Specific effects of KChIP3/calsenilin/DREAM, but not KChIPs 1, 2 and 4, on calcium signalling and regulated secretion in PC12 cells

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The KChIPs (K+ channel-interacting proteins) are members of the NCS (neuronal calcium sensor) protein family of Ca2+-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 (downstream regulatory element) motifs and all KChIPs may share this function. In the present study, we have compared the function of isoforms of the four KChIPs. KChIP1–4 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, KChIPs 1, 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 Ca2+ signal resulting in a delay in recovery after the peak Ca2+ elevation and also specifically resulted in down-regulation of the Na+/Ca2+ exchanger NCX3, which could explain the effects on the Ca2+ signal and secretion. Regulation of NCX3 by KChIP3 has been shown to occur via its DREAM (DRE antagonist modulator) function [Gomez-Villafuertes, Torres, Barrio, Savignac, Gabellini, Rizzato, Pintado, Gutierrez-Adan, Mellstrom, Carafoli and Naranjo (2005) J. Neurosci. 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 Ca2+-regulated secretion and also suggest that the functions of each of the KChIPs may be more specialized than previously appreciated.

Key words: calcium, EF-hand, exocytosis, Na+/Ca2+ exchanger, neuronal calcium sensor (NCS) protein, neurotransmitter release.

INTRODUCTION

The transduction of intracellular Ca2+ signals into physiological responses is mediated by Ca2+ sensor proteins that interact with specific target proteins to modify their function [1]. Many of these Ca2+ sensors bind Ca2+ via EF-hand domains. One such family of proteins are the NCS (neuronal calcium sensor) proteins consisting of NCS-1, neurocalcin δ, hippocalcin, VILIPs (visin-like proteins) 1–3, recoverin, GCAPs (GC-activating proteins) 1–3 and the KChIPs (K+ channel-interacting proteins) 1–4 [2,3]. Certain 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 the 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]. However, it is still unclear why so many different NCS proteins are required.

In mammals, KChIP proteins are encoded by four genes (KChIPs 1–4) 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 K+ channels of the Kv4 family [6–8]. The physiological significance of KChIPs 2 and 3 in the heart and the brain has been established from the study of mouse knockouts [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]. Secondly, as DREAM (downstream-regulatory-element-antagonist modulator), based on its ability to act as a repressor of transcription when it is Ca2+-free but not Ca2+ 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 knockout of KChIPs 2 and 3 leads to non-compensated phenotypes [9–11]. However, the non-redundancy may be due 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. In the present study 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 (where EGFP is enhanced green fluorescent protein) [23] was a gift from Dr Christian Haas (Adolf-Butenandt-Institute, Department of Biochemistry, Ludwig-Maximilians-University, Munich, Germany). The KChIP splice variants used in the present study were KChIP1.2, KChIP2.3, KChIP3.1 and KChIP4.1 but these will be referred to henceforth as KChIPs 1–4. All KChIP isoforms used are human, with the GenBank\textsuperscript{®} accession numbers as follows: DQ148477 (1.2), DQ148481 (2.3), DQ148485 (3.1) and DQ148487 (4.1). The KChIP1–ECFP (where ECFP is enhanced cyan fluorescent protein), ECFP–Kv4.2 plasmids have been described previously [15,24]. The KChIP2–ECFP construct was made by inserting the KChIP2 sequence into the pECFP-N1 vector following amplification from existing constructs. For FLAG–KChIP1, the sense primer was 5′-CCCTAGAGTACAATTTCAAAAGAGGAGGA-TGCGGGGCCAGGGCCGC-3′ (BglII) and antisense, 5′-TACCGTCGAC-TAGCAATTTCAAAAGAGGAGGA-TGCGGGGCCAGGGCCGC-3′ (BglII) (SalI). The KChIP3–ECFP construct was made by inserting the KChIP3 sequence into the pECFP-N1 vector following amplification from a KChIP4 cDNA clone (OriGene), with the following primers: sense, 5′-ACTCTAGATCTCAAGATGCAGATGCAGGCAATGAATGAGATGAGGAGGTG-3′ (BglII) and antisense, 5′-TACCGTCGAC-TAGCAATTTCAAAAGAGGAGGA-TGCGGGGCCAGGGCCGC-3′ (SalI). The KChIP4–ECFP construct was made by insertion of the KChIP4 sequence into the pECFP-N1 vector following amplification from KChIP4–EYFP with the following primers: sense, 5′-ACTCTAGATCTCAAGATGCAGATGCAGGCAATGAATGAGATGAGGAGGTG-3′ (BglII) and antisense, 5′-TACCGTCGAC-TAGCAATTTCAAAAGAGGAGGA-TGCGGGGCCAGGGCCGC-3′ (BglII) (SalI). All primers used in PCR amplification were produced by Genosys Biotechnologies and the sequences of all of the constructs were confirmed by sequencing with DBS Genomics.

