Specific effects of KChIP3/calsenilin/DREAM but not KChIPs1, 2 and 4 on calcium signalling and regulated secretion in PC12 cells.

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Abbreviations : DREAM, downstream regulatory element antagonistic modulator ; GH, growth hormone, KChIP, K^+ channel interacting proteins; NCS, neuronal calcium sensor; NCX Na^+/Ca^{2+} exchanger; PBS, phosphate-buffered saline, PBT, phosphate buffered saline containing 0.1% Triton X-100 and 0.3% BSA.

ABSTRACT

The K^+ channel interacting proteins (KChIPs) are members of the neuronal calcium sensor protein family of Ca^{2+}-binding proteins. It is unclear to what extent the KChIPs have distinct functions although they all interact with Kv4 K^+ channels. KChIP3 has also been shown to repress transcription of specific genes via binding to DRE motifs and all KChIPs may share this function. We have compared the function of isoforms of the four KChIPs. KChIPs1-4 were found to stimulate the traffic of Kv4.2 channels to the plasma membrane. KChIP3 expression in PC12 cells resulted in an increase in exocytosis evoked by activation of purinergic receptors. In contrast, KChIPs1, 2 and 4, although expressed to the same extent, had no effect on secretion. In addition, KChIP3 but not KChIPs 1, 2 and 4 modified the ATP-induced Ca^{2+} signal resulting in a delay in recovery after the peak Ca^{2+} elevation and also specifically resulted in down regulation of the NCX3 Na/Ca^{2+} exchanger NCX3 that could explain the effects on the Ca^{2+} signal and secretion. Regulation of NCX3 by KChIP3 has been shown to occur via its DREAM function (Gomez-Villafuertes et al. J Neurosci. (2005) 25, 10822-10830) suggesting that this activity might depend on the cellular context of expression of the various KChIPs. These results reveal a new role for KChIP3 in the regulation of Ca^{2+}-regulated secretion and also suggest that the functions of each of the KChIPs may be more specialised than previously appreciated.

INTRODUCTION

The transduction of intracellular Ca^{2+} signals into physiological responses is mediated by Ca^{2+} sensor proteins that interact with specific target proteins to modify their function [1]. Many of these Ca^{2+} sensors bind Ca^{2+} via EF-hand domains. One such family of proteins are the neuronal calcium sensor (NCS) proteins consisting of NCS-1, neurocalcin δ, hippocalcin, VILIPs1-3, recoverin, GCAPs1-3 and the K^+ channel interacting proteins (KChIPs)1-4 [2, 3]. Certain of the NCS proteins are expressed only in the retina (recoverin and GCAPs)[4], whereas others are expressed predominantly in the brain or in the case of NCS-1 in a wide range of neuronal and non-neuronal cell types. Mammals have at least 26 different NCS proteins generated by expression of 14 genes and by differential splicing [5]. The distinct cellular expression patterns of the NCS proteins, their distinct cellular and molecular properties and in particular their specific target proteins, allows them to regulate a diverse range of neuronal functions in a non-redundant manner [2]. The latter point is supported by the presence of clear phenotypes in organisms with genetic disruption of the expression of only a single NCS protein [2]. It is still unclear however, why so many different NCS proteins are required.

In mammals, KChIP proteins are encoded by four genes (KChIPs1-4) each of which can give rise to multiple splice variants [5]. The KChIPs were so named based on their discovery as proteins that can interact with the pore forming subunits of potassium channels of the Kv4 family [6, 7] [8]. The physiological significance of KChIPs 2 and 3 in the heart and the brain has been established from study of mouse knock-outs [9-11]. All KChIP proteins examined so far are able to become constitutive subunits of Kv4 channels [12, 13], stimulate Kv4 channel traffic to the plasma membrane[14] [15] and modify the gating properties of the channels [6]. Different KChIPs or their isoforms have, however, subtly different effects on Kv4 channel gating properties [8, 16, 17]. KChIP3 was independently discovered in two other contexts. First, as calsenilin a protein able to interact with the presenilins and affect the processing of amyloid precursor protein [18]. Second, as downstream regulatory element antagonistic modulator (DREAM) based on its ability to act as a repressor of transcription when it is Ca^{2+}-free but not Ca^{2+} -bound [19]. In addition, to the common interaction with Kv4 channels, isoforms of all four KChIPS have been shown to have DREAM activity [20] and at least KChIP4, in addition to KChIP3, can also interact with presenilins [7]. The KChIPs may not all have overlapping functions as knock-out of KChIP2 and 3 leads to non-compensated phenotypes [9-11]. The non-redundancy may be due, however, to differential expression of KChIPs in different cell types. The requirement for multiple KChIPs may relate to specific neuronal expression patterns [21, 22] allowing differences in fine tuning of Kv4 channel function, for example, in different neuronal cell types. Nevertheless, it is still unclear to what extent the KChIPs have fully overlapping or distinct functional roles. We have set out to compare the functions of representative isoforms of each of the four KChIPs and this has revealed a specific effect of KChIP3/calsenilin/DREAM in modifying agonist-evoked Ca^{2+} signals and in enhancing secretion from PC12 cells.

