Effects of elevated expression of inositol 1,4,5-trisphosphate 3-kinase B on Ca^{2+} homoeostasis in HeLa cells

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Ins(1,4,5)P_3 3-kinase (IP3K) phosphorylates the Ca^{2+}-mobilizing second messenger Ins(1,4,5)P_3 to yield the putative second messenger Ins(1,3,4,5)P_4. A HeLa cell line was established expressing the rat B isoform of IP3K under the control of an inducible promoter. The IP3KB-transfected cell line possessed 23-fold greater IP3K activity than untransfected cells after induction of IP3KB expression, but only 0.23-fold greater activity when IP3KB expression was not induced. Elevating IP3KB expression significantly reduced levels of Ins(1,4,5)P_3 and increased levels of Ins(1,3,4,5)P_4 after stimulation of cells with histamine, but had no effect on basal levels. Histamine- and ATP-evoked cytosolic Ca^{2+} responses were dramatically reduced upon elevation of IP3KB expression. On stimulation with a supramaximal dose of histamine, 67 % of cells induced to express IP3KB gave no detectable elevation in cytosolic Ca^{2+}, compared with 3 % of uninduced cells. The quantity of Ca^{2+} within thapsigargin-sensitive and -insensitive stores was unaffected by elevation of IP3KB expression, as was capacitative Ca^{2+} entry. These data suggest that IP3KB may play a significant role in the regulation of Ins(1,4,5)P_3 levels, and consequently in Ca^{2+} responses following stimulation of cells with Ins(1,4,5)P_3-elevating agonists.

Key words: calcium, endoplasmic reticulum, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, signalling.

INTRODUCTION

Activation of numerous cell-surface receptors stimulates the hydrolysis of PtdIns(4,5)P_2 by phosphatidylinositol-specific phospholipase C to yield the second messengers Ins(1,4,5)P_3 and diacylglycerol [1]. By binding to a specific receptor, Ins(1,4,5)P_3 activates the release of Ca^{2+} into the cytosol from stores in the endoplasmic reticulum (ER). Ins(1,4,5)P_3 is metabolized by one of two pathways; either dephosphorylation by a 5-phosphatase to yield Ins(1,4)P_2 or phosphorylation by a specific Ins(1,4,5)P_3 3-kinase (IP3K) to yield Ins(1,3,4,5)P_4 [2,3]. There are data to suggest that Ins(1,3,4,5)P_4 has a role in Ca^{2+} homoeostasis via regulation of Ca^{2+} entry into the cell or release of Ca^{2+} from intracellular stores [4]. In addition, a Ras GTPase-activating protein has been cloned which binds Ins(1,3,4,5)P_4 with high affinity and high selectivity compared with other inositol phosphates, and has been proposed to function as an Ins(1,3,4,5)P_4 receptor [5]. Ins(1,3,4,5)P_4 is dephosphorylated by a 5-phosphatase to yield Ins(1,3,4)P_3, which can be metabolized via two pathways, one of which leads to the formation of several other inositol phosphates which have been proposed to perform signalling functions [6].

It is currently unclear whether the function of IP3K is principally to generate Ins(1,3,4,5)P_4 and its metabolites, or whether its action also serves to regulate Ins(1,4,5)P_3 levels. Since Ins(1,4,5)P_3 can also be metabolized by a 5-phosphatase, the action of IP3K could conceivably have little influence on Ins(1,4,5)P_3 levels. Mammalian IP3Ks are activated by Ca^{2+} to a greater extent than the 5-phosphatase responsible for the dephosphorylation of Ins(1,4,5)P_3, suggesting that the proportion of Ins(1,4,5)P_3 metabolized to Ins(1,3,4,5)P_4 by IP3K will increase when cytosolic Ca^{2+} is elevated [7,8]. This has been demonstrated in *Xenopus* oocytes, where IP3K has been shown to make a significant contribution to Ins(1,4,5)P_3 removal, particularly at high cytosolic [Ca^{2+}] [9].

To date, two mammalian isoforms of IP3K, designated A and B, have been cloned and characterized [10–12]. The two isoforms differ significantly in terms of their expression pattern, localization and regulation. The B isoform has been shown to exist in the cytosol and also peripherally, where it is associated with the cytosolic face of ER membranes, whereas the A isoform is cytosolic [13]. The method by which IP3KB associates with ER membranes is currently unknown. Both IP3K isoforms are activated on binding Ca^{2+}-bound calmodulin (CaM) and also by Ca^{2+}-bound CaM-dependent protein kinase II phosphorylation; however the extent of activation is significantly greater for the B isoform in both cases [7,8]. Interestingly, phosphorylation by c-AMP activated protein kinase has been shown to result in an increase in the activity of IP3KA and a decrease in the activity of IP3KB [14]. IP3KA expression is limited to the brain and testis, whereas IP3KB expression is widespread, having been detected in heart, brain, spleen, lung, skeletal muscle, kidney, testis, thymus and liver ([15]; T. H. Millard, S. Thomas and G. Banting, unpublished work). This suggests that the A isoform may perform a specialized, cell-type-specific function, and the B isoform has a more general role.