**Culture and transfection of HeLa and COS-7 cells**

HeLa and COS-7 cells were grown in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) containing 5% FCS (foetal calf serum; Gibco) and 1% non-essential amino acids (Gibco) and were maintained in 75 cm\(^2\) flasks at 37°C in an atmosphere of 5% CO\(_2\). At 16–24 h prior to transfection, cells were seeded on to glass coverslips in a 24-well plate at 40000 cells/well or 100000 cells/well of a 6-well plate. Cells were then transfected at approaching 50% confluency using 100 μl of transfection reaction mixture. The transfection mixture typically contained 1 μg of plasmid DNA (2 μg for double transfections) and 3 μl (per μg of plasmid DNA) of FuGENETM (Roche) and was made up to 100 μl with DMEM. The transfection reaction mixture was incubated at room temperature (24°C) for 30 min before being added dropwise to the cells. Cells were then maintained at 37°C for an additional 48 h before being used in experiments.

**Culture and transfection of PC12 cells**

PC12 cells were grown in suspension in RPMI 1640 medium (Gibco) containing 5% (v/v) FCS, 10% (v/v) horse serum (Gibco), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma) at 37°C in an atmosphere of 5% CO\(_2\). At 16–24 h before transfection, freshly trypsinized cells were plated on to collagen-coated glass coverslips in a 24-well plate at a density of 200000 cells/well or 350000 cells/well for larger coverslips. Cells were then transfected using 100 μl of transfection reaction mixture consisting of 1 μg of plasmid DNA, 3 μl of Lipofectamine\textsuperscript{TM} 2000 transfection reagent (Invitrogen) per μg of plasmid DNA and made up to 100 μl in opti-MEM (Gibco) The transfection mixture was incubated at room temperature for 30 min before being added dropwise to the cells. The cells were then incubated for 4–6 h at 37°C with the transfection reaction mixture before removing the transfection reaction mixture and replacing with growth medium. Cells were then maintained at 37°C, 5% CO\(_2\) for an additional 48 h before being used in experiments.

**Imaging of cells and immunofluorescence staining**

For imaging of transfected cells expressing fluorescent constructs, the cells were washed twice in PBS and fixed in PBS containing 4% formaldehyde for 30 min. Cells were then washed in PBS before air-drying the coverslips and mounted on to glass slides using anti-fade Vectashield (Vector Laboratories). For immunostaining of cells attached to coverslips, these were washed twice in PBS before fixation in PBS containing 4% formaldehyde for 30 min. The cells were incubated in PBT (PBS containing 0.1% Triton X-100 and 0.3% BSA) for 30 min. The PBT was removed, replaced by the primary antibody diluted in PBT and the coverslips were incubated for 1–2 h. For staining of Kv4.2 channels after transfection of COS-7 cells, rabbit anti-Kv4.2 antiserum (Exalpha Biologicals) was used at a 1:500 dilution. For staining of endogenous NCX2 (Na\(^{+}/Ca\(^{2+}\) exchanger 2) and NCX3 proteins, PC12 cells were incubated with a mouse monoclonal anti-NCX2 antibody at 1:50 or a rabbit polyclonal anti-NCX3 antibody at 1:50. Anti-NCX2 and anti-NCX3 antibodies were a gift from Professor Kenneth Philipson, (Cardiovascular Research Laboratories, UCLA School of Medicine, Los Angeles, CA, U.S.A.). The cells were washed three times in PBT followed by incubation with an appropriate species-specific Dylight-649-conjugated secondary antibody (1:200: Pierce) for 30 min prior to air drying and mounting of coverslips on to glass slides.
Confocal laser scanning microscopy

