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Underexpression of the 43 kDa inositol polyphosphate 5-phosphatase is associated with spontaneous calcium oscillations and enhanced calcium responses following endothelin-1 stimulation

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SUMMARY

The 43 kDa inositol polyphosphate 5-phosphatase (5-phosphatase) hydrolyses the signalling molecules inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) and thereby regulates cellular transformation. To investigate the role $Ins(1,4,5)P_3$-mediated Ca^{2+} oscillations play in cellular transformation, we studied $Ins(1,4,5)P_3$-mediated Ca^{2+} responses in cells underexpressing the 43 kDa 5-phosphatase. Chronic reduction in 43 kDa 5-phosphatase enzyme activity resulted in a 2.6-fold increase in the resting $Ins(1,4,5)P_3$ concentration and a 4.1-fold increase in basal intracellular Ca^{2+}. The increased $Ins(1,4,5)P_3$ levels resulted in partial emptying (40%) of the $Ins(1,4,5)P_3$-sensitive Ca^{2+} store, however, store-operated Ca^{2+} influx remained unchanged. In addition, $Ins(1,4,5)P_3$ receptors were chronically down-regulated in unstimulated cells, as shown by a 53% reduction in $[^3H]Ins(1,4,5)P_3$ binding to microsomal receptor sites. Agonist stimulation with endothelin-1 resulted in the rapid rise and fall of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ levels, with no significant differences in the rates of hydrolysis of these second messengers in antisense- or vector-transfected cells. These studies indicate, in contrast to its predicted action, the 43 kDa 5-phosphatase does not metabolise $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ post agonist stimulation. Cells with decreased 43 kDa 5-phosphatase activity exhibited spontaneous Ca^{2+} oscillations in the absence of any agonist stimulation, and increased sensitivity and amplitude of intracellular Ca^{2+} responses to both high and low dose endothelin-1 stimulation. We conclude the 43 kDa 5-phosphatase exerts a profound influence on $Ins(1,4,5)P_3$-induced Ca^{2+} spiking, both in the unstimulated cell and following agonist stimulation. We propose the enhanced Ca^{2+} oscillations may mediate cellular transformation in cells underexpressing the 43 kDa 5-phosphatase.

Key words: Inositol 1,4,5-trisphosphate, 5-Phosphatase, Calcium, Signal transduction

INTRODUCTION

The hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) by phospholipase C to produce the two second messengers inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol, is a ubiquitous intracellular signalling mechanism for the mobilisation of intracellular Ca^{2+} and activation of protein kinase C (Berridge, 1983, 1993; Berridge and Irvine, 1984; Majerus, 1992). $Ins(1,4,5)P_3$-induced increases in intracellular Ca^{2+} are responsible for a large number of receptor-initiated signalling pathways in many cell types, thereby regulating secretion, cytoskeletal rearrangement, fertilisation and gene transcription (Berridge, 1993, 1995a). $Ins(1,4,5)P_3$ mediates intracellular Ca^{2+} release by binding to specific receptors primarily located on the endoplasmic reticulum. In most cells the rise in intracellular Ca^{2+} is associated with oscillatory changes in the free Ca^{2+} concentration, that are both graded in response and spatially organised (Berridge, 1993; Clapham, 1995; Thomas et al., 1996). Sub-maximal doses of $Ins(1,4,5)P_3$ result in quantal or incremental releases of Ca^{2+} from the endoplasmic reticulum. In addition, $Ins(1,4,5)P_3$-mediated Ca^{2+} release is also affected by the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Increasing cytosolic Ca^{2+} potentiates $Ins(1,4,5)P_3$-mediated Ca^{2+} release, so that at Ca^{2+} concentrations between 10 nM and 1 μM they act as co-agonists (Finch et al., 1991; Dawson, 1997).

There are two major pathways for the metabolism of $Ins(1,4,5)P_3$, which differ in relative importance depending on the cell type. $Ins(1,4,5)P_3$ can be phosphorylated by an $Ins(1,4,5)P_3$ 3-kinase to form inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$), which has been implicated in the regulation of Ca^{2+} homeostasis in certain cell types (Batty et al., 1985). In addition, $Ins(1,3,4,5)P_4$ binds with high affinity to Gap1, synaptotagmin I and II, centaurin α, Btk and a high affinity $Ins(1,3,4,5)P_4$-binding protein, which has recently been shown

to encode a GTPase-activating protein (Cullen et al., 1995a,b; Fukuda et al., 1994, 1996; Hammonds-Odie et al., 1996).

Both second messengers, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, are inactivated by removal of the 5-position phosphate from the inositol ring by a family of enzymes known as the inositol polyphosphate 5-phosphatases (5-phosphatase) (Downes et al., 1982; Connolly et al., 1985; Mitchell et al., 1989). Nine family members have been cloned to date and all are characterised by the presence of a '5-phosphatase domain' (Majerus, 1996; Mitchell et al., 1996; Woscholski and Parker, 1997). This domain of approximately 300 amino acids contains two distinct motifs, WXGDXNXR and PXWCDRXL, located 60 amino acids apart, which may represent the catalytic or substrate binding site (Jefferson and Majerus, 1995, 1996; Communi et al., 1996; Communi and Erneux, 1996).

The 5-phosphatase family includes the 43 kDa 5-phosphatase (5-phosphatase I), which hydrolyses only Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Connolly et al., 1985; Erneux et al., 1989; Verjans et al., 1992; Laxminarayan et al., 1993). Other 5-phosphatases such as the 75 kDa 5-phosphatase (5-phosphatase II), the 5-phosphatase mutated in Lowe's syndrome and synaptojanin hydrolyse Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Mitchell et al., 1989; Matzaris et al., 1994; Jackson et al., 1995; Zhang et al., 1995; McPherson et al., 1996; Woscholski et al., 1997). More recently a novel 5-phosphatase designated SHIP (for SH2-containing 5-phosphatase), that only hydrolyses Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 has been characterised (Damen et al., 1996; Kavanaugh et al., 1996; Lioubin et al., 1996; Jefferson et al., 1997).

The 43 kDa 5-phosphatase (also called 5-phosphatase I, or the Type I 5-phosphatase) is the major cellular 5-phosphatase terminating the signalling function of Ins(1,4,5)P_3. Although the intracellular site for Ins(1,4,5)P_3 hydrolysis appears to be predominantly in the plasma membrane, whether the 43 kDa 5-phosphatase regulates basal Ins(1,4,5)P_3 levels, or mediates the rapid fall in Ins(1,4,5)P_3 following agonist stimulation has yet to be shown. Erneux and colleagues have demonstrated that overexpression of the 43 kDa 5-phosphatase in Chinese hamster ovary cells (CHO-K1) abrogates ATP-induced Ca^{2+} oscillations (De Smedt et al., 1997). Studies in our laboratory have shown that a chronic reduction in 43 kDa 5-phosphatase enzyme activity is associated with a transformed phenotype (Speed et al., 1996). The presumed mechanism mediating cellular transformation may be enhanced Ins(1,4,5)P_3-induced Ca^{2+} oscillations, but this has yet to be shown. Furthermore, correlation between the levels of 43 kDa 5-phosphatase activity and the size and sensitivity of the Ca^{2+} oscillatory response has not yet been demonstrated. In this study we have extended our characterisation of cell lines that underexpress the 43 kDa 5-phosphatase. Decreased 43 kDa Ins(1,4,5)P_3 5-phosphatase activity is associated with increased Ins(1,4,5)P_3 concentration in unstimulated cells, resulting in Ins(1,4,5)P_3 receptor activation and subsequent down-regulation. The increased basal Ins(1,4,5)P_3 levels result in spontaneous Ca^{2+} oscillations, in the absence of agonist stimulation, and increased sensitivity and amplitude of Ca^{2+} response to both low- and high-doses of agonist. Collectively, these results show the 43 kDa 5-phosphatase critically controls the intensity and sensitivity of Ins(1,4,5)P_3-induced Ca^{2+} oscillations and we propose thereby regulates cellular transformation.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and tetracycline-HCl were purchased from ICN Biomedicals, Inc. Geneticin was obtained from Gibco. Normal rat kidney cells (NRK, clone 49F) were obtained from American Type Culture Collection. Ortho[^{32}P]phosphoric acid, [^{3}H]*myo*-inositol (20.5 Ci/mmol), [^{3}H]Ins(1,4,5)P_3 (21 Ci/mmol) and the inositol 1,4,5-trisphosphate [^{3}H] radioreceptor assay kit were obtained from Dupont-New England Nuclear. Endothelin-1 (ET-1) was obtained from Peptide Institute Inc., Japan. Bradykinin and thapsigargin were from Calbiochem. Fura-2 AM was obtained from Molecular Probes. All other chemicals were obtained from Sigma.

