation of Na^+, K^+ ATPase expression, but the unavailability of samples throughout human gestation, precluded

464 an investigation of this. The rat myometrium, which behaves very similarly to the
465 human myometrium for excitation-contraction coupling (Wray et al., 2015) provides
466 a good model for testing the hypothesis that gestation will produce specific changes
467 in Na^+, K^+ ATPase isoforms expression and distribution. That changes might be
468 anticipated with the alterations in membrane potential, ion channel and exchanger
469 expression and regulation, and increased contractility, necessary between early to
470 late gestation, underlay our hypotheses. It has also been reported that the excitatory
471 effect of PGE and PGF is followed by hyperpolarization due to Na^+, K^+ ATPase
472 stimulation and that this decreases the frequency of subsequent contractions
473 (Parkington et al., 1999). The amplitude of the hyperpolarization decreases during
474 labour, allowing contraction frequency to increase. Its persistence at this time
475 ensures complete relaxation between each single robust contraction, preventing
476 spasm of the uterus that would restrict blood flow to the fetus during delivery.
477 Changes in Na^+, K^+ ATPase activity were also considered due to literature showing
478 that sex steroids can influence Na^+, K^+ ATPase activity and isoform expression in
479 cardiac and uterine tissue; estradiol has a direct stimulatory on the cardiac Na^+, K^+
480 ATPase via K (Dzurba et al., 1997), due to increased expression, and phosphorylation
481 of the α1 subunit (Obradovic et al., 2014). Furthermore, previous studies suggest
482 that changes in α3 isoform expression can have functional consequences in uterine
483 smooth muscle, where decreased expression was correlated with reduced
484 contractility in estradiol-treated rats (Tsai et al., 2000). Progesterone can upregulate
485 the β1 isoform in mouse uterus (Deng et al., 2013), and α isoforms in rat uterus
486 (Chinigarzadeh et al., 2015), with these same authors also reporting that oestrogen
487 had the opposite effect and might lower reabsorption of uterine fluid Na. Although
488 the Na^+, K^+ ATPase is expressed in other smooth muscle cells, these studies are not
489 extensive or detailed (Dostanic et al., 2005, Shelly et al., 2004b, Burke et al., 1991,
490 Baker Bechmann et al., 2016, Mobasheri et al., 2003a, Mobasheri et al., 2003b), so it
491 is not possible to extrapolate from them to the uterus. The Na^+, K^+ ATPase current
492 has been directly measured in smooth muscle cells from mesenteric artery
493 (Nakamura et al., 1999), and it has also been reported that the smooth muscles
494 have fewer Na^+, K^+-ATPases than striated muscles, which also makes them harder to
495 study (Allen et al., 1991). These data and conclusions were largely gathered from

vascular smooth muscles and were not focussed on the different subunit isoforms. The uterus, especially at term is much more muscular and active than any other smooth muscle, with cells up to 0.5 mm long and large (nA) L-type Ca currents, and so may be expected to be closer to striated muscle than other smooth muscles in terms of activity and ionic demands, (Shmigol et al., 1998, Burke et al., 1991). Burke et al (1991) in colonic smooth muscle found that Na^+, K^+ ATPase isoenzymes were differentially expressed in electrically dissimilar regions of the muscle (Burke et al., 1991).

The constant expression of α1 and β1 and detection in all myometrial tissues underlines their primary role in maintaining intracellular Na and K levels. Given the fundamental and constant requirement of the myometrium to use electrical and chemical gradients, set up by the Na^+, K^+ ATPase, for its rhythmic activity, these findings underlie how important these subunits are to function. The functional effects of inhibiting α1 are discussed later. These findings concerning the α1 and β1 subunits are consistent with findings in other electrically excitable tissues, especially the heart, where the constant expression of the α1 subunit in hearts from all mammals examined, has been related to its constant requirement to maintain ionic gradients as it constantly beats (Orlowski and Lingrel, 1988a, Zahler et al., 1996, Zahler et al., 1993). They differ however from the findings on peri-implanatation mouse uterus where β1 was upregulated by progesterone (Deng et al., 2013), but this may reflect activity in endometrium and glands at this special period in development, and not the myometrium.

We also found the β3 isoform to be unchanged with gestation. Although not extensively studied in intact preparations, this isoform is thought to influence ion transport by modifying ionic affinities (Jaisser et al., 1994, Hilbers et al., 2016). Our data would point to these being essential properties for the Na^+, K^+ ATPase expressed in myometrium at all stages of pregnancy.

Alpha 2 and 3 were both found to be increased significantly (especially α3) in early- and mid- pregnancy and decreased significantly at term. This could be related to the need to maintain the myometrial membrane in a more hyperpolarized state and where Ca transients decreased throughout pregnancy until parturition. The localisation of α2 to the plasma membrane juxtaposed to the SR, is proposed to have

