Evidence for secretion-like coupling involving pp60^{src} in the activation and maintenance of store-mediated Ca^{2+} entry in mouse pancreatic acinar cells

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Store-mediated Ca^{2+} entry (SMCE) is one of the main pathways for Ca^{2+} influx in non-excitable cells. Recent studies favour a secretion-like coupling mechanism to explain SMCE, where Ca^{2+} entry is mediated by an interaction of the endoplasmic reticulum (ER) with the plasma membrane (PM) and is modulated by the actin cytoskeleton. To explore this possibility further we have now investigated the role of the actin cytoskeleton in the activation and maintenance of SMCE in pancreatic acinar cells, a more specialized secretory cell type which might be an ideal cellular model to investigate further the properties of the secretion-like coupling model. In these cells, the cytoskeletal disrupters cytochalasin D and latrunculin A inhibited both the activation and maintenance of SMCE. In addition, stabilization of a cortical actin barrier by jasplakinolide prevented the activation, but not the maintenance, of SMCE, suggesting that, as for secretion, the actin cytoskeleton plays a double role in SMCE as a negative modulator of the interaction between the ER and PM, but is also required for this mechanism, since the cytoskeleton disrupters impaired Ca^{2+} entry. Finally, depletion of the intracellular Ca^{2+} stores induces cytoskeletal association and activation of pp60^{src}, which is independent on Ca^{2+} entry. pp60^{src} activation requires the integrity of the actin cytoskeleton and participates in the initial phase of the activation of SMCE in pancreatic acinar cells.

Key words: actin cytoskeleton, Ca^{2+} influx, cytochalasin D, jasplakinolide.

INTRODUCTION

In pancreatic acinar cells, the main mechanism for Ca^{2+} influx is store-mediated Ca^{2+} entry (SMCE), where the filling state of the intracellular Ca^{2+} stores [endoplasmic reticulum (ER)] modulates the entry of Ca^{2+} across plasma membrane (PM) channels [1]. The mechanism by which store depletion activates Ca^{2+} entry is not fully understood. Several hypotheses have considered both indirect and direct coupling mechanisms. Indirect coupling assumes the existence of a diffusible messenger generated by the Ca^{2+} storage organelles that gates Ca^{2+} channels in the PM. Alternatively, direct coupling (conformational coupling) suggests a physical interaction between the ER and the PM [2,3]. Recently, a secretion-like coupling model has been proposed in smooth muscle cell lines [4], human platelets [5] and Xenopus oocytes [6], on the basis of a physical and reversible interaction of the ER with the PM, which involves trafficking of portions of the ER towards the PM.

In the secretion-like coupling model, a close interaction between the ER and PM, where the actin cytoskeleton plays an important regulatory role, is essential for Ca^{2+} entry, as it does in secretion. Especially important is the cortical actin cytoskeleton, which prevents the coupling between the ER and PM [4,5,7]. Although the inhibitory role of condensation of cortical cytoskeleton in SMCE has been demonstrated extensively [4,5,7,8], disruption of the actin filament network has provided conflicting results in several cell types. In this context, inhibition of actin polymerization abolished (in vascular endothelial cells or astrocytes [9,10]), reduced (in platelets or HepG2 cells [5,11]) or had no effect (in NIH 3T3 or smooth muscle cells [12,13]) on SMCE. These discrepancies are probably due to the variability in the intrinsic properties of actin cytoskeleton as a function of the cell type.

In pancreatic acinar cells, tyrosine kinases, such as those of the Src family, are involved in many cellular events, including exocrine secretion, cell adhesion, cell growth and differentiation [14,15]. In addition, tyrosine phosphorylation has been suggested to be required for the activation of SMCE in different cell types, including pancreatic acinar cells [16,17]. The cytoplasmic tyrosine kinase pp60^{src} has been shown to be involved in a complex with caveolin [18], a major component of caveolae, where Ca^{2+} signalling complexes, including transient receptor potential ('Trp') channels, have been reported to be located [19,20].