Transfection of NRK fibroblasts with the 43 kDa inositol polyphosphate 5-phosphatase

Stable NRK cell lines underexpressing the 43 kDa 5-phosphatase were generated as previously described (Speed et al., 1996). Briefly, the 2.6 kb cDNA encoding the 43 kDa membrane-associated 5-phosphatase was cloned in the antisense orientation into the response plasmid, pUHD 10-3 of the tetracycline-regulatory expression system (Gossen and Bujard, 1992). The construct, along with a neomycin resistance cassette, was stably transfected into NRK cells which expressed the regulator plasmid pUHD 15-1. Control cells were simultaneously co-transfected with the pUHD 10-3 plasmid alone and a neomycin resistance cassette. Cells were cultured in selection medium containing 0.4 μg/ml geneticin and 2 μg/ml tetracycline-HCl and antibiotic-resistant clones selected 3-4 weeks later. The presence of tetracycline (2 μg/ml) in the cell culture medium renders the system inactive, hence the gene of interest is not expressed. Induction of the cloned gene is obtained by withdrawing tetracycline from the cell culture medium for 24 hours.

Cell culture

Cell lines were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin and 0.1% streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Following stable transfection, NRK cells were cultured in the presence of 2 μg/ml tetracycline-HCl.

Determination of inositol polyphosphate 5-phosphatase enzyme activity

Cell culture media was aspirated and the adherent cells were washed three times in imidazole buffer (10 mM imidazole, pH 7.2, containing 1 mM $MgCl_2$, 1 mM EDTA, 0.3 M sucrose, 5 mM 2-mercaptoethanol, 50 μg/ml phenylmethylsulfonyl fluoride). The cells were scraped from the plate, resuspended in 200 μl imidazole buffer containing 1% Triton X-100 and sonicated on ice for 60 seconds. The total cell homogenate was extracted at 4°C with mixing for 2 hours. The sample was centrifuged at 15,000 ***g*** for 10 minutes at 4°C and the supernatant collected as the Triton-soluble membrane fraction, which was then assayed for Ins(1,4,5)P_3 5-phosphatase activity.

Hydrolysis of Ins(1,[^{32}P]4,[^{32}P]5)P_3 was measured by extraction of released $^{32}PO_4$ as previously described (Connolly et al., 1985). The concentration of Ins(1,[^{32}P]4,[^{32}P]5)P_3 used in assays was 30 μM, and was isolated from erythrocyte ghosts as previously described (Downes et al., 1982).

Measurement of Ins(1,4,5)P_3 concentration

Cells were seeded onto 60 mm dishes at a density of 1×10^5 cells/ml and cultured for 48 hours in the absence of tetracycline in DMEM containing 10% FCS. Cells were harvested to measure unstimulated Ins(1,4,5)P_3 concentrations, or stimulated with either 60 nM endothelin-1 (ET-1) or 10 μM bradykinin in Ca^{2+}-containing medium (20 mM Hepes, pH 7.4, 4 mM KCl, 140 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, 1.8 mM $CaCl_2$) for various times at 37°C, prior to harvesting. The solution was aspirated and the reaction terminated by

the addition of 500 mM trichloroacetic acid. The cells were scraped from the dishes in 500 mM trichloroacetic acid and incubated on ice for 20 minutes prior to centrifugation at 5,000 ***g*** for 10 minutes at 4°C. The supernatant was washed three times with ten volumes of water-saturated diethyl ether and then neutralised with $NaHCO_3$. The concentration of Ins(1,4,5)P_3 was determined by competition with [^{3}H]Ins(1,4,5)P_3 for microsomal receptor binding sites (Dupont-New England Nuclear inositol 1,4,5-trisphosphate [^{3}H] radioreceptor assay kit). Protein measurements were made on unlabelled cells cultured under identical conditions.

Extraction and separation of [^{3}H]inositol phosphates in [^{3}H]inositol-labelled fibroblasts

Cells were seeded onto 60 mm dishes at a density of 1×10^5 cells/ml and cultured for 24 hours in the absence of tetracycline in DMEM containing 10% FCS. Cells were then labelled with 5 μCi/ml [^{3}H]inositol in inositol-free DMEM containing 0.5% FCS for 48 hours. Cells were washed twice in Ca^{2+}-containing medium, followed by the addition of fresh medium containing 60 nM ET-1 for 15, 30, 60 seconds or 2.5 minutes. The incubation was stopped by removal of the medium, followed by the addition of 2 ml 3% (v/v) perchloric acid. The [^{3}H]inositol phosphates were extracted and separated according to the method described by De Smedt et al. (1997).

Intracellular Ca^{2+} measurements

Cell monolayers (on coverslips) were cultured for 24-48 hours in the absence of tetracycline in DMEM containing 10% FCS. Cells were loaded with 6 μM fura-2 AM in 10% Pluronic detergent for 40 minutes at 37°C in Ca^{2+}-containing medium. The cells on coverslips were washed 3 times and kept in the dark at room temperature until use, which was within 1 hour of loading. For population $[Ca^{2+}]_i$ measurements, the coverslip was inserted into a standard cuvette with Ca^{2+}-containing medium and a stirring device. In the experiments where Ca^{2+} was omitted from the medium, 0.1 mM EGTA was added. The cuvette was placed into a thermostatically controlled chamber at 37°C in a SPEX dual wavelength 1681 fluorolog spectrometer. Excitation wavelengths were 340 nm and 380 nm, and emitted light collected at 505 nm. Readings were recorded using dM3000 software (Spex Industries, Inc., Edison, NJ). Agonists were added by direct injection of 10 μl volumes of stock solutions into the cuvette. Ca^{2+} values were corrected for cell autofluorescence at each wavelength by the addition of 2 μM ionomycin and 2 mM $MnCl_2$. Lanthanum chloride ($LaCl_3$) was added to a final concentration of 500 μM to block Ca^{2+} entry (Cooper et al., 1994; Aussel et al., 1996). $[Ca^{2+}]_i$ was determined as previously described (Neylon et al., 1992).

Single cell $[Ca^{2+}]_i$ measurements were performed in Ca^{2+}-containing medium, using a Nikon Diaphot-TMD fluorescence microscope equipped with a ×40 oil immersion objective coupled to the SPEX instrument. The temperature was maintained at 37°C using a thermostatically controlled microscope stage. Cells were alternately illuminated at 340 and 380 nm, and the light emitted at 505 nm was collected by use of a dichroic mirror. Changes in $[Ca^{2+}]_i$ were assessed by calculating the ratio of emitted fluorescence at the excitation wavelengths, 340 and 380 nm.

Ins(1,4,5)P_3 binding assay

Cell lines were cultured to near confluence in 90 mm dishes and then scraped from the dishes in 10 mM Hepes, pH 7.4, 155 mM NaCl, 1 mM EDTA. A microsomal fraction was prepared by a modification to the method described by Wojcikiewicz (1995). Cells were centrifuged at 400 ***g*** for 2 minutes at 4°C and resuspended in ice-cold homogenisation buffer (10 mM Tris, pH 7.4, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μM leupeptin) prior to disruption with a Dounce homogeniser (1× 10 seconds). Cell suspensions were centrifuged at 38,000 ***g*** for 15 minutes at 4°C and the microsomal pellets were resuspended in ice-cold homogenisation buffer.