528 a significant role in regulating Ca via the NCX in smooth muscle, as microdomains of
529 Na are formed (Juhaszova and Blaustein, 1997). Changes in expression of the α2
530 isoform have been shown to have critical function in both cardiac and neuronal
531 activity (James et al., 1999, Moseley et al., 2003, Muller-Ehmsen et al., 2002). In
532 work on neonatal rats cerebellum, the ongoing hyperpolarization associated with
533 development has been directly related to stimulation of the Na^+, K^+ ATPase and
534 selective upregulation of α3 (Biser et al., 2000). Although we can find no direct
535 measurements on how these isoform changes affect membrane potential in the
536 uterus or any other smooth muscle, it is tempting to speculate the changes
537 contribute to the mechanisms maintaining resting membrane potential, but further
538 studies are required. The decrease in these isoforms at term is also consistent with
539 the known decrease in membrane polarization at this time, as excitability is
540 increased. In vascular smooth muscle, chronically reduced α2 in genetically modified
541 mice lead to increased vascular resistance and blood pressure, as Na^+, K^+ ATPase
542 activity was reduced, leading to increased Ca via entry on NCX (Zhang et al., 2005).
543 These findings are also consistent with our suggestion that the increased α2
544 expression in early and midterm myometrium may contribute to uterine relaxation.
545 Finally, Maxwell and colleagues reported decreased α2 protein expression in pre-
546 eclamptic women's myometria (Maxwell et al., 1998a). Preeclampsia is marked by
547 elevated blood pressure and constriction of vessels, our finding of increased α2
548 expression in mid-pregnancy is again consistent with it contributing to myometrial
549 relaxation at this time.

550 The only β isoform to change was β2 and this was the only isoform to
551 increase towards term. It has been suggested that this subunit isoform is important
552 for membrane trafficking and caveolae localization. It may therefore be that this is
553 used by the myometrium to increase the drive on contractility near term. As
554 mentioned earlier, caveolae are present in the myometrium in abundance and affect
555 signalling in phasic muscles (Babiychuk et al., 2004). There is however, little known
556 about which signalling pathways in any smooth muscle may be linked directly to the
557 Na^+, K^+ ATPase acting as a receptor for endogenous cardiotonic steroids (Xie and
558 Askari, 2002, Wasserstrom and Aistrup, 2005). We found that FXYD1 is the primary
559 isoform expressed in all myometrial samples. It is likely that the FXYD2a and b gene

560 is expressed in all uteri, as shown by the mRNA data, but may not be translated
561 (unless needed functionally).

562 The physiological studies presented here offer evidence of functional changes
563 occurring in the uteri of non-pregnant rats and those at different stages of gestation.
564 The three α-subunit isoforms, have been characterized, with, low (mM), high (μM)
565 and very high (nM), affinities for ouabain in the order α1, 2 then 3, (Blaustein, 1993,
566 Blaustein et al., 2002). Our finding that the myometrium showed increased
567 sensitivity to inhibition of the Na^+, K^+ ATPase with ouabain at days 10 and 16 day of
568 pregnancy, correlates with increases in expression of ouabain sensitive isoforms of
569 the Na^+, K^+ ATPase, i.e. α2 and α3, at these stages of gestation. These data also are
570 consistent with our biochemical and immunohistochemical findings. In the
571 myometrium, we found that active force and Ca transients significantly increase with
572 ouabain. The frequency of contractions and transients were the most obvious effects
573 of ouabain, suggesting that the membrane was becoming more depolarized. We did
574 not observe tonic contraction or rise in basal Ca. Data from a previous study on rat
575 myometrial preparations showed that 100-300μM ouabain induces a significant
576 increase in rhythmic contractions and no increase in resting force (Ausina et al.,
577 1996a). These findings are in broad agreement with ours. However, if, as functional
578 studies suggest (Monteith and Blaustein, 1998), that 100μM ouabain is sufficient to
579 inhibit even the highly resistant rat α1 isoform, the previous study could not shed
580 light on differ isoform contributions to these effects, and no gestational studies were
581 made and Ca was not measured. Interestingly it has been observed that high
582 circulating levels of endogenous ouabain are found during human pregnancy
583 (Vakkuri et al., 2000) and postnatally (Di Bartolo et al., 1995).

584 In summary, the different Na^+, K^+ ATPase subunit expression that we have
585 found with gestation, allows its activity to be optimized to its role in the
586 myometrium. These findings agree with and extend findings from earlier, preliminary
587 studies reporting isoform switching in pregnancy in rat and human myometrium
588 (Esplin et al., 2003a, Maxwell et al., 1998b, Floyd et al., 2010b). This is because
589 trafficking, membrane domain, posttranslational modifications and ionic affinities
590 and susceptibility to glycosides can all be changed via the isoforms expressed. We
591 have also found functional difference, especially on contraction and Ca transient

592 frequency, to Na^+, K^+ ATPase inhibition, which are gestation dependent. There
593 remains much work to be done on directly linking these changes to problems of
594 uterine function, such as preterm birth and dysfunctional labours, but findings in
595 preeclampsia and hypertension in pregnancy, suggest this would be worthwhile.
596

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955 **Figure Legends**

956 Figure 1 Analysis of mRNA transcripts corresponding to α 1-3, β 1-3 and FXYD2 a, b
957 and c isoforms of Na, K-ATPase in uteri from non-pregnant (NP) rats and gestation
958 days 10, 16 and 21 pregnant rats.

959 Figure 2 Quantitative Western blot analysis of Na, K-ATPase α, β and FXYD isoform
960 protein expression in uteri from non-pregnant (NP) rats and gestation days 10, 16
961 and 21 in pregnant rats. Blots are expressed as a % of relative intensity of beta actin
962 lane-controls; error bars are SD of 4 technical replicates; where dotted line $P<0.05$
963 filled line $P<0.001$

964

965 Figure 3 Distribution of Na, K-ATPase α 1-3 isoforms in uteri from non-pregnant (NP)
966 rats and gestation days 10, 16 and 21 pregnant rats, was determined by
967 immunohistochemistry using isoform specific antibodies on tissue microarrays.