For confocal laser scanning microscopy, either a Leica AOBS microscope system (Leica) or a Leica TCS-SP-MP microscope was used to examine transfected cells using an optimal pin hole of 1 airy unit and a 63× water-immersion objective with a 1.2 numerical aperture. For imaging of live cells, cells were maintained in Krebs–Ringer buffer [20 mM Hepes (pH 7.4); 145 mM NaCl, 10 mM glucose, 5 mM KCl, 3 mM CaCl2, 1.3 mM MgCl2 and 1.2 mM NaH2PO4]. For imaging ECFP-tagged constructs, cells were excited at 430 nm and light collected at 460–510 nm. EGFP was imaged using excitation at 488 nm with collection at 500–550 nm. 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-NCX2 and anti-NCX3 fluorescence staining in PC12 cells, Leica-Lite post-capture analysis software (Leica Microsystems) was used to draw regions of interest around the perimeter of cells. The fluorescence intensity of anti-NCX2 or anti-NCX3 staining in KChIP–ECFP-expressing cells was directly compared with control non-transfected cells on the same coverslips. For comparison of expression levels of KChIPs 1–4, 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 100 μl of Laemmli buffer (Sigma). Cell samples were then boiled for 10 min. Typically, 20 μl of sample was separated on SDS/PAGE (10% gel) before overnight transfer on to nitrocellulose. Nitrocellulose blots were washed twice with PBS for 15 min before blocking with 3% (w/v) non-fat dried skimmed milk in PBS for 45 min. The primary antibody was added at the appropriate dilution (anti-GFP at 1:5000) in 3% (w/v) non-fat dried skimmed milk in PBS and was incubated for 1 h at room temperature or overnight at 4°C. The antibody solution was then removed and the blot was washed three times in PBS. The blot was then incubated with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibody (Sigma) diluted 1:400 in 3% (w/v) non-fat dried skimmed milk and 0.5% Tween in PBS for 1 h. The blot was then washed once in PBS containing 0.5% Tween, washed three times in PBS, once in 0.3 M NaCl in PBS for 30 min and once in distilled water for 15 min. The blot was then developed using ECL® (enhanced chemiluminescence) reagents (Amersham).

Assay of GH (growth hormone) secretion from PC12 cells

For an assay of GH secretion from transfected cells [25,26], freshly trypsinized PC12 cells were plated out on to poly-D lysine-coated 24-well plates (VWR International) at a density of 350000 cells/well at 16–24 h prior to transfection. Cells were transfected using 100 μl of transfection reaction mixture consisting of 1 μg of control/test plasmid DNA, 1 μg of human GH plasmid and 3 μl of Lipofectamine® 2000 transfection reagent (Invitrogen) per μg of plasmid DNA, made up to 100 μl in opti-MEM (Gibco). The transfection mixture was incubated at room temperature for 30 min before being added dropwise to the cells. The cells were then incubated for 4–6 h at 37°C with the transfection reaction mixture before removing the transfection reaction mixture and replacing with growth medium supplemented with 10 μM CdCl2. Cells were then maintained at 37°C, 5% CO2 for a further 48 h before use in experiments. The secretion assay was performed on either permeabilized or intact cells depending upon the experiment. In the case of the former, cells were washed once with KGE buffer [20 mM Pipes (pH 6.5); 139 mM Kglutamate, 5 mM EGTA, 2 mM ATP and 2 mM MgCl2] before permeabilization with KGE buffer containing 20 μM digitonin for 6 min. Cells were stimulated by addition of 300 μl of KGE buffer containing no added CaCl2 or with CaCl2 added to give a calculated free Ca2+ concentration of 10 μM and incubated for 15 min. For experiments on intact cells, cells were washed with Krebs–Ringer buffer and then stimulated by the addition of 300 μl of buffer with either 0 or 300 μM 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 (300 μl/well for 15 min) to give corresponding samples containing unsecreted GH. GH was assayed following the manufacturer’s instructions in the hGH (human GH) ELISA kit (Roche Diagnostics). The levels of GH were calculated and secreted GH was expressed as a percentage of the total GH for each well.

Imaging of intracellular [Ca2+]i in PC12 cells

For analysis of changes in the intracellular free Ca2+ concentration ([Ca2+]i) in PC12 cells, the cells were plated on to collagen-coated glass coverslips (350000 cells/well) and transfected with 2 μg of control plasmid or 2 μg of test plasmid using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol. At 48 h after transfection, the cells were washed in Krebs–Ringer buffer and then incubated with 1 μM X-rhod-1 AM (rhod is Rhodamine and AM is acetoxymethyl ester; Molecular Probes) in Krebs–Ringer buffer for 30 min 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 543 nm and light collected at 600–650 nm. The X-rhod-1 fluorescence was diffusely and uniformly distributed, suggesting cytosolic localization of the probe. Before and after the application of 300 μM ATP as an agonist, the fluorescence of X-rhod-1 was monitored and the fluorescence values for the cells was expressed after normalization 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 a C-terminal ECFP- or N-terminal FLAG-tagged construct. 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 section but will refer to them henceforth as KChIPs 1–4 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
Figure 1 Co-expression of KChIPs 1–4 with Kv4.2 in COS-7 cells stimulates traffic of Kv4.2 to the plasma membrane