The Ins(1,4,5)P_3 binding assay was performed using 100, 200 or 500 μg of microsomal protein and 2.5 or 5 nM [^{3}H]Ins(1,4,5)P_3 in 50 mM Tris-HCl, pH 8, 2 mM EDTA for 15 minutes on ice in a 200 μl final volume. Bound and free radioligand were separated by centrifugation at 15,000 ***g*** for 5 minutes at 4°C and the pellet washed once in ice-cold water. Microsomal pellets were solubilised in 1% Triton X-100 for 15 minutes at 37°C. [^{3}H]Ins(1,4,5)P_3 binding was quantitated by liquid scintillation counting. The specificity of the assay was determined by competition with 20 μM Ins(1,3,4)P_3. Non-specific binding was determined in the presence of 20 μM Ins(1,4,5)P_3.

Miscellaneous methods

Protein concentrations were performed using Bio-Rad protein assay with bovine serum albumin as a standard.

RESULTS

Characterisation of cell lines underexpressing the 43 kDa 5-phosphatase

Normal rat kidney (NRK) cells were stably transfected with the cDNA encoding the 43 kDa 5-phosphatase, cloned in the antisense orientation into the pUHD 10-3 vector of the tet-regulatory system, and control cells were transfected with the pUHD 10-3 vector alone (Speed et al., 1996). We have observed stable cell lines underexpressing the 43 kDa 5-phosphatase gradually lose their phenotype with repeat passaging, due to the incomplete suppression of the antisense 43 kDa 5-phosphatase gene by tetracycline (Speed et al., 1996). Therefore we have chosen to use vector-transfected clones as the appropriate controls for all experiments. Cell lines underexpressing the 43 kDa 5-phosphatase (antisense) were selected on the basis of a reduction in Ins(1,4,5)P_3 5-phosphatase enzyme activity in the membrane fraction of the cell, which is the major cellular location for the 43 kDa 5-phosphatase (Fig. 1A). The Triton-soluble membrane fraction of antisense-transfected cells, in the absence of tetracycline, demonstrated a 75% reduction in Ins(1,4,5)P_3 5-phosphatase activity, as compared to control vector-transfected cells (0.41±0.06 nmol Ins(1,4,5)P_3 hydrolysed/minute/mg (n=8) versus 1.6±0.25 nmol Ins(1,4,5)P_3 hydrolysed/minute/mg (n=7), respectively, P<0.01). Investigation of the Ins(1,4,5)P_3 concentration in unstimulated cells was undertaken, as described in Materials and Methods. The Ins(1,4,5)P_3 concentration in antisense-transfected cells was increased approximately 2.6-fold over that observed in cells transfected with vector alone (21±2 pmol/mg (n=7) versus 8±1 pmol/mg (n=6) respectively, P<0.01; Fig. 1B).

Assessment of intracellular Ca^{2+} levels in antisense- or vector-transfected NRK cells

The effect of increased cellular Ins(1,4,5)P_3 levels on the basal $[Ca^{2+}]_i$ was determined as described in Materials and Methods. Fura-2 loaded antisense-transfected cells demonstrated a basal $[Ca^{2+}]_i$ of 334±33 nM (n=7) in Ca^{2+}-containing medium with 500 μM $LaCl_3$, a significant increase (4.1-fold, P<0.01) over vector-transfected cells (81±6 nM, n=13; Fig. 2). $LaCl_3$ was added to the Ca^{2+}-containing medium to block Ca^{2+} entry, thus excluding any contribution of extracellular Ca^{2+} to the basal $[Ca^{2+}]_i$. In Ca^{2+}-free medium the validity of the elevated basal $[Ca^{2+}]_i$ of antisense-transfected cells (296±31 nM (n=8) versus

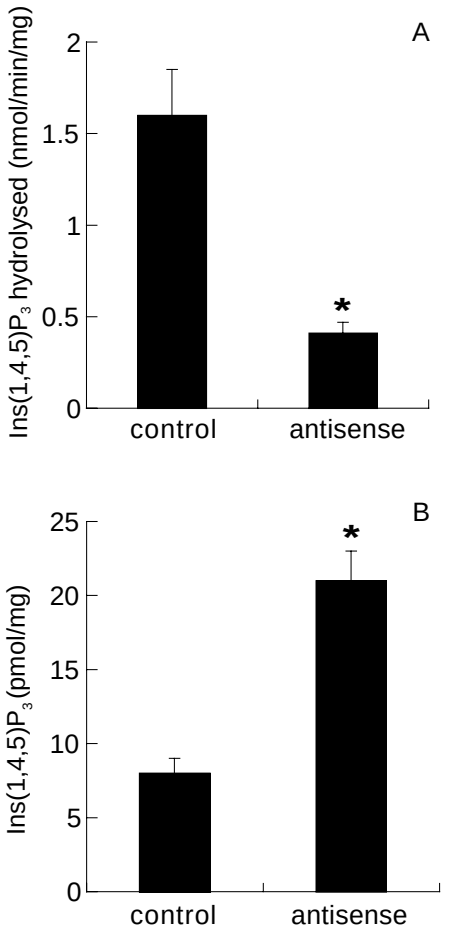


Fig. 1. Characterisation of cell lines stably underexpressing the 43 kDa 5-phosphatase. (A) Isolated clones, transfected with the antisense 43 kDa 5-phosphatase construct (antisense) or with vector alone (control), were withdrawn from tetracycline 24 hours prior to the measurement of $Ins(1,4,5)P_3$ 5-phosphatase activity in the Triton-soluble membrane fraction of the cell. Results are the mean and s.e.m. of three separate experiments using three independent antisense- or vector-transfected clones. *P<0.01. (B) The concentration of $Ins(1,4,5)P_3$ in unstimulated antisense or control cells was measured as described in Materials and Methods. Results are the mean and s.e.m. from at least six experiments, each performed in duplicate, using three independent antisense- or vector-transfected clones. *P<0.01.

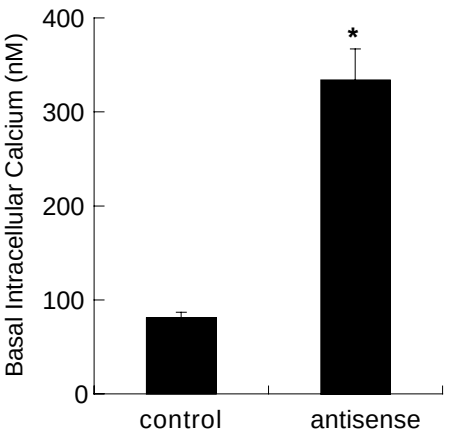


Fig. 2. Measurement of basal $[Ca^{2+}]i$ in NRK cells. Cells were loaded with 6 μM fura-2 AM and the basal cytosolic Ca^{2+} levels of antisense or control cells were measured as described in Materials and Methods. Measurements were made in Ca^{2+}-containing medium with 500 μM $LaCl_3$. Results are the mean and s.e.m. of at least seven measurements, using three independent antisense- or vector-transfected clones. *P<0.01.

control cells 83±8 nM (n=7)) was confirmed (3.6-fold, P<0.01). In addition the basal $[Ca^{2+}]_i$ was measured using Ca^{2+}-free medium containing 0.1 mM EGTA, which lowered the basal $[Ca^{2+}]_i$ of antisense-transfected cells (126±7 nM, n=14). However, due to the constitutive elevation in $Ins(1,4,5)P_3$ levels, the basal $[Ca^{2+}]_i$ of antisense-transfected cells still remained significantly higher (1.7-fold, P<0.01) than vector-transfected cells (74±5 nM, n=13).