In the present study, we sought to expand our understanding of the mechanisms of activation and maintenance of SMCE in pancreatic acinar cells, a secretory cell type where exocytosis and SMCE might share components and characteristics and, therefore, is an ideal model to investigate the properties of the secretion-like coupling model for Ca^{2+} entry. Our results indicate that the secretion-like coupling is the most likely model to explain the activation and maintenance of SMCE in pancreatic acinar cells. In these cells, as for secretion [21], the cortical actin network acts as a clamp which blocks the interaction between the ER and PM. However, the actin cytoskeleton also has a positive role, since actin depolymerizing agents inhibited store depletion-induced Ca^{2+} entry. We have also investigated the cytoskeletal association and activation of the protein-tyrosine kinase pp60^{src} after depletion of the internal Ca^{2+} stores in pancreatic acinar cells.

MATERIALS AND METHODS

Animals and materials

Adult male Swiss mice were obtained from the Animal Farm, Veterinary Faculty, University of Extremadura, Cáceres, Spain.

Abbreviations used: [Ca^{2+}]$_i$, intracellular free Ca^{2+} concentration; CCK, cholecystokinin; ER, endoplasmic reticulum; HBS, Hepes-buffered saline; PM, plasma membrane; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; SMCE, store-mediated Ca^{2+} entry; TBS, Tris-buffered saline; TBST, TBS containing 0.1% Tween 20.

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Collagenase (CLSPA grade) was obtained from Worthington Biochemicals (Lakewood, NJ, U.S.A.). Fura 2 acetoxymethyl ester and jasplakinolide were from Molecular Probes (Leiden, The Netherlands). BSA, paraformaldehyde, leupeptin, benzamidine, PMSF, Nonidet P40, FITC-labelled phalloidin, cholecystokinin (CCK)-8 and thapsigargin were from Sigma (Madrid, Spain). Cytochalasin D and latrunculin A were from Calbiochem (Madrid, Spain). 4-Amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo[3,4-d]-pyrimidine (PP1) was from Alexis Corporation (Nottingham, U.K.). The phospho-specific antibody to c-Src phosphorylated at Tyr[416] [anti-(phospho-c-Src Tyr[416]) antibody] and horseradish peroxidase-conjugated goat anti-(rabbit IgG) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-pp60[src] monoclonal antibody (GD11) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and horseradish peroxidase-conjugated ovine anti-(mouse IgG) antibody (NA931) was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Enhanced chemiluminescence detection reagents were from Pierce (Tattenhall, Cheshire, U.K.). All other reagents were purchased from Panreac (Barcelona, Spain).

Preparation of isolated pancreatic acinar cells

Animals were killed by cervical dislocation, the pancreas was rapidly removed and the acinar cells were isolated as described previously [22]. Briefly, the pancreas was incubated in the presence of collagenase for 5–10 min at 37 °C under gentle agitation. The enzymic digestion of the tissue was followed by gently pipetting the cell suspension through tips of decreasing diameter for mechanical dissociation of the acinar cells. After centrifugation, cells were resuspended in Hepes-buffered saline [HBS; 10 mM Hepes, 140 mM NaCl, 4.7 mM KCl, 1 mM $MgCl_2$, 10 mM glucose and 1.1 mM $CaCl_2$ (pH 7.4)]. In experiments performed in Ca^{2+}-free medium, cells were resuspended in HBS containing 0.2 mM $CaCl_2$, and 250 μM EGTA was added at the time of experiment. Cell viability, monitored with Trypan Blue, was always greater than 95% and this was not significantly reduced by drugs at least for the duration of the experiments.

Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

Freshly isolated mouse pancreatic acinar cells were loaded with fura 2 by incubation with fura 2 acetoxymethyl ester (2 μM) at 23–25 °C for 40 min as described previously [17]. Fluorescence was recorded from 1.5 ml aliquots of magnetically stirred cell suspension at 37 °C using a spectrofluorimeter (Shimadzu, Japan). Samples were alternatively excited at 340 and 380 nm and the resulting fluorescence was measured at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored and presented as the 340/380 fluorescence ratio of fura 2. Thapsigargin- and CCK-8-evoked Ca^{2+} release from internal stores was estimated as the integral of the rise in $[Ca^{2+}]_i$ above basal for 6 min after addition of the agent.

Ca^{2+} influx was estimated using the integral of the rise in the 340/380 fluorescence ratio for 2 min and 30 s after addition of $CaCl_2$, where a sample was taken every second [23]. When cells were preincubated with inhibitors, Ca^{2+} entry was corrected by subtraction of the 340/380 fluorescence ratio due to leakage of the indicator. To calculate the initial rate of Ca^{2+} elevation after the addition of Ca^{2+} to the medium, the traces were fitted to the equation $y = A + KX$, where K is the slope.