We were interested in studying the role of the widely expressed B isoform of IP3K in Ca^{2+} homoeostasis and assessing the significance of Ins(1,4,5)P_3 metabolism and Ins(1,3,4,5)P_4 production by the enzyme. HeLa cells were stably transfected with cDNA encoding IP3KB under the control of an inducible promoter. Elevating recombinant IP3KB expression was found

Abbreviations used: IP3K, Ins(1,4,5)P_3 3-kinase; fura-2 AM, fura 2 acetoxymethyl ester; CaM, calmodulin; ER, endoplasmic reticulum; EM, extracellular medium; CCE, capacitative calcium entry; TOH, Tet-off HeLa.

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to significantly reduce levels of Ins(1,4,5)P_3 and increase levels of Ins(1,3,4,5)P_4 following stimulation of cells with Ins(1,4,5)P_3-elevating agonists. It was found that inducing expression of IP3KB resulted in near total loss of Ca^{2+} mobilization in response to Ins(1,4,5)P_3-elevating agonists but did not affect capacitative calcium entry (CCE) or Ca^{2+}-store content. These data suggest that IP3KB may play a significant role in the regulation of Ins(1,4,5)P_3 levels and consequently Ca^{2+} responses following stimulation of cells with Ins(1,4,5)P_3-elevating agonists.

EXPERIMENTAL

All reagents were purchased from Sigma unless otherwise stated. Restriction enzymes were purchased from Roche Molecular Biochemicals. *myo*-[^{3}H]Inositol and [^{3}H]Ins(1,4,5)P_3 were purchased from NEN Life Science Products and [^{3}H]Ins(1,3,4,5)P_4 was prepared by incubation of [^{3}H]Ins(1,4,5)P_3 with recombinant IP3KB.

Cloning of the rat IP3KB coding region into pTRE

The coding region of rat IP3KB was amplified by PCR using primers 5′-TTGGTACCATATGGAAAGAGGTTCCCCGCG-3′ and 5′-ATAGATCTATCAGGTGAGTGGGCTGCCCT-3′. The PCR product was cloned into pGEM-T (Promega) and sequenced. The IP3KB coding region was excised from pGEM-T using *Ksp*I and *Bgl*II and ligated into pTRE (Clontech) which had been digested with *Ksp*I and *Bam*HI.

Cell culture

pTet-Off-transfected HeLa (TOH) cells (Clontech) were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal-calf serum (Life Technologies, Inc.), 100 units·ml^{-1} penicillin, 100 μg·ml^{-1} streptomycin (Life Technologies Inc.), 100 μg·ml^{-1} G418 (Life Technologies, Inc.) and 2 μg·ml^{-1} tetracycline. Cells were co-transfected with IP3KBpTRE and pMEP (Invitrogen) using Lipofectin (Life Technologies Inc.) according to the manufacturer's instructions. Note that pTRE does not have a mammalian-selectable marker but pMEP confers hygromycin resistance. Stable cell lines were selected using 500 μg·ml^{-1} hygromycin B (Roche Molecular Biochemicals) and maintained in 250 μg·ml^{-1} hygromycin B. When expression of recombinant IP3KB was desired, the medium was replaced with tetracycline-free medium 48 h and 24 h before use of the cells.

Preparation of whole-cell lysate

Cells were scraped from their culture dishes in a buffer consisting of 10 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 0.1 % (v/v) Triton X-100 and 1 mM PMSF. Cells were then lysed by 10 passages through a 25G needle and centrifuged in a Sorvall SS-34 rotor at 17000 ***g*** for 20 min at 4 °C. The supernatants were retained and protein concentration determined according to the method of Bradford [16].

Immunoblotting

Samples (50 μg) of whole-cell lysates were subjected to SDS/PAGE (8 % gel); the separated proteins were then blotted to nitrocellulose (Schleicher and Schuell). The blot was blocked overnight in PBS containing 0.05 % (v/v) Tween 20 and 5 % (w/v) dried skimmed milk, and then incubated for 1 h with antibody p52 (1 : 1000) in blocking buffer; p52 is a polyclonal antibody raised against rat IP3KB [13]. After several washes in blocking buffer, the blot was incubated for 1 h with a horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Sigma) (1 : 10000) in blocking buffer. The blot was washed in PBS/0.02 % (v/v) Tween 20 and antibody bound to the blot was detected by chemiluminescence (Western blotting kit; Roche Molecular Biochemicals).

Assay for IP3K activity

IP3K activity assays were performed at 37 °C in a buffer consisting of 84 mM Hepes (pH 7.5), 1 mg·ml^{-1} BSA, 2 mM ATP, 1 mM EGTA, 20 mM $MgCl_2$, 5 mM 2,3-bisphosphoglycerate and 10 μM Ins(1,4,5)P_3. [^{3}H]Ins(1,4,5)P_3 (5000 c.p.m.) was included in each assay. Assays were commenced by the addition of 7.5 μl of diluted whole-cell lysate to 7.5 μl of buffer, and were terminated by the addition of 10 μl of 80 mM HCl/120 μM Ins(1,4,5)P_3/120 μM Ins(1,3,4,5)P_4 (note that the raw lysate was diluted substantially in lysis buffer prior to the assay to achieve an appropriate IP3K concentration for assay). Samples were dried, resuspended in 0.3 M HCl/0.2 M KH_2PO_4 and then applied to a polyethyleneimine–cellulose TLC plate (Macherey-Nagel), which was developed in 0.5 M HCl. Phosphate-containing molecules on the plate were detected using the method of Clarke and Dawson [17]. Spots corresponding to Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were cut from the plate. Inositol phosphates were extracted from the resulting chips by incubation in 0.5 ml of conc. HCl for 15 min, with shaking, and the ^{3}H content of each extract was determined by scintillation counting. The IP3K activities derived were adjusted for total protein content of the sample.