The effects of chronically elevated $Ins(1,4,5)P_3$ on the Ca^{2+} content of the $Ins(1,4,5)P_3$-sensitive store was assessed using the Ca^{2+}-ATPase inhibitor, thapsigargin (TG). TG has previously been shown to specifically deplete the $Ins(1,4,5)P_3$-sensitive, agonist-responsive Ca^{2+} store in intact cells (Berman et al., 1994). To assess the optimal TG concentration that mediates Ca^{2+} release, a dose-response curve was performed using fura-2 loaded vector-transfected cells. Maximal stored Ca^{2+} was released following the addition of 200 nM TG, with doses up to 1 μM exhibiting an inhibitory effect (results not shown). The Ca^{2+} content of the $Ins(1,4,5)P_3$-sensitive store was quantitated in antisense- or vector-transfected cells using 200 nM TG in Ca^{2+}-free medium containing 0.1 mM EGTA (Figs 3 and 4A). Antisense-transfected cells demonstrated a significant 40% reduction in the Ca^{2+} content of the $Ins(1,4,5)P_3$-sensitive store, as compared to vector-transfected cells (76±6 nM (n=8) versus 121±7 nM (n=8), respectively, P<0.01). These observations were further supported by similar experiments performed in Ca^{2+}-containing medium with 500 μM $LaCl_3$. The $Ins(1,4,5)P_3$-sensitive Ca^{2+} store of antisense-transfected cells was again reduced by 40%, as compared with vector-transfected cells (145±27 nM (n=4) versus 248±49 nM (n=4), respectively).

Assessment of store-operated Ca^{2+} influx in antisense- or vector-transfected cells

The effects of increased cytosolic Ca^{2+} concentration and depleted $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores on store-operated Ca^{2+} influx were assessed as described in Materials and Methods. Much experimental data suggests that $Ins(1,4,5)P_3$ stimulates Ca^{2+} influx either indirectly, as depletion of intracellular Ca^{2+} stores releases a diffusible Ca^{2+} influx factor (CIF), or directly by interaction of the $Ins(1,4,5)P_3$ receptor with Ca^{2+} release activated channels (CRAC) (Berridge, 1995b). Fura-2 loaded cells were treated with 200 nM TG in Ca^{2+}-free medium containing 0.1 mM EGTA (Fig. 4A). The addition of extracellular Ca^{2+} to the TG-treated cells resulted in rapid capacitative Ca^{2+} influx, with a subsequent sustained elevation in $[Ca^{2+}]_i$. However, the rise in $[Ca^{2+}]_i$ following the addition of extracellular Ca^{2+} was not significantly different between antisense- or vector-transfected cells (134±18 nM (n=6) versus 149±14 nM (n=7), respectively; Fig. 4B).

As unstimulated antisense-transfected cells demonstrated a 40% reduction in the Ca^{2+} content of the $Ins(1,4,5)P_3$-sensitive

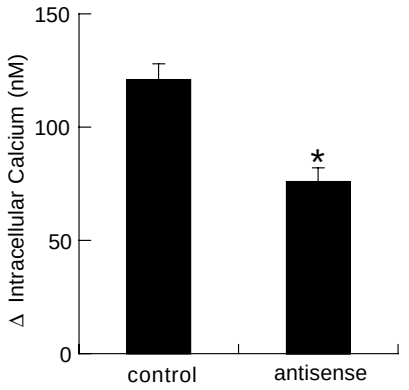


Fig. 3. Assessment of Ins(1,4,5)P_3-sensitive Ca^{2+} store by treatment with thapsigargin. Cytosolic Ca^{2+} responses were measured in fura-2-loaded cells in response to thapsigargin (TG) as described in Materials and Methods. Results are expressed as the rise in $[Ca^{2+}]_i$ following treatment with 200 nM TG in Ca^{2+}-free medium with 0.1 mM EGTA and represent the mean and s.e.m. of eight experiments, using three independent antisense- or vector-transfected clones. *P<0.01.

store, we assessed whether this depletion was sufficient to induce store-operated Ca^{2+} influx, without prior treatment with TG. Antisense- or vector-transfected cells were incubated in Ca^{2+}-free medium containing 0.1 mM EGTA, prior to the addition of extracellular Ca^{2+} (1.8 mM). No apparent Ca^{2+} entry was observed for either cell type, suggesting that a 40% reduction in the Ins(1,4,5)P_3-sensitive Ca^{2+} store is insufficient to induce Ca^{2+} influx (results not shown).

Agonist-induced Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 formation

In most cellular systems Ins(1,4,5)P_3 levels rapidly rise within 1-2 minutes following agonist stimulation and return to basal levels within 5-10 minutes. As the 43 kDa 5-phosphatase is the major cellular Ins(1,4,5)P_3 5-phosphatase, we predicted cells underexpressing this enzyme would demonstrate sustained Ins(1,4,5)P_3 levels following agonist stimulation. We examined agonist-induced Ins(1,4,5)P_3 formation during a time course of stimulation using either 60 nM endothelin-1 (ET-1) (Fig. 5A), or 10 μM bradykinin (Fig. 5B). In both vector- and antisense-transfected cells, Ins(1,4,5)P_3 levels rose rapidly within the first minute following either ET-1 or bradykinin stimulation and returned to pre-stimulation levels within 10 minutes. A second rise in Ins(1,4,5)P_3 concentration was routinely observed in antisense-transfected cells, approximately 5 minutes after the addition of 60 nM ET-1. Although the basal Ins(1,4,5)P_3 concentration was elevated approximately three-fold in antisense-transfected cells, the rate of fall in the Ins(1,4,5)P_3 concentration to pre-stimulation levels was not delayed in cells