(A) COS-7 cells were transfected to express KChIPs 1–4 as ECFP-tagged constructs and expression of the proteins was detected by Western blotting with an anti-GFP antibody. (B) COS-7 cells were transfected to express Kv4.2 and 48 h after transfection the cells were fixed and channel localization was detected by immunostaining with an anti-Kv4.2 antibody. (C–F) COS-7 cells were transfected to co-express Kv4.2, one of the KChIP–ECFPs, as indicated and channel localization was detected by immunostaining with anti-Kv4.2. The colour overlays show the KChIP–ECFPs in green and Kv4.2 in red, with co-localization seen in yellow. In all cases, traffic of the Kv4.2 to the plasma membrane was evident (arrowheads) along with KChIP localization to the plasma membrane. The scale bar represents 10 μm.

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 localizations 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 (results not shown). As reported previously [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 of the KChIPs colocalized 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 Ca2+ signalling and Ca2+-triggered secretion [28]. Overexpression 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 Ca2+ signalling in other cell types [32,33] we aimed to examine the effect of overexpression 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 localizations similar to that seen in COS-7 cells (Figure 2B). KChIP1 showed a punctate pattern of localization, KChIP2 was primarily associated with the plasma membrane and KChIPs 3 and 4 were diffusely distributed in the cytosol and the nucleus. From the Western blot (Figure 2A) analysis, 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 localization. No differences were seen in overall cellular expression levels for the four KChIPs (Figure 2C).

To probe the effect of KChIP overexpression on secretion, we made use of a well-established assay in which cells were cotransfected so that they expressed human 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 response to Ca2+ elevation [26,34]. This system was used to demonstrate a stimulatory effect on regulated secretion due to NCS-1 overexpression [29]. The PC12 cells were cotransfected 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 300 μM 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]. Therefore we also tested the effect of transfection with untagged constructs in the pcDNA3 vector. In a comparison of KChIPs 1 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 stimulated GH release owing to KChIP3 expression was similar to that previously seen with NCS-1 overexpression [29].

The effect of KChIP3 on GH secretion in PC12 cells could be due to a direct effect on the exocytotic machinery. If this
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KChIP3 is known to interact with presenilin-1 and -2 and the presenilins have been shown to potentially affect [Ca\textsuperscript{2+}], through an effect on ER Ca\textsuperscript{2+} by having a Ca\textsuperscript{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\textsuperscript{2+} signals and secretion could have been due to an interaction with presenilin. To test this possibility we examined the effect of expression on presenilin-1 itself on [Ca\textsuperscript{2+}], and secretion and its effect of the enhancement of secretion by KChIP3. Cells were transfected to overexpress 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\textsuperscript{2+} response to ATP stimulation (Figure 6A). In addition, presenilin-1–EGFP expression did not affect GH secretion (Figure 6B). We confirmed that cotransfection 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). Therefore these results did not support an interaction between KChIP3 and presenilin underlying the effects on [Ca\textsuperscript{2+}], 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 (results not shown).

An alternative explanation for the effect of KChIP3 expression on reducing the decline of the Ca\textsuperscript{2+} transient would be by down-regulation of the NCX3 plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. It has been shown that overexpression of wild-type or expression of a Ca\textsuperscript{2+}-insensitive and thus activated mutant of KChIP3 in SH-SY5Y cells results in suppression of transcription of NCX3 through DREAM activity, whereas other NCX transporters are unaffected and expression of the mutant in cerebellar granule

Figure 2 Expression of KChIP–ECFPs in PC12 cells

(A) PC12 cells were transfected to express KChIPs 1–4 as ECFP-tagged constructs and expression of the proteins was detected by Western blotting with an anti-GFP antibody. (B) PC12 cells were transfected to express KChIPs 1–4 as ECFP-tagged constructs and live cells were imaged 48 h after transfection. KChIP1 was localized to punctate structures, whereas 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 in order to compare expression levels.
Figure 3 Expression of KChIP3, but none of the other KChIPs, increases stimulated GH release from PC12 cells

(A) PC12 cells were transfected to express GH and each one of the KChIPs as N-terminally FLAG-tagged constructs with the FLAG vector used as a control. At 2 days after transfection the cells were washed in Krebs–Ringer buffer and incubated with no additions (Basal) or with 300 μM ATP for 15 min. GH release was assayed and expressed as a percentage of the total cellular GH levels (n = 4). (B) PC12 cells were transfected to express GH and KChIPs 1 or 3 in pcDNA3 as untagged constructs with the pcDNA vector used as a control. At 2 days after transfection, the cells were washed in Krebs–Ringer buffer and incubated with no additions (Basal) or with 300 μM ATP for 15 min. GH release was assayed and expressed as a percentage of the total cellular GH levels (n = 9).