[^{3}H]Inositol labelling and separation of [^{3}H]inositol phosphates

Cells were seeded onto 6-well plates and grown to 50 % confluence. Culture medium was removed and replaced with inositol-free Dulbecco's modified Eagle's medium (ICN Pharmaceuticals Ltd.) containing 10 % (v/v) dialysed foetal-calf serum (Labtech International Ltd.) and 10 μCi·ml^{-1} [^{3}H]inositol. The cells were then incubated for 30 h at 37 °C and then washed twice with extracellular medium (EM) consisting of 121 mM NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2$, 6 mM $NaHCO_3$, 9 mM glucose, 25 mM Hepes, 1.8 mM $CaCl_2$ (pH 7.4) [18]. For agonist-stimulated samples, the cells were then incubated in 300 μl of EM for 15 min at 37 °C. At the end of this period, 450 μl of pre-warmed EM containing histamine (final concentration 100 μM) was added rapidly and the reaction was terminated 8 s later by the rapid addition of 750 μl of ice-cold 10 % trichloroacetic acid. For unstimulated samples, cells were incubated in 750 μl of EM for 15 min at 37 °C and 750 μl of ice-cold trichloroacetic acid then added. The samples were incubated for 10 min on ice, and the well contents were transferred to a microfuge tube and centrifuged at 12000 ***g*** for 3 min. The supernatant containing inositol phosphates was removed and extracted five times with water-saturated diethyl ether; the samples were then neutralized using $NaHCO_3$ and frozen at −20 °C, after evaporation of residual diethyl ether. Inositol lipids were extracted from the pellet according to the method of Bligh and Dyer [19], and ^{3}H content was determined by scintillation counting. Inositol phosphates in the acid-soluble extracts were separated by HPLC using a 20 cm × 4.6 mm Partisil 10-SAX column (Phenomenex). A separation protocol based on that described by Cullen et al. [20] was used. The column was pre-equilibrated with 350 mM NaH_2PO_4 (pH 3.8). Inositol trisphosphate isomers were separated by isocratic elution with 550 mM NaH_2PO_4, and higher inositol phosphates were sequentially eluted by a linear gradient of [NaH_2PO_4], to 1.5 M.

Population Ca^{2+} measurements

Cells cultured to confluence in plastic dishes were detached by brief treatment with trypsin and pelleted by centrifugation at 150 ***g*** for 5 min. Cells were then resuspended in EM containing 2 μM fura 2 acetoxymethyl ester (fura-2 AM; Molecular Probes Inc.) and incubated at room temperature for 30 min. The cells were centrifuged, washed with EM and re-centrifuged, and finally resuspended in EM. Population Ca^{2+} measurements were then performed (LS-5B fluorimeter; Perkin–Elmer) and all recordings were made at 20–22 °C.

Single-cell Ca^{2+} measurements

Cells were grown to confluence on 22 mm coverslips, then washed with 2 ml of EM and incubated in 1 ml of EM containing 2 μM fura-2 AM for 30 min at room temperature. Cells were then washed twice with 2 ml of EM. Ca^{2+} imaging was performed using the Merlin ratiometric Ca^{2+}-imaging system (Perkin–Elmer), an UltraPix FKL300 digital camera and an Olympus IX50 inverted microscope fitted with a 40 × oil-immersion objective lens. Data were processed using Merlin software. All imaging was performed at 37 °C with constant perfusion. [Ca^{2+}] calibrations were calculated with Merlin software, using 340/380 nm ratio maxima and minima obtained by treatment of cells with 2 μM ionomycin followed by 20 mM EGTA.

RESULTS

Stable, inducible expression of recombinant IP3KB in HeLa cells

TOH cells were transfected with cDNA encoding rat IP3KB and clones isolated as described in the Experimental section. Whole-cell lysate was prepared from each clone following 48 h culture in the presence or absence of 2 $\mu g \cdot ml^{-1}$ tetracycline. Recombinant IP3KB expression was detected by immunoblotting using an antibody (p52) which recognizes rat IP3KB. Note that this antibody is species specific and does not recognize endogenous IP3KB in HeLa cells. Two clones (C6 and C11) were chosen for further study. Both exhibited a high level of expression of recombinant IP3KB when cultured without tetracycline and a high degree of inducibility (Figure 1). No recombinant IP3KB could be detected, by immunoblotting, in lysates prepared from either clone following culture of the cells in the presence of tetracycline.

Lysates prepared from C6 and TOH cells, cultured with or without 2 $\mu g \cdot ml^{-1}$ tetracycline, were assayed for total cellular IP3K activity. IP3K activity was not significantly different in TOH cells cultured with or without tetracycline (1.00 ± 0.07 and 1.01 ± 0.01 respectively); the IP3K activity of the C6-cell lysate was 1.23 ± 0.07 following culture with tetracycline and 22.84 ± 1.00 without tetracycline (i.e. 1.23- and 23-fold that of TOH cells respectively). Thus the increase in expression of recombinant IP3KB on removal of tetracycline, demonstrated by immunoblotting, correlates with an increase in total cellular IP3K activity. Despite the fact that no expression of recombinant IP3KB could be detected by immunoblotting following culture of C6 cells with tetracycline (Figure 1), the small but significant increase in IP3K activity over that of TOH cells suggests that some expression may be occurring. Alternatively, this increase could result from clonal variation in endogenous IP3K activity.