MATERIALS AND METHODS

PLASMIDS

Plasmid encoding presenilin-1-EGFP (PS-1-EGFP) [23] was a gift from Dr Christian Haass (Adolf-Butenandt-Institute, Department of Biochemistry, Ludwig-Maximilians-University, Munich, Germany). The KChIP splice variants used in this study were KChIP1.2, KChIP2.3, KChIP3.1 and KChIP4.1 but these will be referred to henceforth as KChIP1-4. All KChIP isoforms used are human, with the GenBank accession numbers as follows: DQ148477 (1.2), DQ148481 (2.3), DQ148485 (3.1) and DQ148487 (4.1). The KChIP1-ECFP, ECFP-Kv4.2 plasmids were described previously [15, 24]. The KChIP2-ECFP construct was made by inserting the KChIP2 sequence into the pECFP-N1 vector (Clontech, California, USA) following amplification from an existing KChIP2-pcDNA construct (kindly provided by Dr. Robert Bähring, Zentrum für Molekulare Neurobiologie, Hamburg, Germany) with the following primers (endonuclease sites underlined): the sense primer was 5'-ACTC<u>AGATCT</u>CAGGATGCGGGGCCAGGGCCGC-3' (BglII) and the antisense primer was 5'-TACC<u>GTCGAC</u>TGGATGACATTGTCAAAGAG-3' (SalI). The KChIP3-ECFP construct was made by inserting the KChIP3 sequence into the pECFP-N1 vector following amplification from an existing KChIP3-EYFP construct with the following primers: the sense primer used was 5'-ACTC<u>AGATCT</u>CAGGATGCAGCCGGCTAAGGAA-3' (BglII) and the antisense primer was 5'-TGCA<u>GAATTC</u>CGATGACATTCTCAAACAGC-3' (EcoRI). The KChIP4-ECFP construct was made by insertion of the KChIP4 sequence into the pECFP-N1 vector following amplification from a KChIP4 cDNA clone (OriGene, Rocksville, USA), with the following primers: the sense primer was 5'-ACTCAGATCTCAGGATGAATGTGAGGAGGGTG-3' (BglII) and the antisense primer was 5'-TACCGTCGACTGAATCACATTTTCAAAGAG-3' (SalI). The pcDNA-KChIP3 construct was made by the insertion of the KChIP3 sequence into the pcDNA3.1 (+) vector (Invitrogen), following amplification from KChIP3-EYFP with the following primers: the sense primer used was 5'-TTC<u>GAATTC</u>AGGATGCAGCCGGCTAAGGAA-3' (EcoRI) and the antisense primer was 5'-CAAC<u>GCGGCCGC</u>TAGATGACATTCTCAAACAGC-3' (NotI). The FLAG tagged KChIP constructs were made by insertion into the pFLAG-CMV-4 expression vector (Sigma, USA), following amplification from existing constructs. For FLAG-KChIP1, the sense primer was 5'-GCTT<u>GCGGCCGC</u>GAGGATGGGGGCCGTCATGGGC-3' (NotI) and the antisense primer was 5'-TATC<u>AGATCT</u>ATTTACATGACATTTTGAAACAG-3' (BglII). For FLAG-KChIP2, the sense primer used was 5'-GCTT<u>GCGGCCGC</u>GAGGATGCGGGGCCAGGGCCGC-3' (NotI) and the antisense primer was 5'-TATC<u>AGATCT</u>ATCTAGATGACATTGACAAAGAG-3' (BglII). For FLAG-KChIP3, the sense primer used was 5'-GCTT<u>GCGGCCGC</u>GAGGATGCAGCCGGCTAAGGAA-3' (NotI) and the antisense primer was 5'-TATC<u>AGATCT</u>CCCTAGATGACATTCTCAAACAGC-3' (BglII). For FLAG-KChIP4, the sense primer used was 5'-GCTT<u>GCGGCCGC</u>GAGGATGAATGTGAGGGTG-3' (NotI), and the antisense primer was 5'-TATC<u>AGATCT</u>ATTTAAATCACATTTTCAAAGAG-3' (BglII). All primers used in PCR amplification were produced by Genosys Biotechnologies (Cambs, UK) and the sequences of all the constructs were confirmed by sequencing by

DBS Genomics (Durham, UK).

CULTURE AND TRANSFECTION OF HELA AND COS-7 CELLS

HeLa and COS-7 cells were grown in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) containing 5% foetal calf serum (Gibco, Paisley, UK) and 1% non-essential amino acids (Gibco, Paisley, UK) and maintained in $75cm^2$ flasks at 37^oC in an atmosphere of 5% CO_2. At 16-24 hours prior to transfection, cells were seeded onto glass cover slips in a 24-well plate at 40,000 cells per well or 100,000 cells per well of a 6 well plate. Cells were then transfected at approaching 50% confluency using 100µl transfection reaction mixture. The transfection mix typically contained 1µg plasmid DNA (2µg for double transfections), 3µl (per µg plasmid DNA) Fugene (Roche, UK) and was made up to 100µl with Dulbecco's modified Eagle's medium (Gibco, Paisley, UK). The transfection reaction mixture was incubated at room temperature for 30 minutes before being added drop-wise to the cells. Cells were then maintained at 37^oC for an additional 48 hours before being used in experiments.

CULTURE AND TRANSFECTION OF PC12 CELLS

PC12 cells were grown in suspension in RPMI 1640 media (Gibco, Paisley, UK) containing 5%foetal calf serum (Gibco, Paisley, UK), 10% horse serum (Gibco, Paisley, UK), 100 units/ml penicillin and 0.1mg/ml streptomycin (Sigma) at 37^oC in an atmosphere of 5% CO_2. At 16-24 hours before transfection, freshly trypsinized cells were plated onto collagen-coated glass cover slips in a 24 well plate at a density of 200,000 cells/well or 350,000 cells/well for larger coverslips. Cells were then transfected using 100µl transfection reaction mixture consisting of 1µg plasmid DNA, 3µl LipofectAMINE 2000 transfection reagent (Invitrogen, UK) per µg of plasmid DNA and made up to 100µl in opti-mem (Gibco, Paisley, UK) The transfection mixture was incubated at room temperature for 30 minutes before being added drop wise to the cells. The cells were then incubated for 4-6 hours at 37^oC with the transfection reaction mixture before removing the transfection reaction mixture and replacing with growth media. Cells were then maintained at 37^oC, 5% CO_2 for an additional 48 hours before being used in experiments.