968

969 Figure 4 Distribution of Na, K-ATPase β 1-3 isoforms in uteri from non-pregnant (NP)
970 rats and gestation days 10, 16 and 21 pregnant rats, was determined by
971 immunohistochemistry using isoform specific antibodies on tissue microarrays.

972

973 Figure 5 Distribution of FXYD1 & FXYD2 isoforms in uteri from non-pregnant (NP) rats
974 and gestation days 10, 16 and 21 pregnant rats, was determined by
975 immunohistochemistry using isoform specific antibodies on tissue microarrays.

976

977 Figure 6 Dose-dependent inhibition of alpha subunit isoforms of Na, K-ATPase with
978 50μM, 75μM and 100μM ouabain in myometrial strips from non-pregnant (NP) rats
979 and gestation days 10, 16, and 21 pregnant rats. Data are expressed as % of 10-
980 minute control period immediately preceding ouabain exposure where * denotes
981 $P<0.05$.

982

983 Figure 7 Relationship between force and calcium during ouabain inhibition of alpha
984 subunit isoforms of Na, K-ATPase with 50μM (A), 75μM (B) and 100μM (C) ouabain in
985 myometrial strips from non-pregnant (NP) rat.

986

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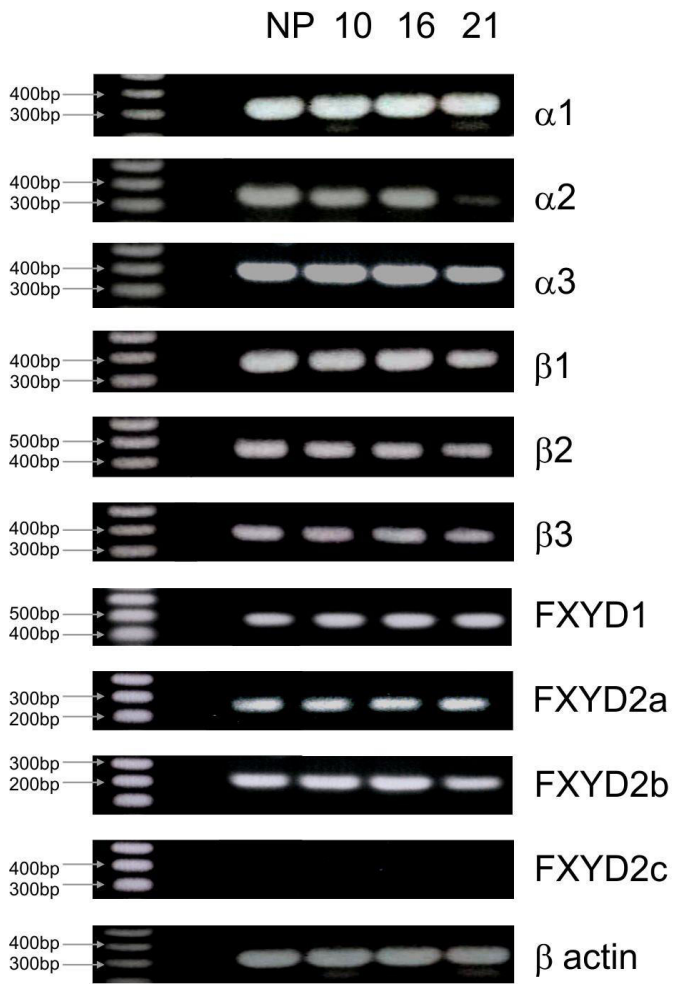


Figure 1 Analysis of mRNA transcripts corresponding to α 1-3, β 1-3 and FXYD2 a, b and c isoforms of Na, K-ATPase in uteri from non-pregnant (NP) rats and gestation days 10, 16 and 21 pregnant rats. Relative abundance was determined by comparing to the beta actin control.