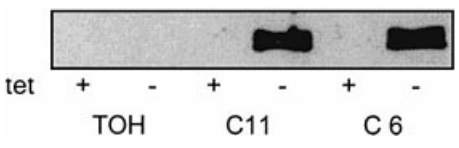

Figure 1 Tetracycline-regulated expression of IP3KB in transfected HeLa clones

Whole-cell lysates were prepared from TOH, C11 and C6 cells cultured with or without 2 $\mu g \cdot ml^{-1}$ tetracycline (tet) for 48 h. Lysate proteins (50 μg total protein/lane) were separated by SDS/PAGE (8% gel) and were then blotted on to nitrocellulose. The blot was probed with antibody p52, which recognizes rat IP3KB.

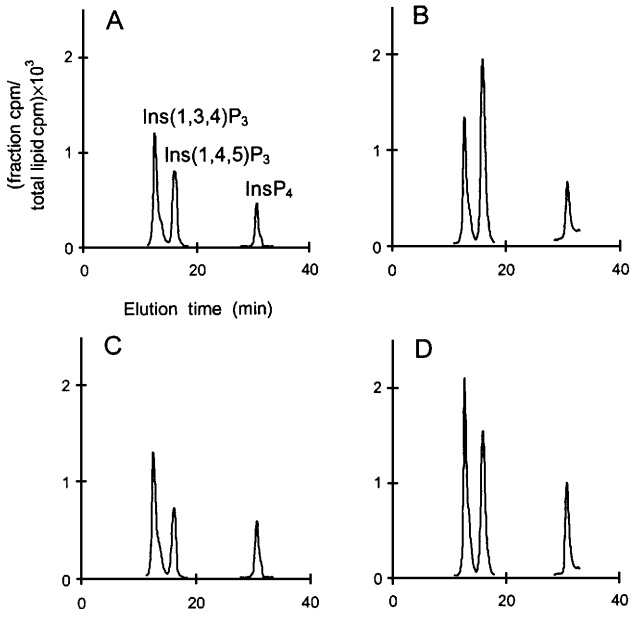

Figure 2 Analysis of Ins(1,4,5)P_3, InsP_4 and Ins(1,3,4)P_3 levels in C6 cells cultured with or without tetracycline

C6 cells were cultured in the presence of [^{3}H]inositol with or without 2 $\mu g \cdot ml^{-1}$ tetracycline. Inositol phosphates were then extracted. Extracted samples were subjected to HPLC and ^{3}H-label in the fractions was monitored. (**A**) Unstimulated C6 cells cultured with tetracycline. (**B**) C6 cells cultured with tetracycline and stimulated with 100 μM histamine for 8 s immediately before extraction. (**C**) Unstimulated C6 cells cultured without tetracycline. (**D**) C6 cells cultured without tetracycline and stimulated with 100 μM histamine for 8 s immediately before extraction. Axes labels in (**A**) apply to (**B**, **C** and **D**). The data shown were derived from a single representative experiment ($n = 3$).

Inositol phosphate levels in C6 cells cultured with or without tetracycline

Experiments were performed in order to investigate the effect of elevating IP3KB expression on cellular inositol phosphate levels. C6 cells cultured for 48 h with or without 2 $\mu g \cdot ml^{-1}$ tetracycline were labelled with [^{3}H]inositol, and HPLC was used to separate [^{3}H]inositol phosphates. HPLC elution times of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were identified using ^{3}H-labelled standards. Ins(1,3,4)P_3 has been shown previously to elute immediately before Ins(1,4,5)P_3 using this separation protocol [20]. Different isomeric forms InsP_4 are not separated using this protocol. For each HPLC run, counts of radioactivity were divided by total counts in inositol lipids for each sample, to control for labelling efficiency. Levels of Ins(1,3,4)P_3, Ins(1,4,5)P_3 and InsP_4 were found to be virtually identical in unstimulated C6 cells cultured with or without tetracycline (Figures 2A and 2C). ^{3}H-Labelled inositol phosphates were isolated from C6 cells following stimu-

lation of the cells with a supramaximal dose (100 μM) of histamine for 8 s. Histamine activates Ins(1,4,5)P_3 production and consequently Ca^{2+} mobilization in HeLa cells by binding to the G-protein-linked H1 receptor [21]. Histamine stimulation resulted in an increase in Ins(1,4,5)P_3 levels in C6 cells cultured both with and without tetracycline (Figures 2B and 2D). However, in C6 cells cultured without tetracycline (Figure 2D), the increase was reduced to approx. 75% of that observed for C6 cells cultured with tetracycline (Figure 2B). InsP_4 and Ins(1,3,4)P_3 levels were also increased on histamine stimulation, and for both inositol phosphates the increase was greater for C6 cells cultured without tetracycline; approx. 1.6-fold greater for InsP_4 and approx. 5-fold greater for Ins(1,3,4)P_3 (Figures 2B and 2D). These data indicate that [Ins(1,4,5)P_3] elevation following histamine stimulation is reduced when IP3KB expression is elevated and that this is caused by an increase in flux through IP3K, resulting in increased production of Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3.