To compare the rate of decay of $[Ca^{2+}]_i$ to basal values, after treatment of pancreatic acinar cells with thapsigargin and between different treatments, we used the constant of the exponential decay. Traces were fitted to the equation $y = A(1 - e^{-K_1 T})e^{-K_2 T}$, where K_1 and K_2 are the constants of exponential increase and decay respectively, T is temperature and A is the span.

Confocal microscopy

For actin filament localization studies, samples of FITC-labelled phalloidin-stained cells were placed on to a coverslip attached to the bottom of a perfusion chamber on the stage of a confocal microscope (eclipse TE300; Nikon, Kanagawa, Japan). Actin filaments were visualized using a confocal laser-scanning system (MRC 1024; Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) with excitation wavelength of 488 nm and emission at 515 nm.

pp60[src] activation

Autophosphorylation of pp60[src] at Tyr[416] and thus activation was detected by gel electrophoresis and Western blotting using the anti-(phospho-c-Src Tyr[416]) antibody [24]. Stimulation of pancreatic acinar cells was terminated by addition of Laemmli's buffer [25] with 5% (v/v) dithiothreitol, followed by heating for 5 min at 95 °C. One-dimensional SDS/PAGE was performed with 12.5% (w/v) polyacrylamide minigels, and separated proteins were electrophoretically transferred for 2 h at 0.8 mA/cm² using a semi-dry blotter (Hoefer Scientific, Newcastle-under-Lyme, Staffs., U.K.) on to nitrocellulose for subsequent immunodetection. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline [TBS; 20 mM Tris/HCl (pH 7.4) and 137 mM NaCl] with 0.1% Tween 20 (TBST) to block residual protein binding sites. Immunodetection of pp60[src] phosphorylated at Tyr[416] was achieved using the anti-(phospho-c-Src Tyr[416]) antibody diluted 1:600, according to the manufacturer's instructions, in TBST for 2 h at 20 °C. The primary antibody was removed and blots were washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated goat anti-(rabbit IgG) antibody diluted 1:10000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were exposed to photographic films and the integrated absorbance of the blots was estimated using scanning densitometry.

Analysis of cytoskeleton-associated pp60[src]

Cell fractionation was carried out as described previously [26]. Briefly, resting or thapsigargin-treated pancreatic acinar cells were immediately lysed with an equal volume of 2 × Triton buffer [2% (w/v) Triton X-100, 2 mM EGTA, 100 mM Tris/HCl (pH 7.2), 100 μg/ml leupeptin, 2 mM PMSF, 10 mM benzamidine and 2 mM Na_3VO_4] at 4 °C for 30 min. Cell lysates were centrifuged at 16000 g for 5 min, the pellet (cytoskeleton-rich fraction) was solubilized into the original volume in Laemmli's buffer [25], followed by heating for 5 min at 95 °C and was subjected to Western blotting, as described above, using the anti-pp60[src] monoclonal antibody GD11.

Measurement of F-actin content

The F-actin content of resting and stimulated pancreatic acinar cells was determined as described previously [23]. Briefly, acinar cells were stimulated in HBS and samples of acinar cell suspensions (200 μl) were transferred to 200 μl of ice-cold 3% (w/v) formaldehyde in PBS for 10 min. Fixed cells were permeabilized by incubation for 10 min with 0.025% (v/v)

Nonidet P40 in PBS. Cells were then incubated for 30 min with FITC-labelled phalloidin (0.5 μM) in PBS containing 0.5% (w/v) BSA. After incubation, the cells were collected by centrifugation for 60 s at 3000 $\boldsymbol{g}$ and resuspended in PBS. Staining of actin filaments was measured using a Shimadzu spectrofluorimeter. Samples were excited at 496 nm and emission was recorded at 516 nm.

Statistical analysis

Analysis of statistical significance was performed using Student's t test and only values with $P < 0.05$ were accepted as significant.