IMAGING OF CELLS AND IMUNOFLUORESCENCE STAINING

For imaging of transfected cells expressing fluorescent constructs, the cells were washed twice in phosphate buffered saline (PBS): 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM NaH_2PO_4, and fixed in PBS containing 4% formaldehyde for 30 minutes. Cells were then washed in PBS before air-drying the coverslips and mounted on glass slides using antifade Vectashield (Vector Laboratories Inc.). For immunostaining of cells attached to coverslips, these were washed twice in PBS before fixation in PBS containing 4% formaldehyde for 30 minutes. The cells were incubated in PBT (PBS, 0.1% Triton X-100, 0.3% BSA) for 30 min. The PBT was removed, replaced by the primary antibody diluted in PBT and the coverslips incubated for 1-2 hours. For staining of Kv4.2 channels after transfection of COS7 cells, rabbit anti-Kv4.2 antiserum (Exalpha Biologicals Inc, Maynard MA, USA) was used at a 1:500 dilution. For staining of endogenous NCX-2 and NCX-3 proteins, PC12 cells were incubated with mouse monoclonal anti-NCX2 at 1:50 or rabbit polyclonal anti-NCX3 at1:50. Anti-NCX2 and NCX3 were a kind gift from Professor

Kenneth Philipson, (Cardiovascular Research Laboratories, UCLA School of Medicine, LA, CA, USA). The cells were washed three times in PBT followed by incubation with an appropriate species-specific Dylight-649 conjugated secondary antibody (1:200; Pierce, Cramlington, UK) for 30 min prior to air drying and mounting of coverslips onto glass slides.

CONFOCAL LASER SCANNING MICROSCOPY
For confocal laser scanning microscopy, either a Leica AOBS microscope system (Leica, Heidelberg, Germany) or a Leica TCS-SP-MP microscope was used to examine transfected cells using an optimal pin hole of 1 airy unit and a 63x water immersion objective with a 1.2 numerical aperture. For imaging of live cells, cells were maintained in Krebs-Ringer buffer (20mM HEPES (pH7.4), 145mM NaCl, 10mM glucose, 5mM KCl, 3mM $CaCl_2$, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4). For imaging ECFP-tagged constructs, cells were excited at 430nm and light collected at 460-510nm. EGFP was imaged using excitation at 488nm with collection at 500-550nm. . For dual imaging of ECFP tagged KChIP proteins and Kv4.2 or endogenous NCX proteins labelled with Dylight-649, cells were excited with 405 nm and 633 nm lasers and light was collected at 465–500 nm (ECFP) and 680-750 nm (Dylight 649). Images were exported as Tiff files and compiled in CorelDraw.

For quantification of total ECFP fluorescence or anti-NCX-2 and NCX-3 fluorescence staining in PC12 cells, Leica-Lite post-capture analysis software (Leica Microsystems, Heidelberg, Germany) was used to draw regions of interest around the perimeter of cells. The fluorescence intensity of anti-NCX2 or NCX3 staining in KChIP-ECFP expressing cells was directly compared to control non-transfected cells on the same coverslips. For comparison of expression levels of KChIP1-4, transfected cells were examined for all constructs on the same day using identical imaging conditions with the same settings for magnification, laser power and gain.

WESTERN BLOTTING
Cells to be used for Western blotting were washed in PBS followed by lysis in 100µl Laemmli buffer (Sigma). Cell samples were then boiled for 10 minutes. Typically 20µl of sample was separated on a 10% SDS gel before overnight transfer onto nitrocellulose. Nitrocellulose blots were washed twice with PBS for 15 minutes before blocking with 3% milk in PBS for 45 minutes. The primary antibody was added at the appropriate dilution (anti-GFP at 1:5000) in 3% milk in PBS and incubated was added for 1 hour at room temperature or overnight at 4^oC, then removed and the blot washed three times in PBS. The blot was then incubated with the appropriate HRP-conjugated secondary antibody (Sigma) diluted to 1 in 400 in 3% milk, 0.5% Tween, in PBS for 1 hour. The blot was then washed once in PBS containing 0.5% Tween, washed 3 times in PBS, once in 0.3M NaCl in PBS for 30 minutes and once in distilled water for 15 minutes. The blot was then developed using Enhanced Chemiluminescence reagents (Amersham, Bucks, UK).

ASSAY OF GROWTH HORMONE SECRETION FROM PC12 CELLS
For assay of growth hormone (GH) secretion from transfected cells [25, 26], freshly trypsinized PC12 cells were plated out onto poly-D lysine-coated 24 well plates (VWR International, UK) at a density of 350,000 cells/well at 16-24 hours prior to transfection.