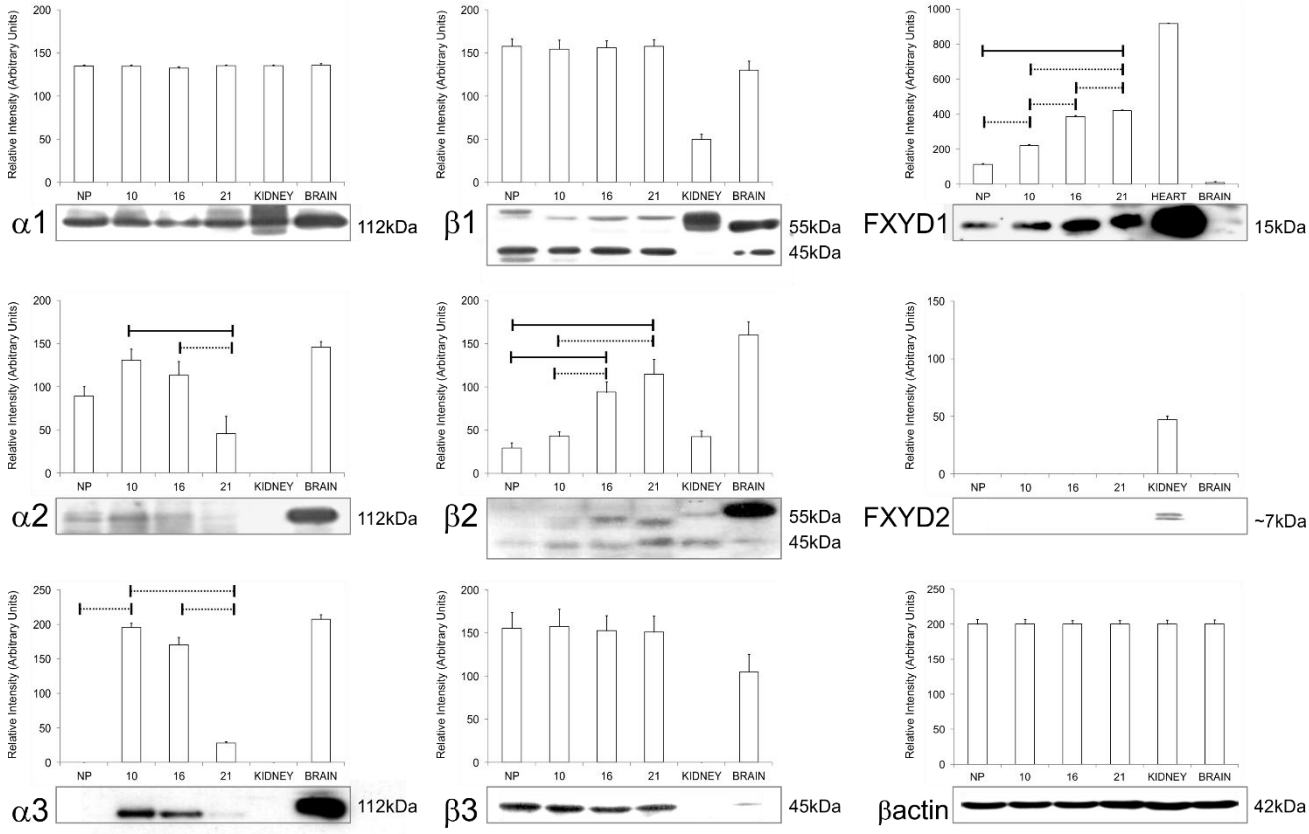


Figure 2 Quantitative Western blot analysis of Na, K-ATPase α, β and FXYD isoform protein expression in uteri from non-pregnant (NP) rats and gestation days 10, 16 and 21 in pregnant rats. Blots are expressed as a % of relative intensity of beta actin lane-controls; error bars are SD of 4 technical replicates; where dotted line P<0.05 filled line P<0.001