Agonist-evoked Ca^{2+} mobilization in C6, C11 and TOH cells cultured with or without tetracycline

Experiments were performed in order to study the effect of elevating the expression of IP3KB on Ca^{2+} mobilization evoked in response to Ins(1,4,5)P_3-elevating agonists in cell populations. C6, C11 and TOH cells were cultured for 48 h with or without 2 $\mu g \cdot ml^{-1}$ tetracycline. Following detachment from the dishes in which they were cultured, cells were loaded with fura-2 AM and were maintained at 20–22 °C in EM buffer containing 1.8 mM $CaCl_2$ throughout the experiment. It was not possible to record cytosolic [Ca^{2+}] at temperatures greater than 22 °C, as leakage of fura-2 into the extracellular medium became significant and resulted in baseline drift. After recording basal [Ca^{2+}] for 30 s, cells were stimulated by the addition of 100 μM histamine, a supramaximal dose. For TOH cells (Figures 3A and 3B), a typical HeLa response to histamine was observed, consisting of a rapid rise in cytosolic [Ca^{2+}] followed by decline and plateau phases [18]. Results obtained for TOH cells cultured with or without tetracycline were not significantly different. For C6 cells cultured with tetracycline, a trace similar to that recorded for TOH cells was obtained, although the cytosolic [Ca^{2+}] elevations were somewhat reduced (Figure 3C). It is unclear whether this reduction was the result of clonal variation or a slight increase in IP3K levels. For C6 cells cultured without tetracycline, the Ca^{2+} response was substantially reduced when compared with C6 cells cultured with tetracycline (Figures 3C and 3D). Instead of exhibiting a large, distinct peak there was a small increase in the 340/380 ratio, which returned to basal levels within 50 s. Similar data were obtained when the experiments were repeated using a different clone, C11 (Figures 3E and 3F). In order to test that this effect was not limited to histamine responses, the experiments were repeated using ATP as the agonist. ATP stimulates Ins(1,4,5)P_3 production in HeLa cells via binding to a 5′-nucleotide receptor [22]. ATP was applied at 100 μM, a supramaximal dose. In order to examine the effect of sequential addition of two agonists, 100 μM histamine was added 200 s after ATP. C6 cells cultured without tetracycline exhibited a significantly reduced elevation in cytosolic Ca^{2+} in response to both agonists when compared with C6 cells cultured with tetracycline (Figures 3G and 3H).

Experiments were performed in order to study the effect of elevating IP3KB expression on agonist-evoked Ca^{2+} mobilization in single C6 cells. C6 cells were cultured on coverslips with or without 2 $\mu g \cdot ml^{-1}$ tetracycline for 48 h, loaded with fura-2 AM and then imaged using the Merlin Ca^{2+} imaging system. Cells

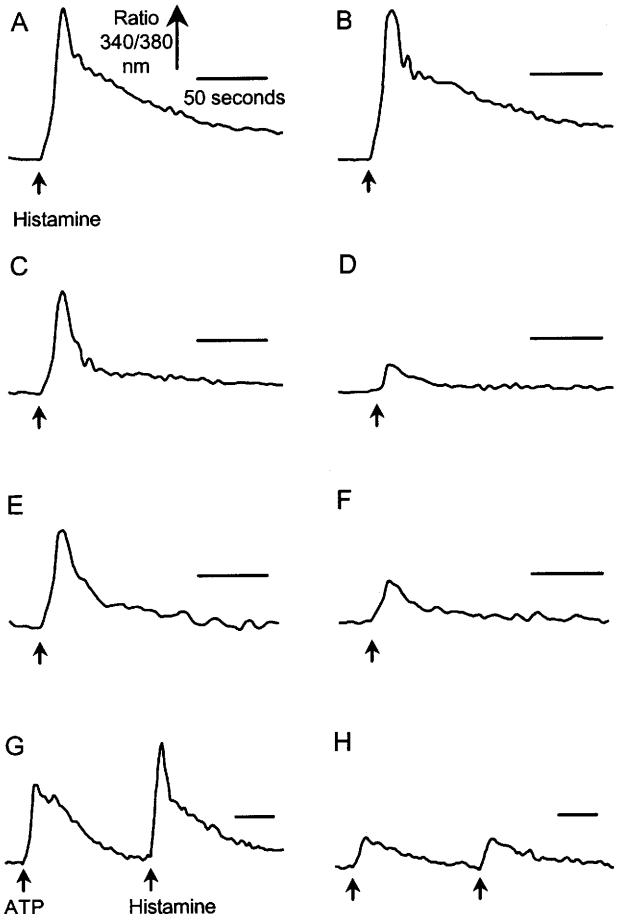

Figure 3 Agonist-evoked Ca^{2+} responses in populations of cells cultured with or without tetracycline

C6 cells were cultured with or without 2 $\mu g \cdot ml^{-1}$ tetracycline for 48 h and then loaded with 2 μM fura-2 AM. Changes in cytosolic [Ca^{2+}] for cell populations were measured using ratiometric fluorimetry. (**A**, **C**, **E**, **G**) Cells cultured with tetracycline. (**B**, **D**, **F**, **H**) Cells cultured without tetracycline. (**A**–**F**) Cells stimulated with 100 μM histamine at the times indicated by arrows: (**A** and **B**) TOH cells. (**C** and **D**) C6 cells. (**E** and **F**) C11 cells. (**G** and **H**) C6 cells stimulated with 100 μM ATP at the time indicated by the first arrow followed by 100 μM histamine at the time indicated by the second arrow. Scale bars = 50 s. Representative traces are shown ($n \geqslant 4$).