RESULTS

Effect of actin filament disruption on the activation and maintenance of SMCE in pancreatic acinar cells

We have reported previously [5,11,23] in human platelets and the human hepatocellular carcinoma cell line HepG2 that inhibition of actin polymerization prevents thapsigargin-induced Ca²⁺ entry. To investigate further whether the actin cytoskeleton is important for thapsigargin-induced Ca²⁺ influx in pancreatic acinar cells, latrunculin A and cytochalasin D were used to disrupt the cytoskeletal network by blocking the formation of actin microfilaments. Latrunculin A is a cell-permeant toxin that disrupts actin filament organization by the formation of complexes with monomeric G-actin [27], whereas cytochalasin D inhibits actin polymerization by binding to the growing end of actin filaments and blocking the further addition of monomeric G-actin [28]. Resting or treated pancreatic acinar cells were fixed, stained with FITC-labelled phalloidin and examined by confocal laser microscopy. In resting cells, actin filaments were mainly organized in a thick cortical layer located beneath the apical membrane and a weak staining was also detected near the basolateral membrane and in the cytoplasmic space (Figure 1A). Pretreatment of pancreatic acinar cells at 37 °C for 1 h with 3 μM latrunculin A or for 40 min with 10 μM cytochalasin D significantly reduced the intensity of actin filament staining in both the basolateral and apical area (Figures 1B and 1C). Figure 1(D) shows non-treated acinar cells that, after fixation and permeabilization, maintained their original shape and organization.

To ascertain whether the actin cytoskeleton is involved in the activation of SMCE, pancreatic acinar cells were preincubated in the presence of cytoskeletal disrupters and thapsigargin-induced SMCE was analysed. As expected, in the absence of extracellular Ca²⁺, addition of thapsigargin (1 μM) to fura 2-loaded pancreatic acinar cells in stirred cuvettes at 37 °C induced an elevation in $[Ca^{2+}]_i$, due to the release of Ca²⁺ from intracellular stores. As shown in Figure 2(A), treatment of pancreatic acinar cells with 1 μM thapsigargin completely depleted the agonist-releasable Ca²⁺ stores ($n = 4$). Subsequent addition of $CaCl_2$ (2 mM) to the external medium resulted in a sustained increase in $[Ca^{2+}]_i$, indicative of SMCE (Figure 2B, control). Treatment of fresh pancreatic acinar cells at 37 °C for 1 h with 3 μM latrunculin A or for 40 min with 10 μM cytochalasin D significantly decreased thapsigargin-induced Ca²⁺ influx by 78.8 ± 8.8 and 46.6 ± 9.8 % respectively (Figure 2B and Figure 3; $P < 0.01$; $n = 5$–10). The initial rate of Ca²⁺ elevation was also decreased by disruption of the actin cytoskeleton. The initial slope was significantly decreased from 0.0612 ± 0.0060 in control cells to 0.0037 ± 0.0009 and 0.0391 ± 0.0046 in latrunculin A- or cytochalasin D-treated cells respectively ($P < 0.01$; $n = 5$–10).