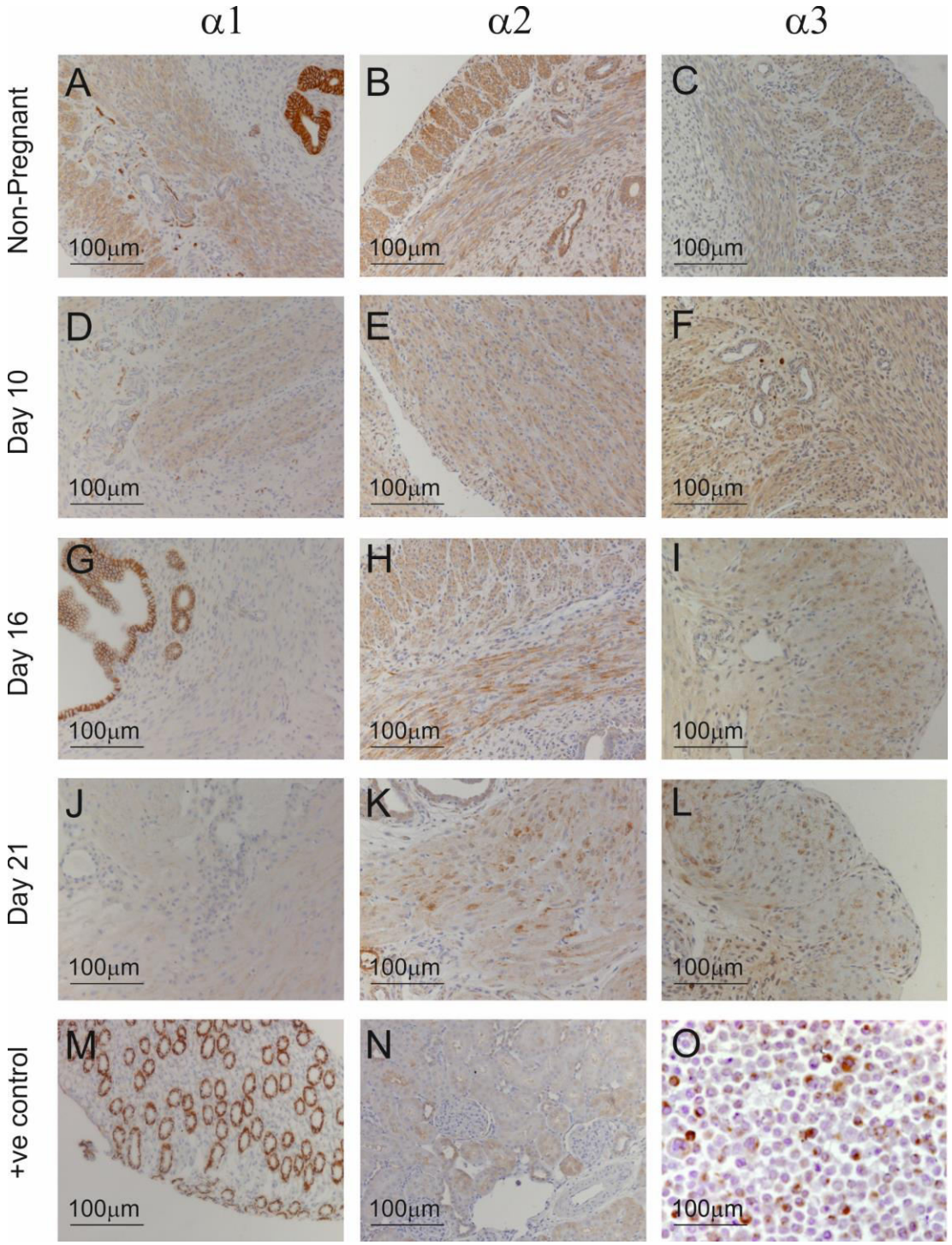
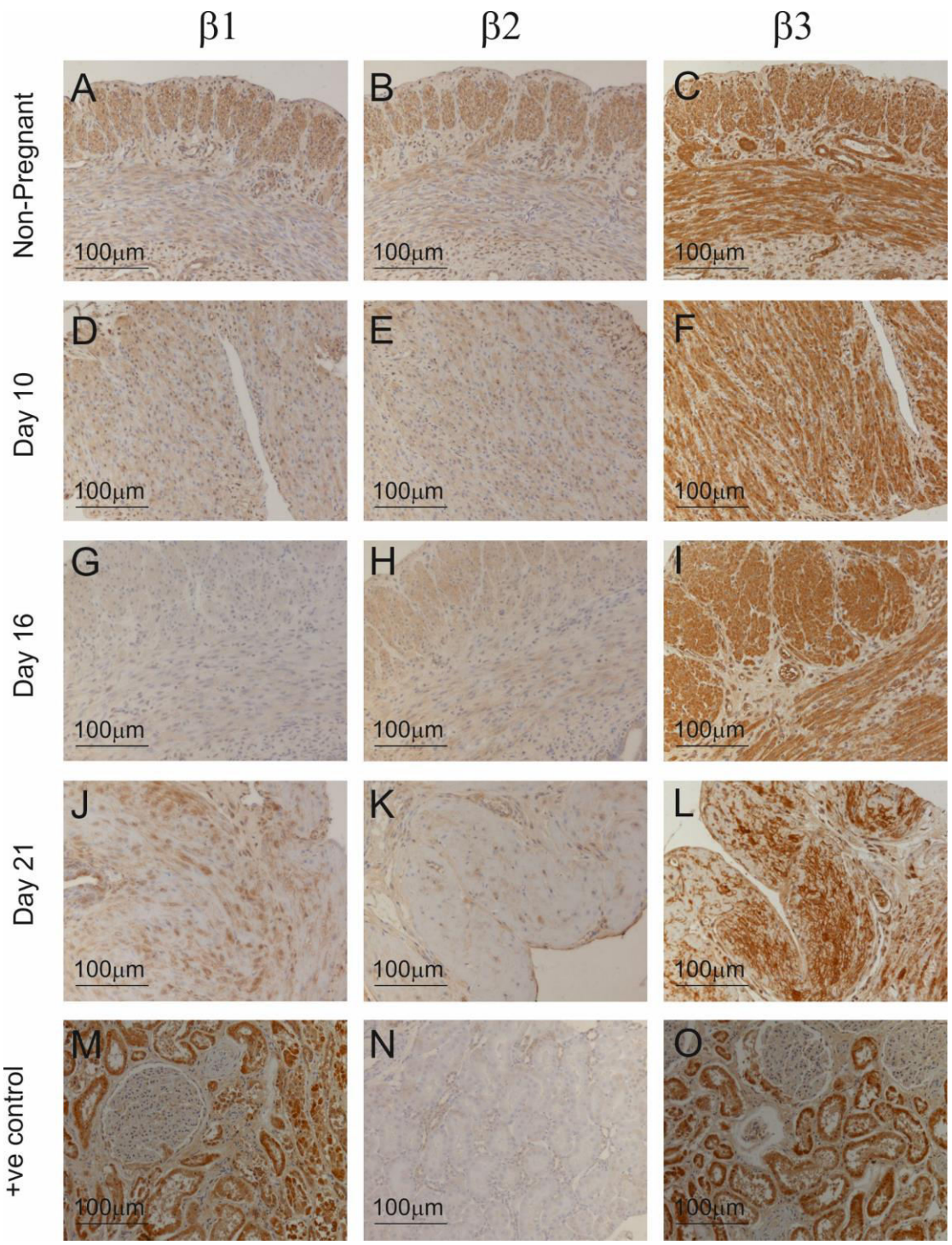


Figure 3 Distribution of Na, K-ATPase α 1-3 isoforms in uteri from non-pregnant (NP) rats and gestation days 10, 16 and 21 pregnant rats, was determined by immunohistochemistry using isoform specific antibodies on tissue microarrays.