Cells were transfected using 100μl transfection reaction mixture consisting of 1μg control/test plasmid DNA, 1μg human Growth hormone (GH) plasmid, 3μl LipofectAMINE 2000 transfection reagent (Invitrogen, UK) per μg of plasmid DNA, made up to 100μl in opti-mem (Gibco, Paisley, UK). The transfection mixture was incubated at room temperature for 30 minutes before being added drop wise to the cells. The cells were then incubated for 4-6 hours at 37^oC with the transfection reaction mixture before removing the transfection reaction mixture and replacing with growth media supplemented with 10μM $CdCl_2$. Cells were then maintained at 37^oC, 5% CO_2 for a further 48 hours before use in experiments. The secretion assay was performed on either permeabilised or intact cells depending upon the experiment. In the case of the former, cells were washed once with KGEP buffer (20mM PIPES (pH 6.5), 139mM Kglutamate, 5mM EGTA, 2 mM ATP 2 mM $MgCl_2$) before permeabilisation with KGEP buffer containing 20μM digitonin (20mM PIPES (pH 6.5), 139mM Kglutamate, 5mM EGTA, 2 mM ATP 2 mM $MgCl_2$ 20μM digitonin) for 6 minutes. Cells were stimulated by addition of 300μl of KGEP buffer containing no added $CaCl_2$ or with $CaCl_2$ added to give a calculated free Ca^{2+} concentration of 10μM and incubated for 15 minutes. For experiments on intact cells, cells were washed with Krebs Ringer buffer and then stimulated by addition of 300μl of buffer with either 0 or 300μM ATP and incubated for 15 min at room temperature. After stimulation, supernatants were collected and stored before lysis of the remaining adherent cells in distilled water containing 0.5% (v/v) Triton X-100 (300μl/well for 15 minutes) to give corresponding samples containing unsecreted growth hormone. GH was assayed following the manufacturer's instructions in the hGH ELISA kit (Roche Diagnostics, Mannheim, Germany). The levels of GH were calculated and secreted GH expressed as a percentage of total GH for each well.

IMAGING OF INTRACELLULAR [Ca^{2+}] IN PC12 CELLS

For analysis of changes in intracellular free Ca^{2+} concentration ([Ca^{2+}]$_i$) in PC12 cells, the cells were plated onto collagen-coated glass coverslips (350,000 cells per well) and transfected with 2μg of control plasmid or 2μg of test plasmid using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. 48 hours after transfection, the cells were washed in Krebs-Ringer buffer and then incubated with 1μM X-rhod-1 AM (Molecular Probes Europe BV, Leiden, The Netherlands) in Krebs-Ringer buffer for 30 minutes at room temperature. Cells were washed in Krebs-Ringer buffer before imaging using laser-scanning confocal microscopy. An ECFP image was taken prior to each experiment to allow distinction between transfected and untransfected cells. X-rhod-1 was imaged using the Leica CS-SP-MP microscope with excitation at 543nm and light collection at 600-650nm. The X-rhod-1 fluorescence was diffusely and uniformly distributed, suggesting cytosolic localization of the probe. Before and after the application of 300μM ATP as an agonist, fluorescence of X-rhod-1 was monitored and fluorescence values for each cells expressed after normalisation to the initial fluorescence level for that cell (F/Fo).

RESULTS

Plasmids were constructed encoding four KChIP isoforms (KChIP1.2, KChIP2.3, KChIP3.1 and KChIP4.1) with each KChIP prepared as C-terminal ECFP or N-terminal FLAG-tagged constructs. Since the terminology used for KChIP isoforms in the literature is inconsistent we have defined these by their accession number in the Materials and Methods but will refer to them henceforth as KChIP1-4 for simplicity. In order to validate the functionality of the constructs and to confirm that the fluorescently-tagged KChIPs behaved as expected for the wild-type proteins, we assessed their expression in cell lines and their ability to stimulate the traffic of Kv4.2 to the plasma membrane based on imaging of expressed Kv4.2 [15]. In initial studies using HeLa cells we successfully found cells expressing KChIPs 1, 2 or 4 but found poor survival of cells expressing KChIP3-ECFP, consistent with the reported apoptotic effect of KChIP3 expression in HeLa cells [27]. In contrast, all four KChIPs were expressed to the same extent in COS-7 cells (Figure 1A) and did not appear to affect cell survival. The four KChIPs expressed as ECFP fusion proteins had differing intracellular localisations consistent with previous results [14] with KChIP1 present on intracellular punctate structures as seen previously [15], KChIP2 being plasma membrane associated and KChIPs 3 and 4 being diffusely distributed throughout the cells (not shown). As reported [15], Kv4.2 when expressed alone trafficked poorly to the plasma membrane and accumulated in the Golgi complex (Figure 1B). In cells co-transfected to express Kv4.2 along with one of the KChIP proteins, all the KChIPs co-localised with Kv4.2 and importantly all four KChIPs stimulated the traffic of the channel to the plasma membrane with all of the KChIPs also being associated with the plasma membrane (Figure 1) demonstrating a similar functional ability of the four KChIP isoforms studied to interact with Kv4.2 as seen in other studies [6, 14].

PC12 cells are a neuronal/neuroendocrine model cell line widely used in the study of Ca^{2+} signalling and Ca^{2+}-triggered secretion [28]. Over-expression of NCS-1 in these cells leads to increased secretion in response to agonist stimulation [29] via activation of phosphatidylinositol-4 kinase-IIIβ [30, 31]. Since KChIP3 has previously been shown to modify Ca^{2+} signalling in other cell types [32, 33] we aimed to examine the effect of over-expression of the KChIPs in PC12 cells using regulated secretion as an initial functional read out. All four KChIPs were expressed in PC12 cells following transfection (Figure 2A) although they had distinct intracellular localisations similar to that seen in COS7 cells (Figure 2B). KChIP1 showed a punctate pattern of localisation, KChIP2 was primarily associated with the plasma membrane and KChIPs3 and 4 were diffusely distributed in the cytosol and the nucleus. From the western blot (Figure 2A), the various KChIPs appeared to be expressed at different levels in PC12 cells. We observed, however, that the numbers of cells expressing the constructs differed between the KChIPs. In order to ensure that individual transfected cells expressed the same levels of each of the KChIP-ECFPs we quantified the average cellular fluorescence intensity of each of the expressed ECFP constructs taking into account total cellular fluorescence so that this would not be affected by differences in localisation. No differences were seen in overall cellular expression levels for the four KChIPs (Figure 2C).