Figure 4 KChIP3–ECFP increases stimulated release of GH in intact cells, but not in response to Ca^{2+} in permeabilized PC12 cells

(A) PC12 cells were transfected to express GH and KChIP3–ECFP with the ECFP vector used as a control. At 2 days after transfection, the cells were washed in Krebs–Ringer buffer and incubated with no additions (Basal) or with 300 μM ATP for 15 min. GH release was assayed and expressed as a percentage of the total cellular GH levels (n = 12). (B) PC12 cells were transfected to express GH and KChIP3–ECFP with the ECFP vector used as a control. At 2 days after transfection the cells were washed, permeabilized with digitonin and incubated in the presence of 0 or 10 μM free Ca^{2+} for 15 min. GH release was assayed and expressed as a percentage of the total cellular GH levels (n = 6).

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 with 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 with 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 knockout 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, although the multiple KChIP isoforms have a common EF-hand-containing domain they have distinct N-termini suggesting the possibility of distinct interactions and functions. In the present study we have demonstrated a distinct effect of expression of KChIP3, compared with 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 we have also shown that this leads to specific physiological consequences in which KChIP3 but not KChIPs 1, 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 an 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. The latter
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Figure 5 Expression of KChIP3, but not KChIPs 1, 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 KChIPs 1–4 as ECFP-tagged constructs and 48 h 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 300 \(\mu\)M 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 normalized to the initial fluorescence for each cell and the results are shown as means ± S.E.M. The numbers of cells for each condition were as follows: KChIP1, 19 control and 17 transfected; KChIP2, 35 control and 29 transfected; KChIP3, 19 control and 21 transfected; KChIP4, 22 control and 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 Student’s t test with P values less than 0.05.

The study also found that overexpression 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\(^{2+}\)], following a Ca\(^{2+}\) transient.

A stimulatory effect of KChIP3 was in response to agonist stimulation but was not seen in permeabilized cells in response to buffered Ca\(^{2+}\) concentration suggesting that the effect was probably 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 (results not shown). The interaction with presenilin
Figure 6  Expression of presenilin-1–EGFP has no effect on Ca$^{2+}$ concentration after stimulation with ATP or on GH release from PC12 cells

(A) PC12 cells were transfected to express presenilin-1–EGFP (PS1–EGFP) and 48 h 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 300 μM 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 normalized to the initial fluorescence for each cell and the values are shown as means ± S.E.M. The numbers of cells for each condition were 28 for control and 26 for transfected conditions. (B) PC12 cells were transfected to express GH and presenilin-1–EGFP (PS1–EGFP) with the ECFP vector used as a control (n = 18). At 2 days after transfection, the cells were washed in Krebs–Ringer buffer and incubated with no additions (Basal) or with 300 μM ATP for 15 min. GH release was assayed and expressed as a percentage of the total cellular GH levels. (C) PC12 cells were transfected to express KChIP3 alone (a), presenilin-1–EGFP alone (b) or both proteins (c and c'). (D) PC12 cells were transfected to express GH and KChIP3–ECFP alone or in combination with presenilin-1–EGFP (n = 12). At 2 days after transfection, the cells were washed in Krebs–Ringer buffer and incubated with no additions (Basal) or with 300 μM ATP for 15 min. GH release was assayed and expressed as a percentage of the total cellular GH levels.

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]. However, our functional experiments did not 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 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 COS-7 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 KChIPs 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, the results of the present study suggest that the
Figure 7 Effect of KChIP expression on the levels of NCX2 and NCX3 Na⁺/Ca²⁺ 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 with non-transfected control cells.

KChIPs may differentially express this function depending on the cellular context. Although this cannot be explained on the basis of expression levels it may be a consequence of the differential intracellular localizations 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 specialization than had been appreciated and in particular that differences in KChIP3 levels may specifically exert modulatory effects on regulated secretion and possibly neurotransmission.

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