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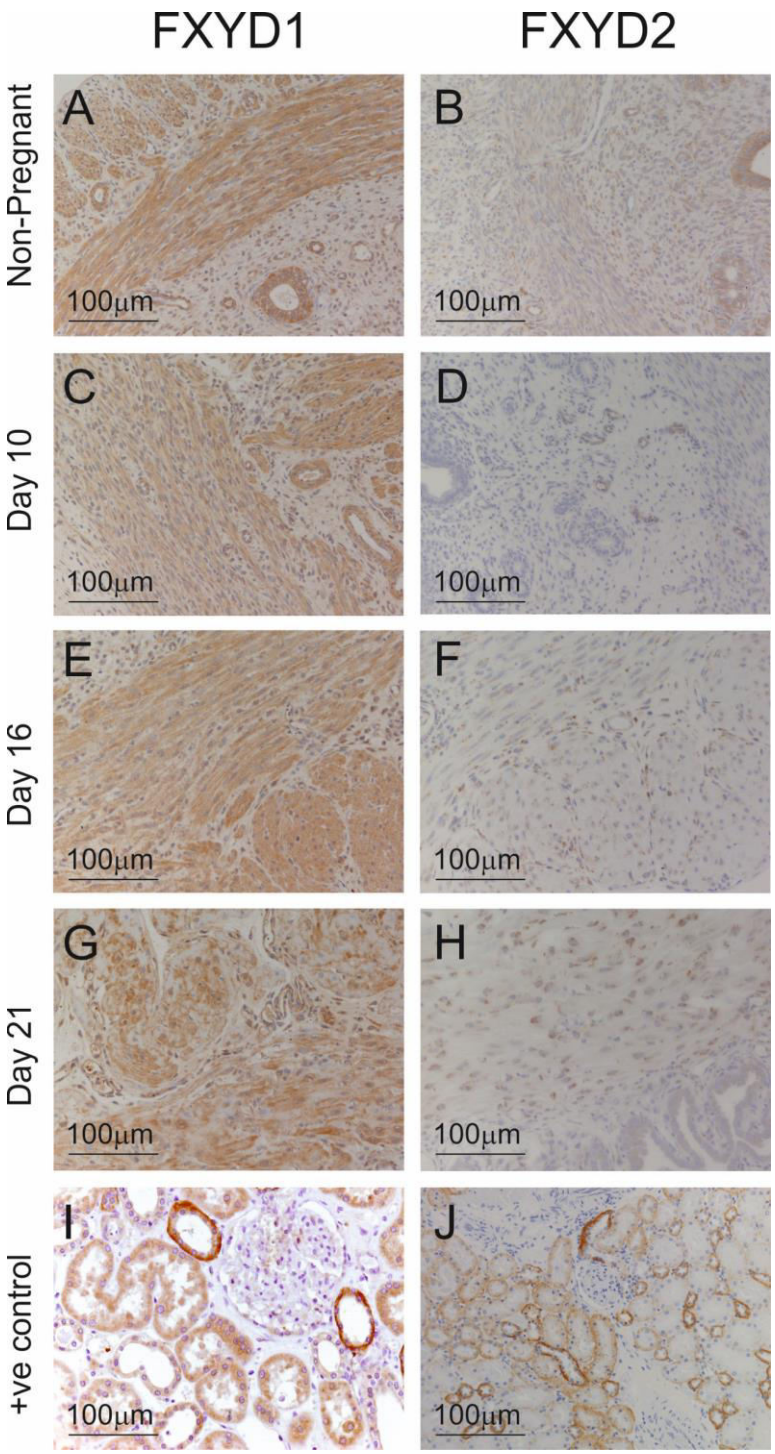

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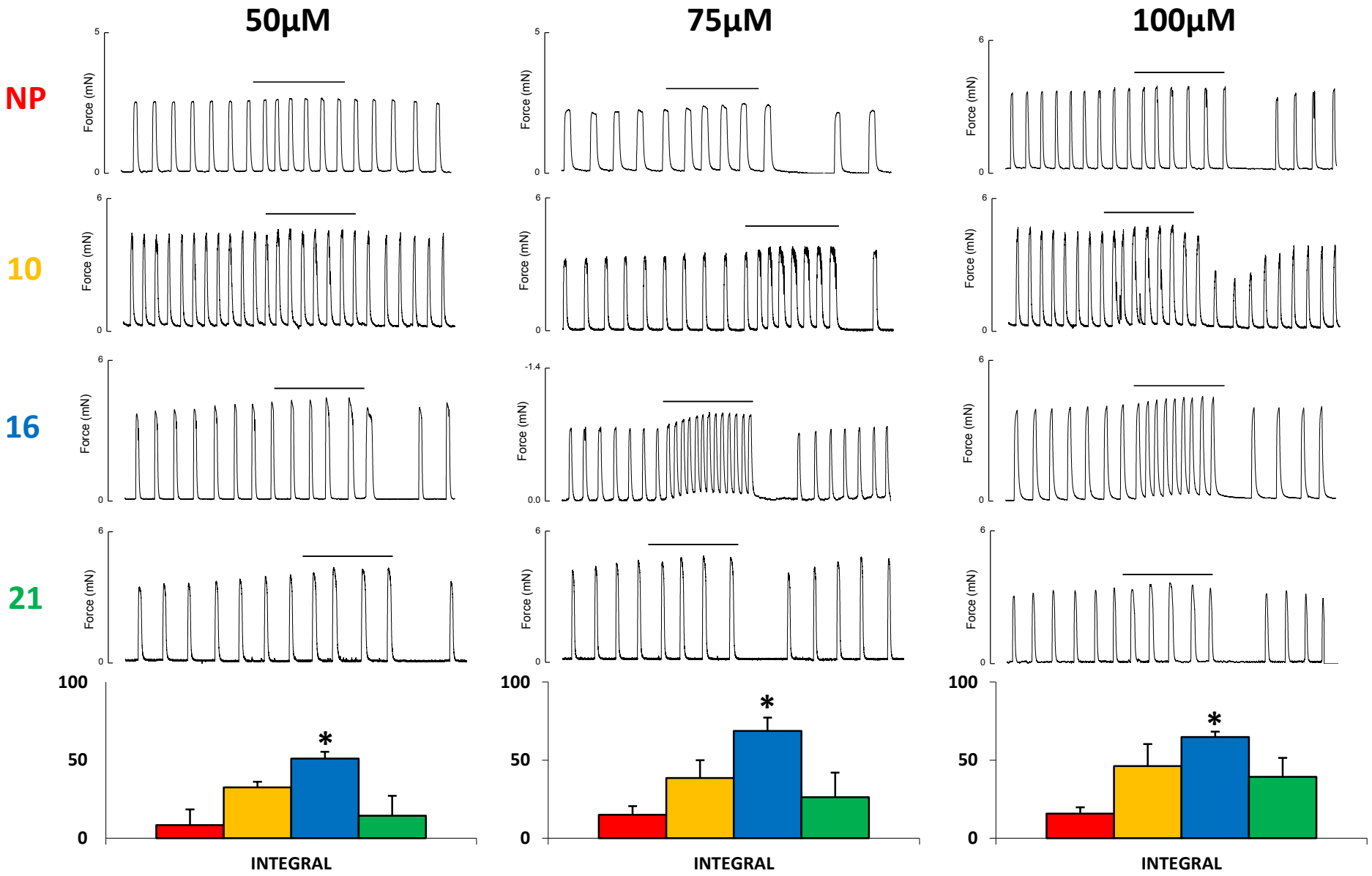