To probe the effect of KChIP over-expression on secretion we made use of a well established assay in which cells were co-transfected so that they expressed human growth hormone (GH) as well as a test protein of choice. This approach is used so that only secretion from the small percentage (~5%) of transfected cells can be assayed. GH is not normally expressed by these cells but in transfected cells is packaged into dense core granules and secreted in response to Ca^{2+} elevation [26, 34] . This system was used to demonstrate a stimulatory effect on regulated secretion due to NCS-1 over-expression [29]. The PC12 cells were co-transfected to express GH and each of the KChIPs as FLAG-tagged constructs. GH secretion was assayed under basal conditions and in response to activation of purinergic receptors following addition of 300μM ATP that primarily activates P2X receptors to stimulate secretion in these cells [35, 36]. None of the KChIPs significantly affected basal release of GH but expression of FLAG-KChIP3 specifically increased the amount of GH release due to ATP-stimulation (Figure 3A). The FLAG tag was at the N-terminus of the proteins where it would prevent myristoylation of KChIP1 which is important for its membrane targeting [24].We also tested, therefore, the effect of transfection with untagged constructs in the pcDNA3 vector. In a comparison of KChIP1 and 3 it was observed that again KChIP3 expression increased ATP-stimulated GH release but KChIP1 did not (Figure 3B). These results also show that the effect of KChIP3 was not affected by the presence of the FLAG tag. In addition, expression of KChIP3 tagged at the C-terminus with ECFP also increased GH release in response to ATP (see below). The magnitude of the increase in evoked GH release due to KChIP3 expression was similar to that previously seen with NCS-1 over-expression [29].

The effect of KChIP3 on GH secretion in PC12 cells could be due to a direct effect on the exocytotic machinery. If this was the case then secretion directly triggered by increasing Ca^{2+} concentration to a fixed level would also be affected by KChIP3 expression. Alternatively, the effect could result from alterations in the Ca^{2+} signal generated in response to ATP. This was tested by examining the effect of KChIP3 on GH release from cells permeabilised with digitonin and challenged with 0 or 10 μM free Ca^{2+} [26]. In cells expressing ECFP tagged KChIP3, GH release was increased following ATP stimulation of intact cells as seen before but unaffected in permeabilised cells (Figure 4) suggesting an indirect effect of KChIP3 expression on exocytosis.

Over-expression of KChIP3 has been shown to result in an increase in the Ca^{2+} content of the ER [33] and to reduce the expression of one of the three [37] plasma membrane Na/Ca^{2+} exchangers NCX3 [32] . It is possible, therefore that the effect of KChIP3 on GH secretion could have been due to a modification in the Ca^{2+} signal following ATP stimulation. To find out if this was the case, and if any effect was specific for KChIP3, PC12 cells were transfected with each of the KChIPs tagged with ECFP, the cells loaded with the calcium indicator X-rhod-1 and changes in $[Ca^{2+}]_i$ in response to ATP monitored [38]. Before and after stimulation, cellular fluorescence levels were monitored from high ECFP-expressing cells and adjacent control non-transfected cells and the data for expressing and non-transfected cells on the same coverslips directly compared. Expression of ECFP alone had no effect on the $[Ca^{2+}]_i$ changes during ATP stimulation (data not shown). Expression of KChIPs1-4 had no significant effect on the peak increase in $[Ca^{2+}]_i$ following ATP addition (Figure 5). KChIPs 1,2 and 4 had no detectable effect on any

other aspect of the Ca^{2+} signal but KChIP3-ECFP resulted in a significantly elevated $[Ca^{2+}]_i$ during later times as the Ca^{2+} concentration was slowly declining (Figure 5C).

KChIP3 is known to interact with presenilin 1 and 2 and the presenilins have been shown to potentially affect $[Ca^{2+}]_i$ through an effect on ER Ca^{2+} by having a Ca^{2+} leak function [39]. Presenilins are widely expressed [40] including in PC12 cells and, therefore, it was possible that the effect of KChIP3 on Ca^{2+} signals and secretion could have been due to an interaction with presenilin. To test this possibility we examined the effect of expression of presenilin-1 itself on $[Ca^{2+}]_i$ and secretion and its effect of the enhancement of secretion by KChIP3. Cells were transfected to over-expression presenilin-1 using a presenilin-1-EGFP construct that has previously been shown to function like the wild –type protein [23]. Expression of presenilin-1-EGFP did not significantly modify the Ca^{2+} response to ATP stimulation (Figure 6A). In addition, presenilin-1-EGFP expression did not affect GH secretion (Figure 6B). We confirmed that co-transfection allowed the expression of both presenilin-1-EGFP and KChIP3-ECFP (Figure 6C). Expression of presenilin-1-EGFP had no effect on the stimulation of GH secretion due to KChIP3 expression (Figure 6D). These data did not, therefore, support an interaction between KChIP3 and presenilin underlying the effects on $[Ca^{2+}]_i$ and secretion. Similarly we did not see any significant effect on GH secretion or the enhancement of secretion by KChIP3 in PC12 cells transfected to express Kv4.2 channels (data not shown).