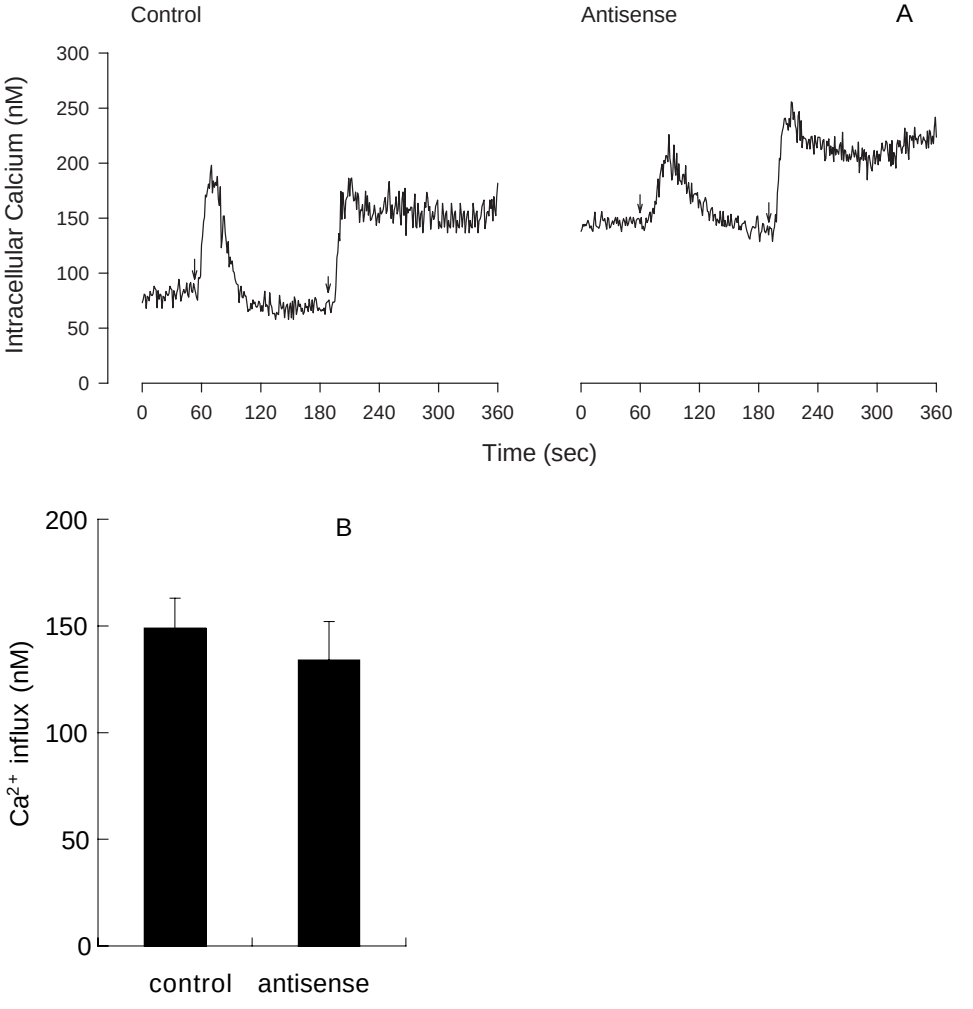


Fig. 4. Assessment of store-operated Ca^{2+} influx in antisense- or vector-transfected cells. Cells were loaded with 6 μM fura-2 AM in Ca^{2+}-containing medium as described in Materials and Methods. The Ca^{2+}-containing medium was replaced with Ca^{2+}-free medium containing 0.1 mM EGTA, immediately prior to the commencement of the trace. (A) TG (200 nM) was added at 60 seconds (left arrow) to deplete the Ins(1,4,5)P_3-sensitive Ca^{2+} store and Ca^{2+} influx was initiated by the addition of 1.8 mM $CaCl_2$ at 200 seconds (right arrow). Each trace is representative of at least six experiments, using three independent antisense- or vector-transfected clones. (B) Rise in $[Ca^{2+}]_i$ (store-operated Ca^{2+} influx) following the addition of 1.8 mM $CaCl_2$. Results are the mean and s.e.m. of at least six measurements, using three independent antisense- or vector-transfected clones.

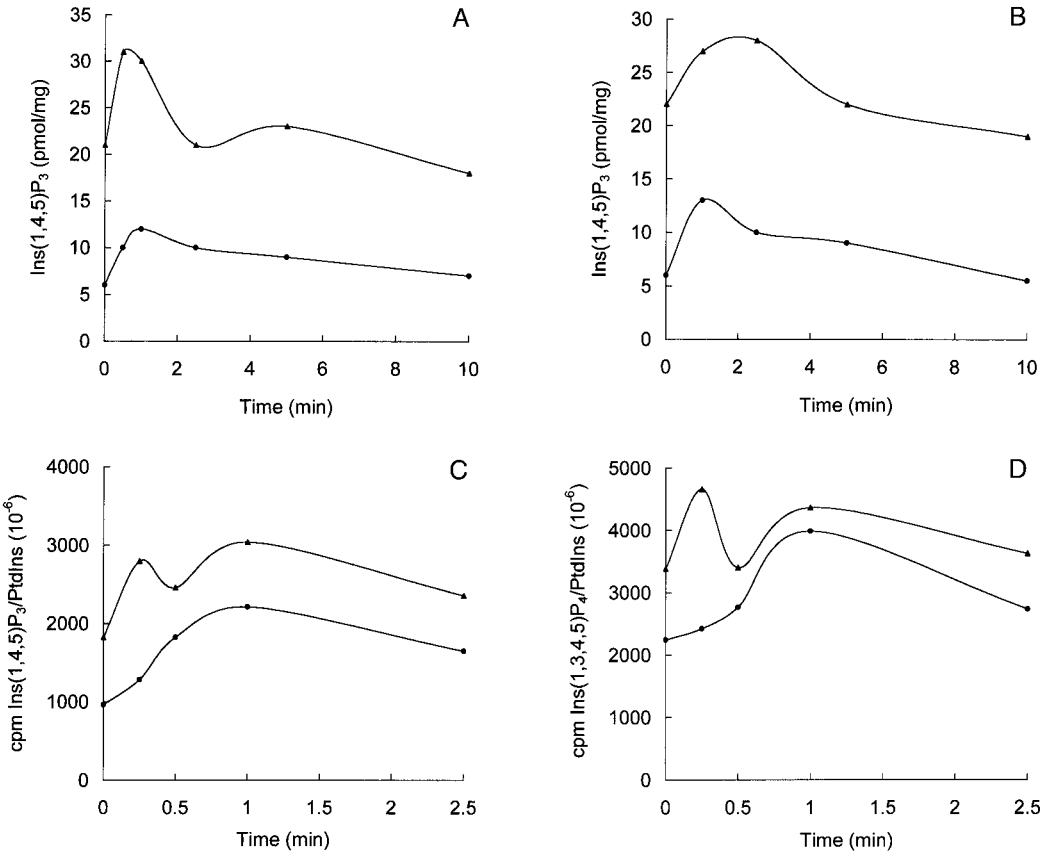


Fig. 5. Time course of agonist-induced Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 formation. (A,B) Time courses of Ins(1,4,5)P_3 formation (pmol/mg) in antisense (▲) or control (●) cells were performed using 60 nM ET-1 (A) or 10 μM bradykinin (B) as described in Materials and Methods. The concentration of Ins(1,4,5)P_3 was measured at the indicated time points. (C,D) Cells were labelled with [^{3}H]inositol and time courses of stimulation of antisense or control cells were performed using 60 nM ET-1 as described in Materials and Methods. The levels of [^{3}H]Ins(1,4,5)P_3 (C) or [^{3}H]Ins(1,3,4,5)P_4 (D) were measured at the indicated time points. The results are normalised with respect to total [^{3}H]PtdIns. Results shown are one representative experiment, from a total of three for each agonist, using two independent antisense- or vector-transfected clones.

underexpressing the 43 kDa 5-phosphatase. In addition, the Ins(1,4,5)P_3 (pmol/mg) released following agonist stimulation did not differ significantly in cells underexpressing the 43 kDa 5-phosphatase, compared to control cells. These findings were further substantiated by the analysis of agonist-induced [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 formation during a time course of stimulation using 60 nM ET-1 (Fig. 5C and D, respectively). Ins(1,3,4,5)P_4 is a major substrate of the 43 kDa 5-phosphatase. Elevated basal [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 levels rose rapidly within the first minute following stimulation and returned to near pre-stimulation levels within 2.5 minutes. The release of [^{3}H]Ins(1,4,5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 following agonist stimulation and the rates of decay of the [^{3}H]inositol phosphates again did not differ significantly in cells underexpressing the 43 kDa 5-phosphatase. Collectively these studies demonstrate that the fall in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 post agonist-stimulation cannot be attributed to the 43 kDa 5-phosphatase, and may result from the action of other Ins(1,4,5)P_3 5-phosphatases, or other mechanisms of Ins(1,4,5)P_3 metabolism.