were bathed in EM containing 1.8 mM $CaCl_2$ at 37 °C and stimulated by perfusion with 100 μM histamine. Each field of cells imaged typically contained 15–20 cells, and changes in cytosolic [Ca^{2+}] following histamine addition were analysed individually for each cell. The data presented were derived from analysis of 127 C6 cells cultured with tetracycline and 118 C6 cells cultured without tetracycline. Individual cells exhibited various responses but, for simplicity, these have been divided into three classes: those exhibiting a clear elevation in cytosolic [Ca^{2+}] on histamine stimulation (full response), those exhibiting no detectable change in cytosolic [Ca^{2+}] on histamine stimulation (no response) and an intermediate class (partial response). Cells classified as giving a partial response exhibited a detectable increase in cytosolic [Ca^{2+}] on histamine stimulation, however, the Ca^{2+} response was of low magnitude (never rising above 100 nM) and consisted of a single elevation which slowly declined back to basal levels rather than appearing as rapid, distinct spikes. Of C6 cells cultured with tetracycline, 91% gave a full response, 6% gave a partial response and only 3% gave no

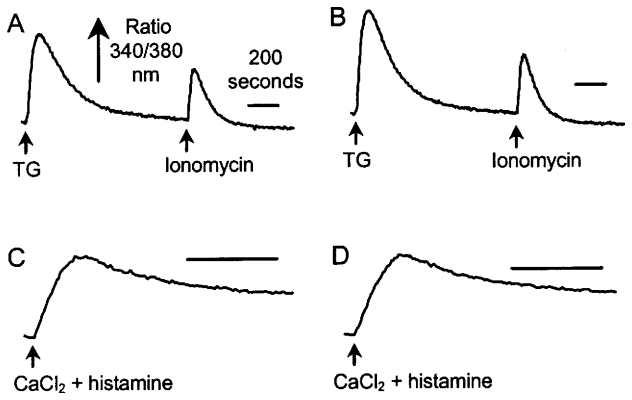

Figure 4 Ca^{2+} storage and CCE in C6 cells cultured with or without tetracycline

C6 cells were cultured with or without 2 $\mu g \cdot ml^{-1}$ tetracycline for 48 h and then loaded with 2 μM fura-2 AM. Changes in cytosolic [Ca^{2+}] for cell populations were monitored by ratiometric fluorimetry. (**A** and **B**) Ca^{2+} store content. Experiments were performed in the absence of extracellular Ca^{2+}. Addition of 2 μM thapsigargin (TG) is indicated by the first arrow; addition of 2 μM ionomycin is indicated by the second arrow. (**A**) C6 cells cultured with tetracycline. (**B**) C6 cells cultured without tetracycline. (**C** and **D**) CCE measurments. Cells were pretreated for 30 min with 2 μM thapsigargin in the absence of extracellular Ca^{2+}. The arrow indicates initiation of CCE by addition of 1.8 mM $CaCl_2$ and 100 μM histamine to the Ca^{2+}-free EM. (**C**) C6 cells cultured with tetracycline. (**D**) C6 cells cultured without tetracycline. Scale bars = 200 s. Representative traces are shown ($n \geqslant 4$).

detectable Ca^{2+} response. Only 8 % of C6 cells cultured without tetracycline gave a full response, with 67 % giving no detectable Ca^{2+} response and 25 % giving a partial response. This suggests that, for the majority of C6 cells cultured without tetracycline, Ca^{2+} mobilization in response to a supramaximal dose of histamine is entirely abolished. Immunofluorescence analysis of C6 cells cultured without tetracycline indicated that there was a substantial variation in the level of recombinant IP3KB expression between individual cells (results not shown). Therefore cells in which histamine elicited a Ca^{2+} response may have been those in which recombinant IP3KB expression was relatively low.

Ca^{2+} storage and CCE in C6 cells cultured with or without tetracycline

Experiments were performed in order to study the effect of elevating the expression of IP3KB on Ca^{2+} storage and CCE in C6 cell populations. C6 cells were cultured with or without 2 $\mu g \cdot ml^{-1}$ tetracycline for 48 h and then loaded with fura-2 AM [23]. Cell-population ratiometric fluorescence measurements were performed at 20–22 °C in Ca^{2+}-free EM buffer containing 1 mM EGTA. Cells were treated with 2 μM thapsigargin, resulting in a transient elevation in cytosolic Ca^{2+} due to the leakage of Ca^{2+} into the cytosol from thapsigargin-sensitive stores [24]. Following the return of cytosolic [Ca^{2+}] to basal levels, cells were treated with the Ca^{2+} ionophore, ionomycin. This caused a further transient elevation in cytosolic [Ca^{2+}] through the release of Ca^{2+} from thapsigargin-insensitive stores. The magnitude and profile of cytosolic [Ca^{2+}] elevation occurring on thapsigargin and ionomycin treatment was found to be not significantly different for C6 cells cultured with or without tetracycline (Figures 4A and 4B).

Cell-population ratiometric fluorescence measurements were performed on fura-2 AM-loaded C6 cells following pretreatment of the cells with 2 μM thapsigargin for 30 min in Ca^{2+}-free EM containing 1 mM EGTA. This depletes Ca^{2+} stores and consequently activates CCE; however, the lack of Ca^{2+} in the extracellular medium prevents Ca^{2+} entry from occurring [24]. CCE was then initiated by the addition of 1.8 mM $CaCl_2$ to the medium together with 100 μM histamine, to stimulate production of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. This resulted in an elevation in cytosolic [Ca^{2+}] followed by a slow decline and a plateau phase. The characteristics of CCE observed for C6 cells cultured with or without tetracycline were not significantly different (Figures 4C and 4D).