An alternative explanation for the effect of KChIP3 expression on reducing the decline of the Ca^{2+} transient would be by down-regulation of the NCX3 plasma membrane Na/Ca^{2+} exchanger. It has been shown that over-expression of wild-type or expression of a Ca^{2+}-insensitive and thus activated mutant of KChIP3 in SH-SY5Y cells results in suppression of transcription of NCX3 through DREAM activity while other NCX transporters are unaffected and expression of the mutant in cerebellar granule cells results in a slowing of the recovery of the $[Ca^{2+}]_i$ following a Ca^{2+} transient [32]. To test whether the specific effect of KChIP3 expression in PC12 cells could be explained by a reduction in NCX3 expression we used immunofluorescence with specific antisera to determine the expression levels of endogenous NCX3 and NCX2 as a control in transfected cells in comparison to non-transfected cells on the same coverslips. No differences were seen in NCX2 levels under any conditions (Figure 7). For NCX3 no differences were observed for cells expressing KChIPs 1, 2 or 4 but in the case of KChIP3 the fluorescence intensity of expressing cells was seen to be lower than that of adjacent non-transfected cells (Figure 7A). Quantification of the fluorescence indicated a specific reduction by KChIP3 of NCX3 levels in transfected cells compared to non-transfected control cells (Figure 7B).

DISCUSSION

From the study of the major known functions of the KChIPs no indications have so far emerged to suggest that they have specific physiological roles but rather have overlapping functions [41]. This is surprising given that the knock-out mice that have been studied for either KChIP2 or KChIP3 have observable phenotypes [9-11], indicating that the KChIPs cannot compensate for one another. One possibility is that KChIPs are non-redundant due to cell type-specific patterns of expression [21, 22]. Nevertheless, while the multiple KChIP isoforms have a common EF-hand containing domain they have distinct N-termini suggesting the possibility of distinct interactions and functions. We have now demonstrated a distinct effect of expression of KChIP3 compared to isoforms of KChIPs 1, 2 and 4 on Ca^{2+} responses to stimulation in PC12 cells that may be due to a specific effect on the expression of the NCX3 plasma membrane Na/Ca^{2+} exchanger and also have shown that this leads to specific physiological consequences in which KChIP3 but not KChIP1, 2 and 4 can enhance stimulus evoked secretion.

Previous work has shown effects of KChIP3 on Ca^{2+} signalling. In one case this was attributed to increased filling of ER Ca^{2+} stores [33]. The plasma membrane Na^+/Ca^{2+} exchangers play crucial roles in the recovery of $[Ca^{2+}]_i$ following transient elevations [42]. Expression of an EF-hand mutant of KChIP3 (Ca^{2+}-independent and thereby locked in an activating state) also resulted in a slowing of the recovery from a Ca^{2+} elevation in cerebellar granule cells [32]. This was attributed to a reduction in the levels of expression of the plasma membrane Na/Ca^{2+} exchanger NCX3 due to a direct repression of its transcription without effects on other exchangers NCX1 and NCX2.. This study also found that over-expression of wild-type KChIP3 in SH-SY5Y cells resulted in down regulation of NCX3. Consistent with these findings we found that expression of KChIP3 resulted in a down-regulation in the levels of NCX3 but not NCX2 in transfected PC12 cells. This finding provides an explanation for our observations on the effects of KChIP3 expression on the recovery of $[Ca^{2+}]_i$ following a Ca^{2+} transient.
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A stimulatory effect of KChIP3 was in response to agonist stimulation but was not seen in permeabilised cells in response to buffered Ca^{2+} concentration suggesting that the effect was most likely related to changes in Ca^{2+} signalling The changes in the Ca^{2+} signal in response to ATP in KChIP3 expressing cells provides an explanation for the increased secretion following stimulation. We can rule out effects of KChIP3 via Kv4 channels and also presenilin interactions. Traffic of Kv4 channels is stimulated by all four KChIPs but we saw no evidence that expression of Kv4.2 could influence exocytosis or the enhancement due to KChIP3. The interaction with presenilin has been described not only for KChIP3 [18] but also for KChIP4 [7] and their interactions have been suggested to modify aspects of Ca^{2+} signalling [43]. Our functional experiments did not, however, implicate a presenilin interaction in the KChIP3 effect on the Ca^{2+} response or secretion in PC12 cells. Expression of KChIP3 has been reported to trigger apoptosis in HeLa cells [27] but other KChIPs do not have this effect. Interestingly, in our initial experiments we found that that HeLa cells transfected with KChIP3 but not KChIPs 1, 2 and 4 failed to survive consistent with distinct physiological effects of these proteins. In other cell types such as PC12 and COS7 cells, KChIP3 does not appear to be apoptotic. There may be a link

between KChIP3-specific effects on intracellular Ca^{2+} as the pro-apoptotic effect of KChIP3 has been attributed to increased filling of EF Ca^{2+} stores [33].

The down regulation of NCX3 but not NCX1 or NCX2 following expression of wild type or mutant KChIP3 in other cell types was ascribed to the DREAM transcriptional regulator function of the protein and indeed the NCX3 gene was found to harbour DRE motifs in its promoter region [32]. It is surprising therefore that we observed down-regulation of NCX3 only as a consequence of KChIP3 expression and saw no effect of KChIP1 1,2 and 4 as the DREAM activity has been suggested to be shared by isoforms of all four KChIPs [20]. Indeed, it has been shown that the EF-hand containing core of KChIP3 is sufficient for Ca^{2+}-dependent binding to the DRE motif [44]. In addition, all four KChIPs were found to bind to DRE sites and to be equally efficient in repressing the transcription from three separate promoters [20]. In contrast, our data suggest that the KChIPs may differentially express this function depending on the cellular context. While this cannot be explained on the basis of expression levels it may be a consequence of the differential intracellular localisations of each of the proteins determined by elements in their variable N-terminal domains including motifs for myristoylation [24] and palmitoylation [45]. Further work will be required to determine the extent to which the DREAM activity in vivo is determined by KChIP3 alone. Overall our findings suggest that the KChIP proteins may have a higher level of functional specialisation than had been appreciated and in particular that differences in KChIP3 levels may specifically exert modulatory effects on regulated secretion and possibly neurotransmission.