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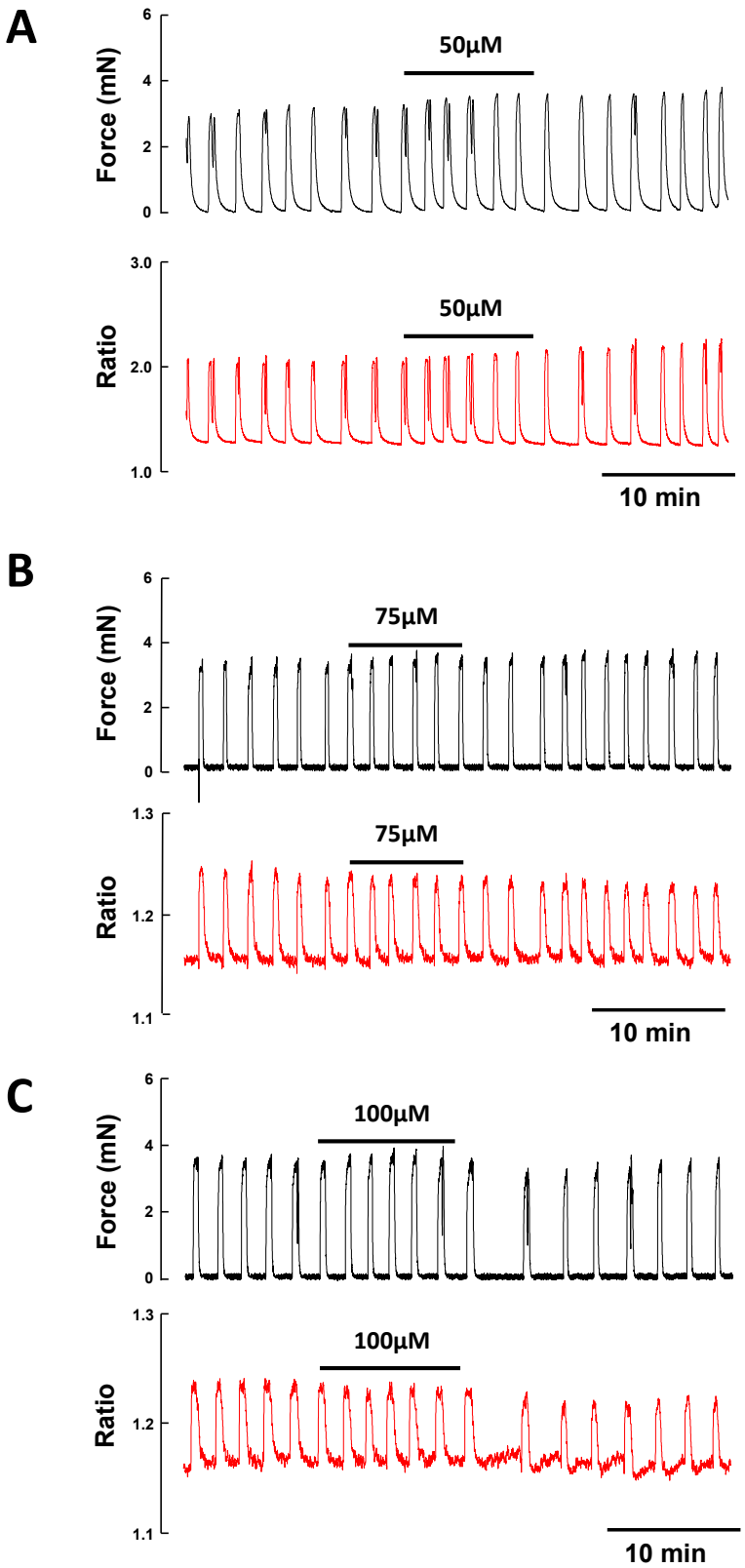
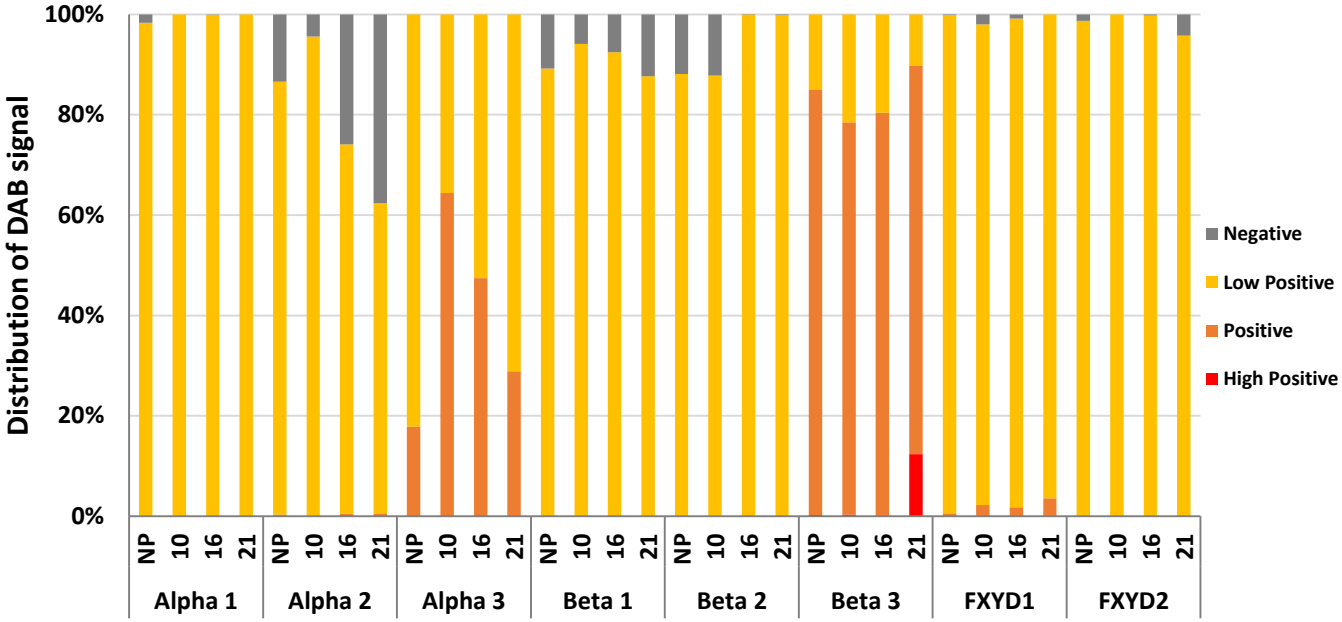