DISCUSSION

In order to investigate the role of IP3KB in Ca^{2+} signalling and homoeostasis, we expressed the enzyme in HeLa cells, a cell line in which Ca^{2+} signalling has been widely studied [18]. The Tet-Off expression system (Clontech) was employed, which allows tightly regulated expression of introduced genes [25]. A clonal cell line (C6) was established, in which total cellular IP3K activity increased from 1.23-fold to 23-fold that of untransfected cells when the cells were cultured in the absence of tetracycline.

Experiments were performed to ascertain the effect of changes in the level of IP3KB expression on the levels of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3. Following its production from Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 is rapidly metabolized to Ins(1,3,4)P_3, and consequently levels of this inositol phosphate rise with flux through IP3K. Inositol phosphate levels were measured both in unstimulated C6 cells and in C6 cells stimulated with a supramaximal dose of histamine, an Ins(1,4,5)P_3 elevating agonist. Elevating the expression of IP3KB resulted in a significant decrease in the level of Ins(1,4,5)P_3 and a significant increase in the levels of Ins(1,3,4)P_3 and InsP_4 following histamine stimulation of C6 cells. This indicates that flux through IP3K following agonist stimulation is increased on elevating IP3KB expression. The levels of the same inositol phosphates in unstimulated C6 cells were found to be virtually unchanged by elevated expression of IP3KB. The fact that a 20-fold increase in total cellular IP3K activity has very little effect on basal Ins(1,4,5)P_3 concentration suggests that IP3KB is not a significant determinant of basal Ins(1,4,5)P_3 concentration in unstimulated cells. This correlates with the data of De Smedt et al. [26], who found that expression of recombinant IP3KA in Chinese hamster ovary cells had little effect on basal Ins(1,4,5)P_3 concentrations. By contrast, in the same study [26], it was reported that expression of recombinant 43 kDa 5-phosphatase caused a 3-fold decrease in basal Ins(1,4,5)P_3 concentrations, and Speed et al. [27] found that underexpression of the 5-phosphatase caused a 2-fold increase in basal Ins(1,4,5)P_3 concentrations. Taken together, these data strongly suggest that the 43 kDa 5-phosphatase is important in controlling basal Ins(1,4,5)P_3 levels, whereas IP3KA and IP3KB play a lesser role. Our data suggest that IP3KB activity is likely to be of more importance following stimulation of cells with Ins(1,4,5)P_3-elevating agonists.

The effect of increased expression of IP3KB on cytosolic Ca^{2+} signals, elicited in response to Ins(1,4,5)P_3-elevating agonists, was then investigated. We found that elevating expression of IP3KB resulted in a profound reduction in histamine-evoked Ca^{2+} mobilization in C6 cells. This was also observed in a different clone and in response to a different Ins(1,4,5)P_3-elevating agonist. Experiments in single cells indicated that for 67 % of C6 cells expressing an elevated level of IP3KB there was no detectable elevation in cytosolic [Ca^{2+}] on stimulation with a supramaximal histamine dose.

Since Ca^{2+} storage and store refilling were unaffected by elevating IP3KB expression, the observed reduction in Ca^{2+}

mobilization is likely to be a result of reduced activation of the Ins(1,4,5)P_3 receptor. This reduction in Ins(1,4,5)P_3-receptor activation could conceivably result from a desensitization of the receptor. We tested Ins(1,4,5)P_3-receptor sensitivity by treatment of cells with a range of concentrations of thimerosal, an agent which sensitizes the Ins(1,4,5)P_3 receptor so that, at high thimerosal doses, the basal Ins(1,4,5)P_3 concentration is sufficient to activate Ca^{2+} release [28]. At all concentrations used, thimerosal-induced cytosolic Ca^{2+} responses were not affected by elevation of IP3KB expression (results not shown), indicating that an increase in IP3KB levels does not affect Ins(1,4,5)P_3-receptor sensitivity. Furthermore, this provides additional evidence that the basal Ins(1,4,5)P_3 concentration is not greatly affected by elevating IP3KB expression.

The most likely explanation for the loss of agonist-evoked Ca^{2+} responses on elevation of IP3KB expression would seem to be a reduction in post-stimulation Ins(1,4,5)P_3 levels. It is perhaps surprising that the small change in post-stimulation Ins(1,4,5)P_3 levels observed on elevation of IP3KB expression could result in such a profound alteration in Ca^{2+} signals. However, the complex manner in which Ins(1,4,5)P_3 and Ca^{2+} act as co-agonists in the control of Ca^{2+} release via the Ins(1,4,5)P_3 receptor means that a small reduction in Ins(1,4,5)P_3 concentration could conceivably have a great effect on Ca^{2+} mobilization [1]. Alternatively, Ins(1,3,4,5)P_4 could influence Ins(1,4,5)P_3-evoked Ca^{2+} release in some way, so that the ratio of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 was a significant factor. Another possible explanation is that there is substantial variation in inositol phosphate levels at specific locations within the cell, which is not apparent in whole-cell mass measurements. Both endogenous and recombinant IP3KB have been shown to localize to the cytosolic face of ER membranes as well as to the cytosol [13]. Since this localization pattern is also observed in C6 cells (results not shown), it is likely that IP3K activity is concentrated at the ER in C6 cells. At the ER, IP3KB is well placed to respond rapidly to Ca^{2+} elevations and specifically reduce Ins(1,4,5)P_3 concentration in the vicinity of Ins(1,4,5)P_3 receptors. De Smedt et al. [26] observed that expression of recombinant 43 kDa 5-phosphatase had a profound effect on Ins(1,4,5)P_3-evoked Ca^{2+} responses, but only if the enzyme was correctly localized to the plasma membrane, indicating that subcellular location of Ins(1,4,5)P_3 metabolism is of importance.