FIGURE LEGENDS

Figure 1 Co-expression of KChIPs1-4 with Kv4.2 in COS-7 cells stimulates traffic of Kv4.2 to the plasma membrane.
A. COS-7 cells were transfected to express KChIP1-4 as ECFP tagged constructs and expression of the proteins detected by western blotting with anti-GFP. B, COS-7 cells were transfected to express Kv4.2 and 48 hours after transfection the cells were fixed and channel localisation detected by immunostaining with anti-Kv4.2 antibody. C-D, COS-7 cells were transfected to co-express Kv4.2 one of the KChIP-ECFPs as indicated and channel localisation detected by immunostaining with anti-Kv4.2. The colour overlays show the KChIP-ECFPs in green and Kv4.2 in red, with co-localisation seen in yellow. IN all cases traffic of the Kv4.2 to the plasma membrane was evident (arrowheads) along with KChIP localisation to the plasma membrane. The scale bar represents 10 μm.

Figure 2. Expression of KChIP-ECFPs in PC12 cells.
A. PC12 cells were transfected to express KChIP1-4 as ECFP tagged constructs and expression of the proteins detected by western blotting with anti-GFP. B, PC12 cells were transfected to express KChIP1-4 as ECFP tagged constructs and live cells were imaged 48 hours after transfection. KChIP1 was localised to punctate structures, whilst KChIP2 was found predominantly on the plasma membrane and KChIPs 3 and 4 appeared to be mainly cytosolic and also present in the nucleus. The scale bar represents 10 μm. C. The average fluorescence intensity of cells expressing each of the four KChIPs (n=12-18) was determined under identical imaging conditions to compare expression levels.

Figure 3 Expression of KChIP3 but none of the other KChIPs increases stimulated growth hormone release from PC12 cells. A. PC12 cells were transfected to express growth hormone (GH) and each one of the KChIPs as N-terminally FLAG-tagged constructs with the FLAG vector used as a control. Two days after transfection the cells were washed in Krebs-Ringer buffer and incubated with no additions (Basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels (n=4). B. PC12 cells were transfected to express growth hormone (GH) and KChIP1 or 3 in pcDNA3 as untagged constructs with the pcDNA vector used as a control. Two days after transfection the cells were washed in Krebs-Ringer buffer and incubated with no additions (Basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels (n=9).

Figure 4 KChIP3-ECFP increases stimulated release of growth hormone in intact but not in response to Ca^{2+} in permeabilised PC12 cells.
A. PC12 cells were transfected to express growth hormone (GH) and KChIP3-ECFP with the ECFP vector used as a control. Two days after transfection the cells were washed in Krebs-Ringer buffer and incubated with no additions (Basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels (n=12). B. PC12 cells were transfected to express growth hormone (GH) and KChIP3-ECFP with the ECFP vector used as a control. Two days after transfection the cells were washed, permeabilised with digitonin and incubated in the presence of 0 or

13

10 μM free Ca^{2+} for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels (n=6).

Figure 5 Expression of KChIP-3 but not KChIP1, 2 or 4 delays the decline of intracellular free Ca^{2+} concentration from the peak after stimulation with ATP.
PC12 cells were transfected to express KChIPs1-4 as ECFP tagged constructs and 48 hours after transfection were loaded with X-rhod-1 AM and then live cells were imaged. An ECFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before stimulation and after stimulation by perfusion with 300μM ATP. Images of ECFP and X-rhod-1 before and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean $\pm$ SEM. The numbers of cells for each condition were as follows: KChIP1, 19 control, 17 transfected; KChIP2, 35 control, 29 transfected; KChIP3, 19 control, 21 transfected; KChIP4, 22 control, 22 transfected. * indicates time points at which the values for KChIP3-ECFP transfected cells were significantly different from control values based on use of a Students' t-test with p values less than 0.05.

Figure 6 Expression of presenilin-1-EGFP has no effect on Ca^{2+} concentration after stimulation with ATP or on growth hormone release from PC12 cells.
A. PC12 cells were transfected to express presenilin-1-EGFP (PS1-EGFP) and 48 hours after transfection were loaded with X-rhod-1 AM and then live cells were imaged. An EGFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of EGFP and X-rhod-1 before stimulation and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean $\pm$ SEM. The numbers of cells for each condition were 28 for control and for 26 transfected conditions B. PC12 cells were transfected to express growth hormone (GH) and presenilin-1-EGFP (PS1-EGFP) with the ECFP vector used as a control (n=18). Two days after transfection the cells were washed in Krebs-Ringer buffer and incubated with no additions (Basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels. C. PC12 cells were transfected to express KChIP3 alone (a), presenilin-1-EGFP alone (b) or both proteins (c,c'). D. PC12 cells were transfected to express growth hormone (GH) and KChIP3-ECFP alone or in combination with presenilin-1-EGFP (n=12). Two days after transfection the cells were washed in Krebs-Ringer buffer and incubated with no additions (Basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels.