Cells previously incubated with latrunculin A or cytochalasin D showed a similar release of Ca²⁺ from the intracellular pools
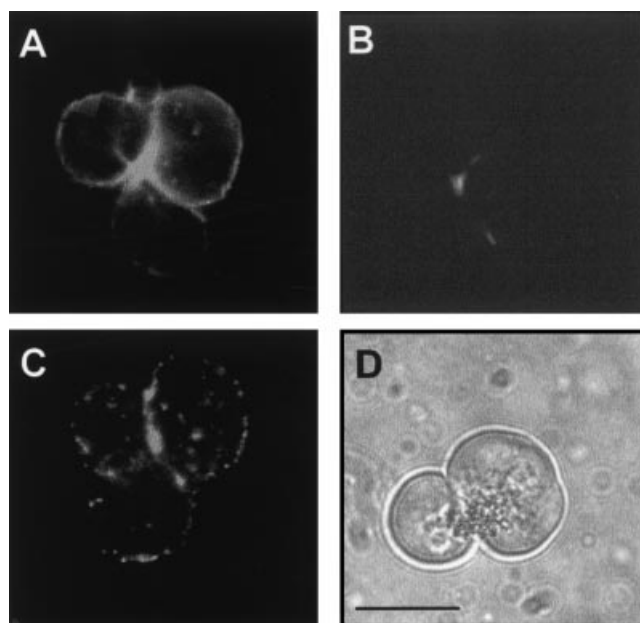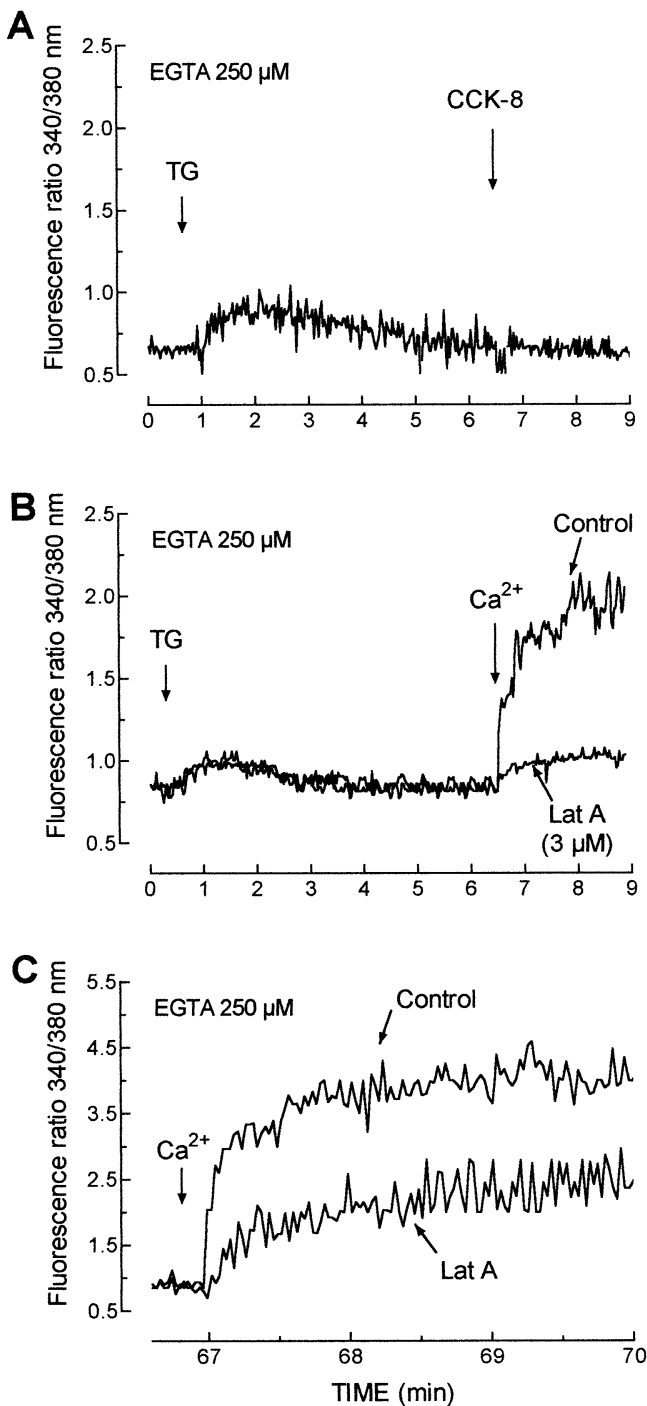

Figure 1   Effect of latrunculin A and cytochalasin D on the actin cytoskeleton in pancreatic acinar cells

Resting pancreatic acinar cells (**A**), cells treated for 1 h with 3 μM latrunculin A (**B**) or for 40 min with 10 μM cytochalasin D (**C**) were fixed, stained with FITC-labelled phalloidin and fluorescence was detected using a confocal microscope, as described in the Materials and methods section. (**D**) Transmission micrograph of the fluorescent images of non-treated pancreatic acinar cells that were fixed and permeabilized as the others. Images shown are representative of 8–10 separate experiments. Scale bar represents 20 μm.

in response to thapsigargin compared with control cells (110.2 ± 4.7 and 101.7 ± 5.26 % of control respectively; Figure 2B and Figure 3). These findings indicate that the accumulation of Ca²⁺ in the internal stores was unaffected by disruption of the actin filament network. To investigate whether the inhibition of SMCE observed after treatment with latrunculin A or cytochalasin D was not due to an increase in Ca²⁺ extrusion, we studied the kinetics of the recovery after treatment with thapsigargin by following a previously published procedure [29]. Treatment of pancreatic acinar cells with latrunculin A or cytochalasin D did not significantly modify the rate of decay of $[Ca^{2+}]_i$ to basal levels. The decay constants were 0.0071 ± 0.0006 in control cells and 0.0063 ± 0.0005 or 0.0073 ± 0.0003 in latrunculin A- or cytochalasin D-treated cells ($n = 5$).