It has been observed that Ins(1,3,4,5)P_4 modifies the characteristics of Ins(1,4,5)P_3-evoked Ca^{2+} mobilization in some cell types [29–31]. A possible explanation for this is that Ins(1,3,4,5)P_4 alters the characteristics of Ca^{2+} storage. CCE is the process whereby depletion of Ca^{2+} stores activates Ca^{2+} entry into the cell in order to replenish stores [32]. Ins(1,3,4,5)P_4 has been shown to stimulate Ca^{2+} entry in certain cell types, and it has been proposed that Ins(1,3,4,5)P_4 contributes to the activation of CCE [33,34]. We were interested in establishing whether Ca^{2+} storage or CCE were altered on elevating the expression of IP3KB. Since agonist-evoked Ca^{2+} mobilization was virtually abolished in cells expressing elevated levels of IP3KB, agonist-evoked store depletion was minimal and hence we were unable to study CCE in this context. Therefore we chose to induce CCE by depletion of stores using thapsigargin. As post-stimulation levels of Ins(1,4,5)P_3 and InsP_4 levels were altered on elevating IP3KB expression, CCE was activated in the presence of histamine in order to establish whether the alteration in Ins(1,4,5)P_3 and InsP_4 levels affected the characteristics of thapsigargin-induced CCE. We found no evidence to suggest that elevated expression of IP3KB or the resulting alteration in post-stimulation inositol phosphate levels had any effect on CCE. We also found no evidence to suggest that the quantity of stored Ca^{2+} or the distribution of Ca^{2+} between store types was altered by elevated expression of IP3KB.

Two groups have investigated the effect of expressing the A isoform of IP3K on Ca^{2+} signals in mammalian cells. Balla et al. [35] observed a significant reduction in the Ca^{2+} response to thrombin stimulation of CCL39 fibroblasts, whereas De Smedt et al. [26] observed little change in the Ca^{2+} response to ATP stimulation of Chinese hamster ovary cells. Given that the characteristics of the B isoform of IP3K are substantially different from those of the A isoform in terms of regulation and subcellular localization, the results of the present work are clearly distinct from those of previous studies. In addition, given its widespread tissue distribution, studies on the B isoform may provide information relating to a more ubiquitous role for IP3K than studies on the brain- and testis-specific A isoform. The widespread expression of IP3KB suggests that it is likely that this enzyme is an important component of the machinery which controls Ca^{2+} signalling and homoeostasis; however, its precise function has remained elusive. Our data are consistent with a model in which the enzyme plays a role in controlling Ca^{2+} signalling via modulation of post-stimulation Ins(1,4,5)P_3 levels. However, we found no evidence to suggest that Ins(1,3,4,5)P_4 contributes to the regulation of Ca^{2+} storage or store refilling in HeLa cells. Our data suggest that the action of IP3KB is likely to be of most significance following agonist stimulation of a cell. IP3KB activity is regulated in a number of ways, most notably by Ca^{2+}-dependent mechanisms, such as in Ca^{2+} CaM binding, which activates the enzyme 20-fold, increasing to 40-fold if the enzyme is also phosphorylated by CaM-dependent protein kinase II [8]. Thus the cellular function of IP3K could be to reduce levels of Ins(1,4,5)P_3 in response to elevated [Ca^{2+}]. Verjans et al. [36] observed little change in the initial magnitude of agonist-evoked Ca^{2+} release on overexpression of IP3KA in *Xenopus* oocytes but found that latter phases of the response were reduced, suggesting that IP3K exerts a greater influence once Ca^{2+} has become elevated. A number of mechanisms exist to ensure that Ins(1,4,5)P_3-evoked Ca^{2+} release occurs in short, discrete spikes, and Ca^{2+}-activated metabolism of Ins(1,4,5)P_3 by IP3K could represent another [37,38]. In the case of IP3KB, localization to the ER could serve to potentiate Ca^{2+}-regulated changes in Ins(1,4,5)P_3 concentration in the immediate vicinity of Ins(1,4,5)P_3 receptors. We were unable to investigate Ca^{2+} oscillations in the present study, as the elevation of IP3KB levels entirely abolished Ca^{2+} signals in most cells; however, it is interesting to speculate how IP3KB might contribute to Ca^{2+} oscillations. Recent data [39] have suggested that concentrations of Ins(1,4,5)P_3 oscillate in synchrony with [Ca^{2+}], and Ca^{2+}-activated metabolism of Ins(1,4,5)P_3 by IP3KB could explain this effect. Introduction of exogenous Ins(1,3,4,5)P_4 into HeLa cells has been shown to increase Ca^{2+}-oscillation frequency; therefore it is possible that IP3KB could contribute to the generation of Ca^{2+} oscillations through both metabolism of Ins(1,4,5)P_3 and production of Ins(1,3,4,5)P_4 [40]. There is evidence for involvement of CaM-dependent protein kinase II in controlling Ca^{2+} oscillations in HeLa cells and this effect could be mediated, at least in part, via modulation of IP3KB activity [41].

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