Changes in $[Ca^{2+}]_i$ in response to a maximal concentration of ET-1

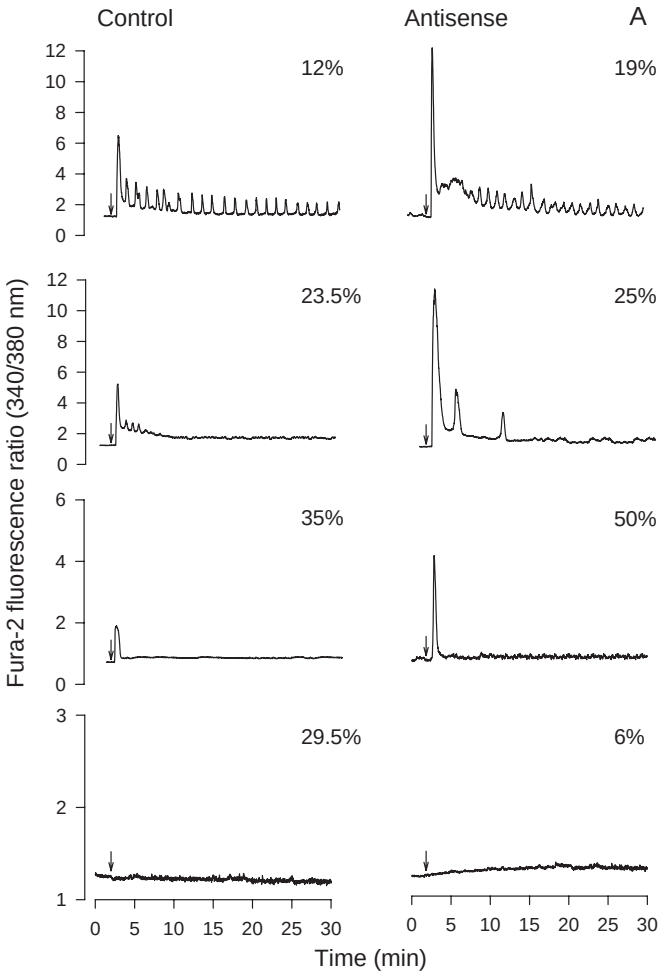
The addition of ET-1 to NRK cells has been shown to result in the rapid generation of Ins(1,4,5)P_3 and an increase in $[Ca^{2+}]_i$ mediated by the ET-A receptors (Suzaki et al., 1997). Single-cell $[Ca^{2+}]_i$ responses to a maximal dose of ET-1 (60 nM) were measured in fura-2 loaded antisense- or vector-transfected cells as described in Materials and Methods (Fig. 6A). Vector-transfected cells demonstrated varying $[Ca^{2+}]_i$ responses to ET-1 stimulation. Approximately 30% of cells showed no response, 35% demonstrated a single Ca^{2+} spike, 23.5% showed an initial Ca^{2+} spike followed by three smaller Ca^{2+} transients and 12% displayed an oscillatory response. The proportion of responders and the oscillatory patterns observed in vector-transfected cells did not alter significantly with increasing doses of ET-1 up to 300 nM (results not shown). In contrast, antisense-transfected cells revealed a marked increase in sensitivity to 60 nM ET-1, with all but 6% of the cells responding. Of the antisense-transfected cells examined, 50% displayed a single Ca^{2+} spike, 25% showed an initial Ca^{2+} peak followed by two-three transients and 19% displayed an oscillatory response. In addition, the amplitude of the Ca^{2+} response was markedly enhanced. The mean rise of the initial Ca^{2+} peak, or Ca^{2+} oscillations following stimulation with 60 nM ET-1, were quantitated and are depicted in Fig. 6B. The initial Ca^{2+} peak of antisense-transfected cells following stimulation was increased 1.7-fold (P<0.05) over vector-transfected cells (fluorescence ratios of 6.1±1.1 (n=15) versus 3.51±0.51 (n=12), respectively). In addition, the Ca^{2+} oscillations that followed the initial peak were significantly larger in amplitude (2.2-fold, P<0.05) in cells underexpressing the 43 kDa 5-phosphatase, than those of vector-transfected cells (fluorescence ratios of 1.46±0.32 (n=7) versus 0.66±0.13 (n=14), respectively).

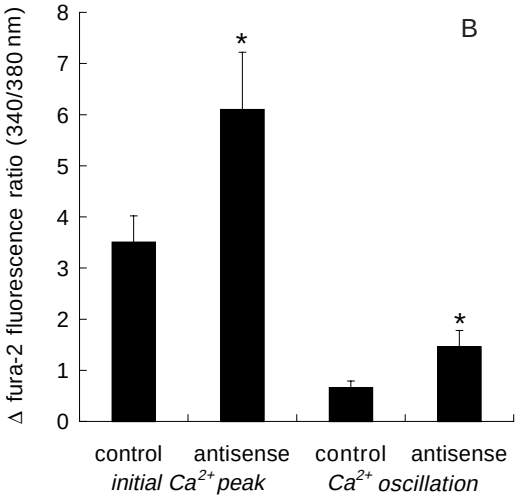
Changes in $[Ca^{2+}]_i$ in response to a sub-maximal concentration of ET-1

Single-cell $[Ca^{2+}]_i$ responses to a sub-maximal dose of ET-1 (1 nM) were also measured in fura-2 loaded antisense- or vector-transfected cells as described in Materials and Methods (Fig.

7A). The antisense-transfected cells showed a similar oscillatory pattern to the control cells, however, the intensity and proportion of responders was markedly enhanced. Of the vector-transfected cells examined, 24% of cells exhibited fewer than 4 Ca^{2+} spikes which occurred at irregular time intervals, while 19% of cells displayed an oscillatory pattern of varying intensity. The majority of vector-transfected cells (57%) failed to respond to 1 nM ET-1. In contrast, 95% of antisense-transfected cells responded to 1 nM ET-1 and displayed three types of oscillatory responses with 21% of cells responding with regular sharp Ca^{2+} spikes, 63% with multiple Ca^{2+} spikes of varying intensity and 11% with an initial Ca^{2+} spike followed by erratic Ca^{2+} transients. In addition, the amplitude of the Ca^{2+} oscillations in response to 1 nM ET-1 were substantially enhanced (1.5-fold, $P<0.05$) in antisense-transfected cells compared to vector-transfected cells (fluorescence ratios of 0.73±0.07 (n=42) versus 0.5±0.05 (n=26), respectively; Fig. 7B). There were no significant differences in the frequency of Ca^{2+} oscillations between antisense- or vector-transfected cells (results not shown). The requirement for Ca^{2+} influx to maintain ET-1-induced Ca^{2+} spiking was assessed by blocking extracellular Ca^{2+} entry using 500 μM $LaCl_3$ (Fig. 8). The blockage of Ca^{2+} influx resulted in the progressive inhibition of ET-1-induced $[Ca^{2+}]_i$ oscillations in both antisense- and vector-transfected cells.

Single-cell $[Ca^{2+}]_i$ responses to low dose ET-1 were also assessed in fura-2 loaded antisense- or vector-transfected cells. Doses of ET-1 in the picomolar range (0.4-0.8 nM) failed to induce Ca^{2+} responses in either antisense- or vector-transfected cells (results not shown).





Cells underexpressing the 43 kDa 5-phosphatase exhibit spontaneous Ca^{2+} oscillations

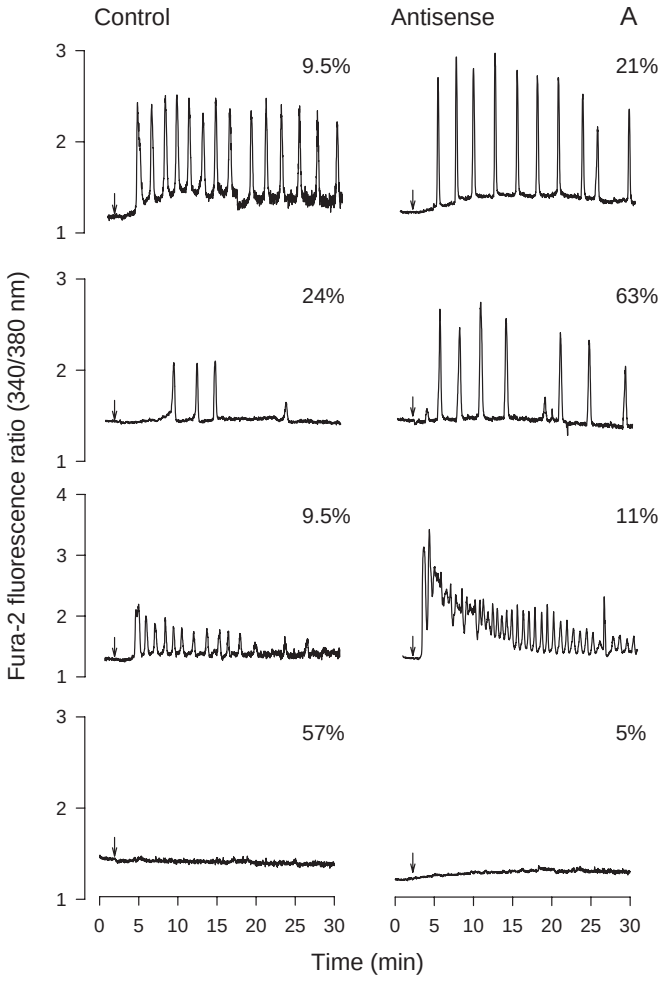
Approximately 14% of cells underexpressing the 43 kDa 5-phosphatase were found to spontaneously oscillate in the absence of agonist stimulation. The oscillatory Ca^{2+} responses varied in frequency and intensity, however the magnitude of the responses were decreased compared to agonist-induced Ca^{2+} oscillations (Fig. 9). None of the vector-transfected cells were found to oscillate spontaneously.