We have reported previously [5] that the integrity of the actin cytoskeleton is essential for the maintenance of SMCE in human platelets. To investigate this possibility further, we examined the effect of latrunculin A and cytochalasin D on Ca²⁺ entry in pancreatic acinar cells once the Ca²⁺ stores had been fully depleted by treatment with thapsigargin.

To examine the effect of addition of latrunculin A or cytochalasin D to store-depleted cells, 3 μM latrunculin A, 10 μM cytochalasin D or vehicles (control) were added 6 min after thapsigargin and cells were then incubated for a further 1 h or 40 min respectively, before the addition of Ca²⁺ to the medium (final concentration 2 mM) to initiate Ca²⁺ entry. At the time when the inhibitors were added, the agonist-sensitive Ca²⁺ stores had already been depleted, as indicated by the lack of further release of Ca²⁺ stimulated by CCK-8 (Figure 2A; $n = 4$) and, therefore, Ca²⁺ entry was already stimulated (Figure 2B and Figure 3). Addition of latrunculin A or cytochalasin D once

A

EGTA 250 μM

TG

CCK-8

B

EGTA 250 μM

Control

Ca²⁺

TG

Lat A
(3 μM)

C

EGTA 250 μM   Control

Ca²⁺

Lat A

TIME (min)

Figure 2   Effect of latrunculin A on the activation and maintenance of SMCE in pancreatic acinar cells

(**A**) Fura 2-loaded pancreatic acinar cells were treated with thapsigargin (TG; 1 μM) in a Ca²⁺-free medium (250 μM EGTA was added) and 6 min later cells were stimulated with CCK-8 (1 nM). (**B**) Fura 2-loaded pancreatic acinar cells were incubated at 37 °C for 1 h in the presence of 3 μM latrunculin A (Lat A) or the vehicle (Control). At the time of the experiment, 250 μM EGTA was added. Cells were then stimulated with thapsigargin (1 μM) and 6 min later CaCl₂ (final concentration 2 mM) was added to the medium to initiate Ca²⁺ entry. (**C**) Fura 2-loaded pancreatic acinar cells were suspended in a Ca²⁺-free medium (250 μM EGTA added) as described in the Materials and methods section. Cells were then stimulated with thapsigargin (TG; 1 μM) and 6 min later 3 μM latrunculin A (Lat A) or the vehicle (Control) were added. CaCl₂ (final concentration 2 mM) was added to the medium 1 h after latrunculin A or the vehicle to initiate Ca²⁺ entry. Changes in [Ca²⁺]ᵢ were monitored as described in the Materials and methods section. Traces are representative of 4–5 independent experiments.

EGTA 250 μM   Control

Ca²⁺

TG

CD
(10 μM)

TIME (min)
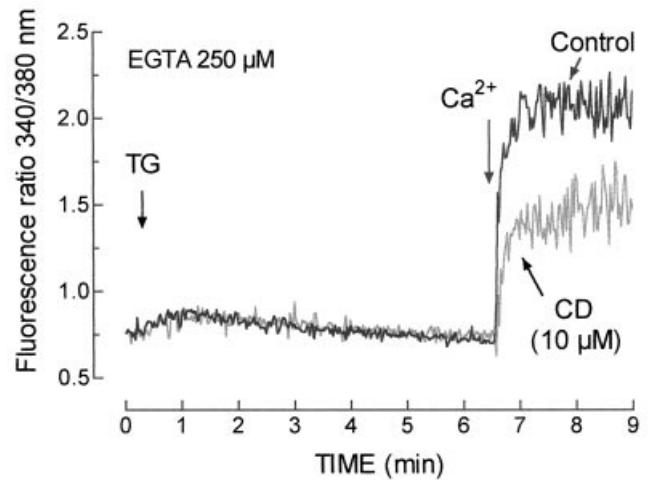
Figure 3   Effect of cytochalasin D on the activation and maintenance of SMCE in pancreatic acinar cells