Figure 7. Effect of KChIP expression on the levels of NCX2 and NCX3 Na/Ca^{2+} exchangers. A. PC12 cells were transfected to express KChIP-ECFP constructs as indicated, fixed and stained with anti-NCX2 or NCX3. Microscope fields were examined that contained both transfected cells and non-transfected cells as seen in the ECFP images.

Cells transfected with KChIP3-ECFP showing lower NCX3 immunofluorescence levels are indicated by arrows. The scale bar represents 10 μm. B. Fluorescence levels from transfected and adjacent control non-transfected cells were determined (n=12) and the values for transfected cells were expressed as a percentage of the appropriate controls. * p<0.001 compared to non-transfected control cells.

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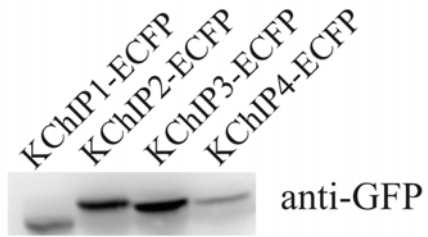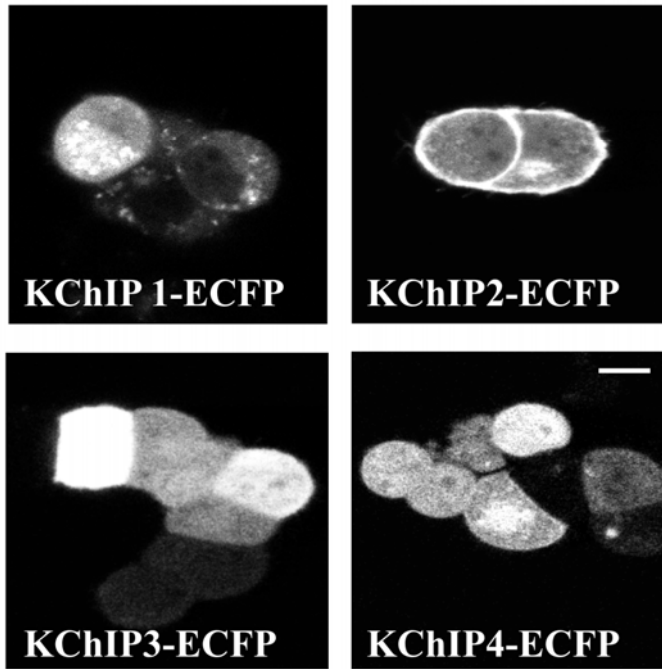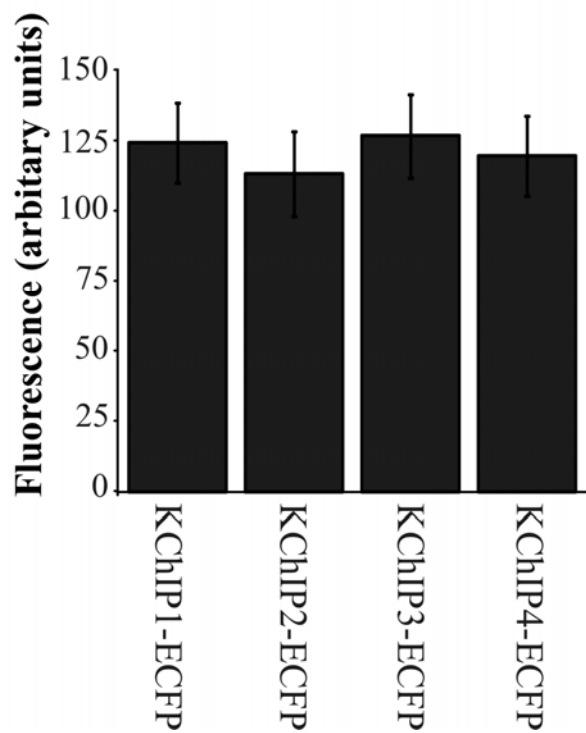
Figure 1

A   KChIP1-ECFP   KChIP2-ECFP   KChIP3-ECFP   KChIP4-ECFP

anti-GFP

B   Kv4.2-pcDNA

C   KChIP1-ECFP   Kv4.2-pcDNA   Overlay

D   KChIP2-ECFP   Kv4.2-pcDNA   Overlay

E   KChIP3-ECFP   Kv4.2-pcDNA   Overlay

F   KChIP4-ECFP   Kv4.2-pcDNA   Overlay

Figure 2

A

anti-GFP

B

KChIP 1-ECFP

KChIP2-ECFP

KChIP3-ECFP

KChIP4-ECFP

C

Figure 3

A

GH release (% of total)

ATP
Basal

p<0.01

FLAG vector   FLAG-KChIP1   FLAG-KChIP2   FLAG-KChIP3   FLAG-KChIP4

B

GH release (% of total)

ATP
Basal

p<0.02

pcDNA3   pcKChIP1   pcKChIP3

Figure 4

A

B

Figure 5

Figure 6

A

PS1-EGFP | X-Rhod-1 Basal | X-Rhod-1 +ATP

Normalised fluorescence (F/F0)
— PS1-EGFP
— Untransfected

Time (s)

B

GH release (% of total)

■ ATP
□ Basal

ECFP   PS1-EGFP

C

a — KChIP3-ECFP
b — PS1-EGFP
c — KChIP3-ECFP
c' — PS1-EGFP

D

GH release (% of total)

■ ATP
□ Basal

p<0.01   p<0.01

ECFP   KChIP3-ECFP   KChIP3-ECFP
                        + PS1-EGFP

Figure 7