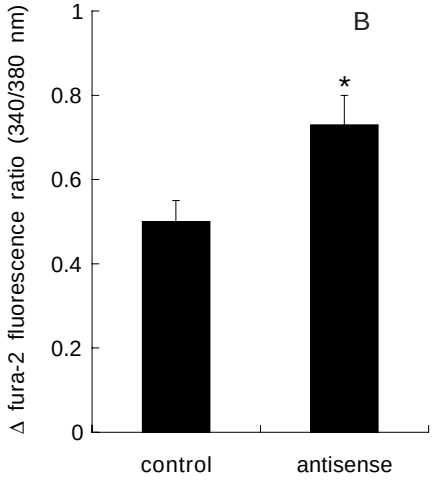
Binding of $[^3H]Ins(1,4,5)P_3$ to the microsomal fraction of antisense- or vector-transfected cells

To analyse the expression of $Ins(1,4,5)P_3$ receptors in the microsomal fraction of antisense- or vector-transfected cells, $[^3H]Ins(1,4,5)P_3$ binding assays were performed as described in Materials and Methods. $Ins(1,4,5)P_3$ binding sites were detected using 2.5-5 nM $[^3H]Ins(1,4,5)P_3$ as a ligand and non-specific binding was determined in the presence of 20 μM unlabelled $Ins(1,4,5)P_3$. To confirm the specificity of the assay, unlabelled $Ins(1,3,4)P_3$ (20 μM) was unable to effectively compete with $[^3H]Ins(1,4,5)P_3$ for microsomal binding sites (results not shown). $Ins(1,4,5)P_3$ binding to the microsomal fraction of antisense-transfected cells was significantly reduced (53%, $P<0.01$), compared to vector-transfected cells (8±1 fmol $Ins(1,4,5)P_3$ bound/mg (n=15) versus 17±3 fmol $Ins(1,4,5)P_3$

Fig. 6. Single-cell $[Ca^{2+}]_i$ responses to 60 nM ET-1. Cells were loaded with fura-2 and single-cell $[Ca^{2+}]_i$ measurements were performed as described in Materials and Methods.
(A) Representative traces are shown together with the percentage of the cells showing the specific response. The time points of ET-1 addition are indicated by the arrows. The results are expressed as a ratio of fura-2 fluorescence. The number of cells examined were 17 control and 16 antisense, from three independent antisense- or vector-transfected cell clones. (B) Amplitude of initial Ca^{2+} peak or Ca^{2+} oscillations following treatment with 60 nM ET-1. Results are expressed as the rise in the ratio of fura-2 fluorescence and represent the mean and s.e.m. of at least seven measurements, using three independent antisense- or vector-transfected clones. *$P<0.05$.

bound/mg (n=9), respectively). These results indicate that the elevation in Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ observed in antisense-transfected cells induces down-regulation of the Ins(1,4,5)P_3 receptors (Hajnoczky and Thomas, 1994; Wojcikiewicz, 1995). We also attempted to analyse Ins(1,4,5)P_3 receptor expression by western blotting the microsomal fractions with antibodies to the three receptor isoforms. Due to a consistently low signal, we were unable to convincingly determine the concentration of each receptor isoform and correlate this with Ins(1,4,5)P_3 binding (results not shown).





DISCUSSION

The precise cellular mechanisms by which the major Ins(1,4,5)P_3 5-phosphatase, the 43 kDa enzyme, regulates Ins(1,4,5)P_3 levels are currently being delineated. The results of the present study demonstrate changes in the expression of the 43 kDa 5-phosphatase lead to dramatic alterations in intracellular Ca^{2+} homeostasis. Reduction in 43 kDa 5-phosphatase enzyme activity results in elevated basal levels of Ins(1,4,5)P_3, leading to spontaneous Ca^{2+} oscillations, in the absence of agonist stimulation, and significantly enhanced sensitivity of receptor agonists to induce cytoplasmic Ca^{2+} oscillations. As these effects do not involve alterations in Ca^{2+} influx, or the rate of fall of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 following agonist stimulation, we conclude that underexpression of the 43 kDa 5-phosphatase plays a critical role in regulating the Ca^{2+} mobilising second messengers in the unstimulated cell.

Erneux and colleagues have shown that overexpression of the 43 kDa 5-phosphatase in CHO-K1 cells results in the abrogation of Ca^{2+} oscillations in response to 1 μM ATP, presumably due to increased hydrolysis of Ins(1,4,5)P_3 by the 43 kDa 5-phosphatase in the plasma membrane and the subsequent inability of Ins(1,4,5)P_3 to reach the receptor located in the endoplasmic reticulum (De Smedt et al., 1996, 1997). However these studies did not demonstrate, as we have shown, that the 43 kDa 5-phosphatase controls both the amplitude of Ca^{2+} responses and sensitivity to agonist-induced Ca^{2+} oscillations, by regulating the basal Ins(1,4,5)P_3 concentration. We propose the increased basal Ins(1,4,5)P_3 concentration activates the Ins(1,4,5)P_3 receptors in the unstimulated cell, leading to spontaneous Ca^{2+} oscillations. Although the subsequent agonist-induced release of Ins(1,4,5)P_3 is not exaggerated in cells underexpressing the 43 kDa 5-phosphatase and its fall to pre-stimulation levels is not delayed, the cumulative increase in Ins(1,4,5)P_3 results in markedly enhanced sensitivity to agonists, and doubling of the amplitude of initial Ca^{2+} spikes and subsequent oscillations.

It is noteworthy that although cells underexpressing the 43 kDa 5-phosphatase clearly have enhanced Ca^{2+} responses in the absence of agonist stimulation, neither complete Ins(1,4,5)P_3 receptor, inactivation or complete emptying of the Ca^{2+} stores was observed. Ins(1,4,5)P_3 receptor activation and subsequent down-regulation clearly occurs in unstimulated cells underexpressing the 43 kDa 5-phosphatase, as

Fig. 7. Single-cell $[Ca^{2+}]_i$ responses to 1 nM ET-1. Cells were loaded with fura-2 and single-cell $[Ca^{2+}]_i$ measurements were performed as described in Materials and Methods. (A) Representative traces are shown together with the percentage of the cells showing the specific response. The time points of ET-1 addition are indicated by the arrows. The results are expressed as a ratio of fura-2 fluorescence. The number of cells examined were 21 control and 19 antisense, from three independent antisense- or vector-transfected cell clones. (B) Amplitude of Ca^{2+} oscillations induced by 1 nM ET-1. Results are expressed as the rise in the ratio of fura-2 fluorescence and represent the mean and s.e.m. of at least 26 measurements, using three independent antisense- or vector-transfected clones. *P<0.05.

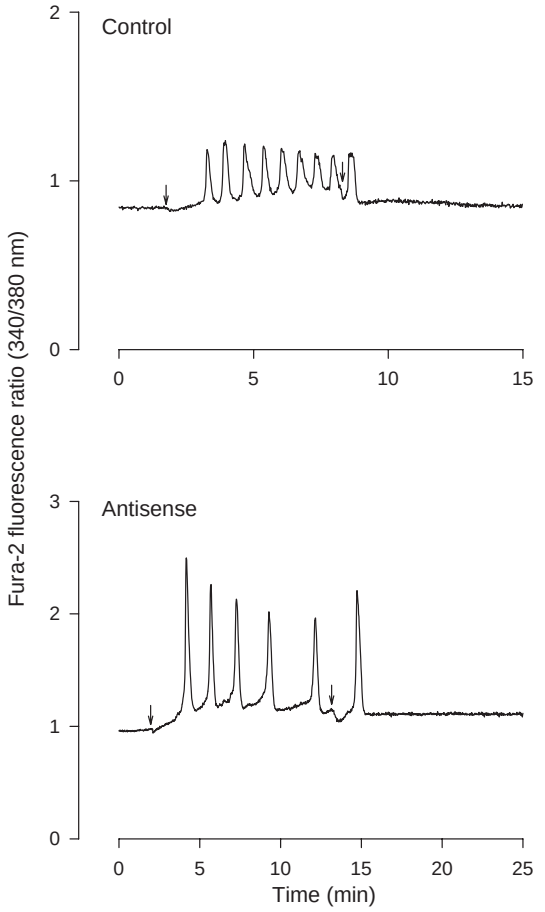


Fig. 8. Ca^{2+} oscillations induced by ET-1 are dependent on extracellular Ca^{2+} entry. Cells were loaded with fura-2 and single-cell $[Ca^{2+}]_i$ measurements were performed as described in Materials and Methods. Addition of ET-1 (1 nM) is indicated by the left arrows. $LaCl_3$ (500 μM) was added subsequently to block Ca^{2+} entry (right arrows). Results are expressed as a fura-2 fluorescence ratio. Each trace is representative of at least four similar traces, using three independent antisense- or vector-transfected clones.