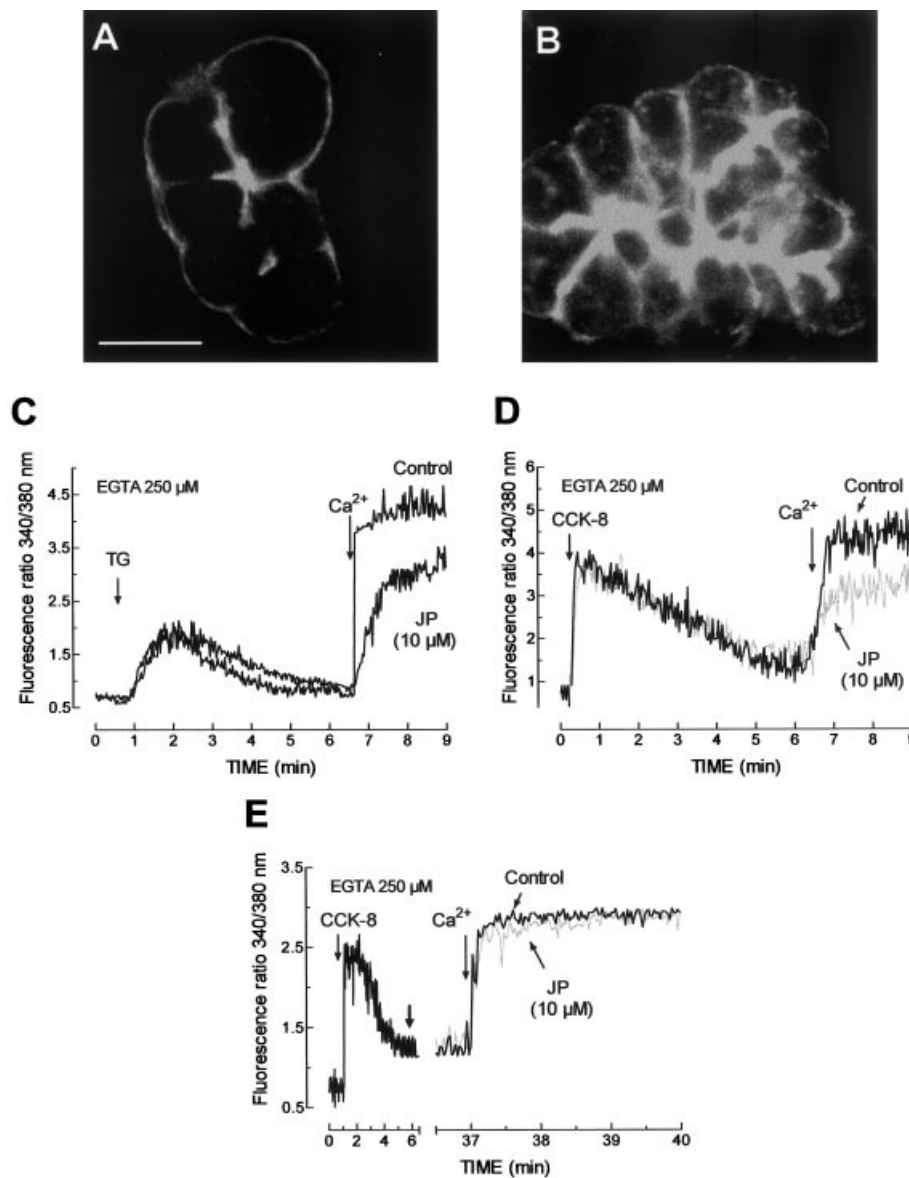(**A**) Fura 2-loaded pancreatic acinar cells were incubated at 37 °C for 40 min in the presence of 10 μM cytochalasin D (CD) or the vehicle (Control). At the time of the experiment, 250 μM EGTA was added. Cells were then stimulated with thapsigargin (TG; 1 μM) and 6 min later CaCl₂ (final concentration 2 mM) was added to the medium to initiate Ca²⁺ entry. Changes in [Ca²⁺]ᵢ were monitored as described in the Materials and methods section. Traces shown are representative of 10 independent experiments.

SMCE had been activated significantly decreased Ca²⁺ entry by $50.5 \pm 4.4\%$ (Figure 2C) and $48.2 \pm 1.0\%$ respectively ($P < 0.01$; $n = 5$–8). In addition, the initial slope was significantly decreased from 0.0693 ± 0.0053 in control cells to 0.0187 ± 0.0019 and 0.0291 ± 0.0022 in latrunculin A- or cytochalasin D-treated cells respectively ($P < 0.01$; $n = 5$–8). Taken together, these findings indicate that the integrity of the actin cytoskeleton is required for the activation and also the maintenance of SMCE in pancreatic acinar cells.