Figure 7 Relationship between force and calcium during ouabain inhibition of alpha subunit isoforms of Na, K-ATPase with 50µM (A), 75µM (B) and 100µM (C) ouabain in myometrial strips from non-pregnant (NP) rat.

A



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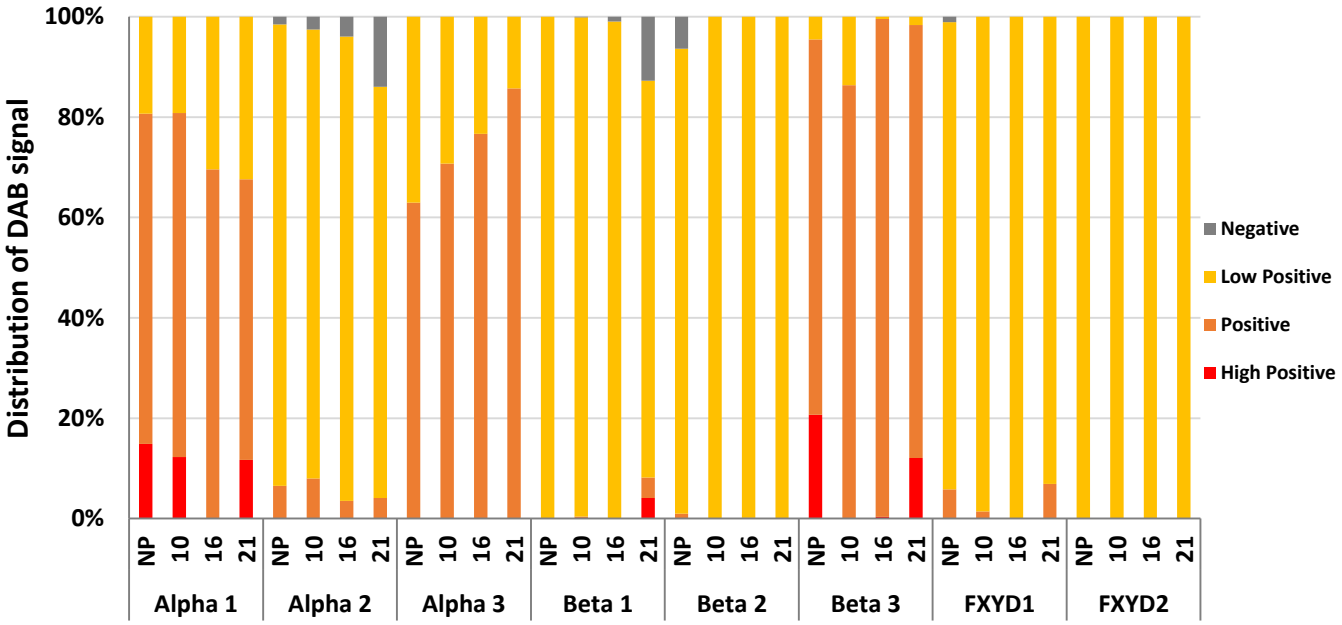

Figure S1 Quantitative evaluation of Na, K ATPase isoform immunoreactivity in (A) smooth muscle and (B) epithelial cell layers using spectral deconvolution