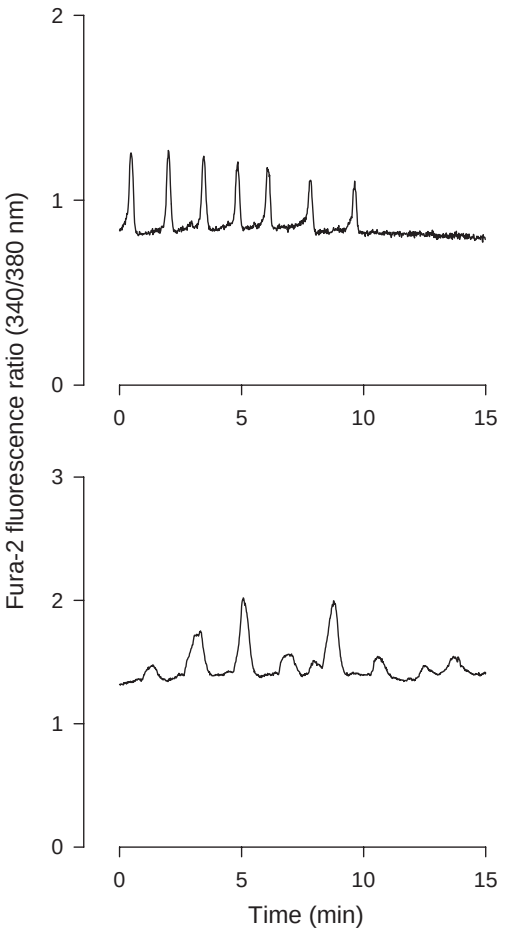


Fig. 9. Cells underexpressing the 43 kDa 5-phosphatase exhibit spontaneous Ca^{2+} oscillations. Single-cell $[Ca^{2+}]_i$ measurements were made on fura-2 loaded cells as described in Materials and Methods. Each trace depicts the typical oscillatory pattern displayed by antisense cells. The results are expressed as a ratio of fura-2 fluorescence. These results were obtained using three independent antisense-transfected clones.

demonstrated by a 53% decrease in $[^3H]Ins(1,4,5)P_3$ binding to the microsomal fraction. In most cellular systems a delayed $Ins(1,4,5)P_3$ receptor inactivation is observed following agonist-induced rises in cytosolic Ca^{2+} (Dufour et al., 1997; Hajnoczky and Thomas, 1997; Marchant and Taylor, 1997). Down-regulation of the $Ins(1,4,5)P_3$ receptor is in part an adaptive process as a consequence of accelerated $Ins(1,4,5)P_3$ receptor degradation. Although all three receptor subtypes (I, II and III) have been shown to be down-regulated, each isoform shows different sensitivity to degradation, with the Type II receptor being relatively resistant (Wojcikiewicz, 1995). We were unable to demonstrate by western analysis using isotype specific antibodies, $Ins(1,4,5)P_3$ receptor expression even in vector-transfected cells. This may relate to relatively low level receptor expression in NRK cells. Despite the decreased expression of the $Ins(1,4,5)P_3$ receptor, exaggerated Ca^{2+} responses were still observed in cells underexpressing the 43 kDa 5-phosphatase following endothelin-1 stimulation. Recent studies have suggested a model for inactivation of the $Ins(1,4,5)P_3$ receptor, proposing this is associated with conversion of the receptor to a low affinity activatable state, rather that complete inactivation of the receptor (Marchant and Taylor, 1997), and thereby Ca^{2+} release still may occur.

The $Ins(1,4,5)P_3$-sensitive Ca^{2+} store was significantly (40%, $P<0.01$) depleted in cells underexpressing the 43 kDa 5-phosphatase. We propose the elevated $Ins(1,4,5)P_3$ concentration increases the sensitivity of the $Ins(1,4,5)P_3$ receptors to induce Ca^{2+} release which partially empties the store, but this is not sufficient to activate Ca^{2+} influx. Recent investigations have suggested that there are two types of functional $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores, one involved in Ca^{2+} release and another primarily responsible for the activation of Ca^{2+} influx (Parekh et al.,1997). In this study dialysis of $Ins(1,4,5)P_3$ (60-600 nM) into rat basophilic leukemia cells significantly reduced the Ca^{2+} content of the stores without activating Ca^{2+} entry, consistent with our observations.

An unexpected finding from our studies was the observation that, despite a profound decrease in Ins(1,4,5)P_3 5-phosphatase enzyme activity, the rate of decline of agonist-induced increases in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 levels was not altered. These results show that the 43 kDa 5-phosphatase does not mediate the fall in these second messengers following agonist stimulation. A 2-3-fold elevation in Ins(1,4,5)P_3 may increase the maximal activity of other 5-phosphatases, such as the 75 kDa enzyme (5-phosphatase II) or synaptojanin, which also hydrolyse Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Mitchell et al., 1989; McPherson et al., 1996; Matzaris et al., 1998). In support of this, we have shown that approximately 20% of the total cellular Ins(1,4,5)P_3 5-phosphatase may be attributed to the recombinant 5-phosphatase II, as assessed by immunoprecipitation of NRK vector-transfected cell lysates using antibodies to this isoform (results not shown). An alternate explanation for the fall in Ins(1,4,5)P_3 concentration post-agonist stimulation may relate to increased conversion of Ins(1,4,5)P_3 into Ins(1,3,4,5)P_4 by the Ins(1,4,5)P_3 3-kinase. Several studies have shown that the 3-kinase is activated by increasing levels of $[Ca^{2+}]_i$ (Rossier et al., 1987; Zilberman et al., 1987; Dupont and Erneux, 1997). [^{3}H]Ins(1,3,4,5)P_4 levels were consistently elevated approximately 1.5-fold in cells underexpressing the 43 kDa 5-phosphatase. However, as the 43 kDa 5-phosphatase hydrolyses Ins(1,3,4,5)P_4, it is unclear whether elevation in Ins(1,3,4,5)P_4 occurs via enhanced activation of the Ins(1,4,5)P_3 3-kinase enzyme, or as a result of decreased hydrolysis of this second messenger.

We have previously shown that cells underexpressing the 43 kDa 5-phosphatase have a transformed phenotype (Speed et al., 1996). Decreased Ins(1,4,5)P_3 5-phosphatase activity is observed in several human acute and chronic leukemia's, although the identity of the 5-phosphatase enzymes involved has not been defined (Nye et al., 1992; Mengubas et al., 1994). Recent studies have revealed that the amplitude, duration and frequency of Ca^{2+} signals contribute to transcriptional activation and specificity (Dolmetsch et al., 1997, 1998; Berridge, 1997; Li et al., 1998; Meldolesi, 1998). Low sustained concentrations of intracellular Ca^{2+} activate the nuclear factor of activated T cells (NFAT), whilst large Ca^{2+} transients activate transcriptional factors such as NF-κB and c-Jun kinase. The frequency of calcium oscillations are also specific for gene transcription, differentially regulating both the efficiency and selectivity of gene activation. In addition, frequency-specific effects of intracellular Ca^{2+} oscillations have been implicated in neuronal differentiation (Gu and Spitzer, 1995). We predict the spontaneous Ca^{2+} oscillations and the enhanced agonist-induced Ca^{2+} spiking observed in cells underexpressing the 43 kDa 5-phosphatase results in differential activation of transcription factors that regulate cellular transformation.

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