Modulatory role of the cortical actin filament network in SMCE in pancreatic acinar cells

As for secretion, where actin filaments beneath the PM act as a barrier preventing docking and fusion of secretory granules with the PM, the cortical actin cytoskeleton might prevent the physical interaction between the ER and PM required for the activation of SMCE in these cells. To investigate the role of the membrane skeleton in SMCE in pancreatic acinar cells we used jasplakinolide, a cell-permeant peptide isolated from *Jaspis johnstoni*, which has been shown to induce polymerization and stabilization of actin filaments exclusively at the cell periphery close to the PM [5,30]. As we have shown previously in human platelets [5], treatment of pancreatic acinar cells with 10 μM jasplakinolide for 30 min at 37 °C resulted in a significant enhancement in the intensity of actin filament staining beneath the basolateral and, mainly, apical membranes compared with untreated cells (Figures 4A and 4B).

Pretreatment of fresh pancreatic acinar cells for 30 min at 37 °C with jasplakinolide significantly decreased thapsigargin-evoked SMCE by $52.5 \pm 3.8\%$ compared with control (Figure 4C; $P < 0.01$; $n = 5$). As with the cytoskeletal disrupters, jasplakinolide also decreased the initial slope from 0.1144 ± 0.0021 in control cells to 0.0504 ± 0.0046 in jasplakinolide-treated cells ($P < 0.01$; $n = 5$). As shown for latrunculin A or cytochalasin D, treatment of pancreatic acinar

**Figure 4   Effect of jasplakinolide on the activation and maintenance of SMCE in pancreatic acinar cells**

Resting pancreatic acinar cells (**A**) or cells stimulated for 30 min with 10 μM jasplakinolide (**B**) were fixed, stained with FITC-labelled phalloidin and fluorescence was detected using a confocal microscope, as described in the Materials and methods section. Scale bar represents 20 μm. (**C** and **D**) Fura 2-loaded pancreatic acinar cells were incubated at 37 °C for 30 min in the presence of 10 μM jasplakinolide (JP) or the vehicle (Control). At the time of the experiment, 250 μM EGTA was added. Cells were then stimulated with 1 μM thapsigargin (TG) (**C**) or 1 nM CCK-8 (**D**) and 6 min later CaCl$_2$ (final concentration 2 mM) was added to the medium to initiate Ca²⁺ entry. (**E**) Fura-2-loaded pancreatic acinar cells were suspended in a Ca²⁺-free medium (250 μM EGTA added) and stimulated with 1 nM CCK-8. Jasplakinolide (JP; 10 μM) or the vehicle (control) was added 6 min later as indicated by the arrow. CaCl$_2$ (final concentration 2 mM) was added to the medium 30 min later to initiate Ca²⁺ entry. Changes in [Ca²⁺]$_i$ were monitored as described in the Materials and methods section. Traces are representative of 3–5 independent experiments.

cells with jasplakinolide had no effect on resting [Ca²⁺]$_i$ nor any effect on thapsigargin-evoked release of Ca²⁺ from the intracellular stores. The effect of jasplakinolide on SMCE was not caused by the activation of Ca²⁺ extrusion since the decay constant for [Ca²⁺]$_i$ recovery after treatment with thapsigargin was 0.0071 $\pm$ 0.0004 in control and 0.0065 $\pm$ 0.0003 in jasplakinolide-treated cells ($n = 5$).

As shown in Figure 4(D), treatment of pancreatic acinar cells with 10 μM jasplakinolide did not modify CCK-8-induced release of Ca²⁺ from the intracellular stores. However, consistent with the above, jasplakinolide reduced CCK-8-induced Ca²⁺

entry by 43.5 $\pm$ 8.7 % compared with the control ($P < 0.01$; $n = 3$).

We have reported previously [5] that disruption of the actin cytoskeleton once SMCE had been activated inhibited this process, suggesting that an actin-provided scaffold is essential for the maintenance of SMCE. To investigate further the role of the actin cytoskeleton in SMCE in pancreatic acinar cells, we added jasplakinolide to the cellular suspension after depletion of the intracellular Ca²⁺ stores by using thapsigargin or the physiological agonist CCK-8. Treatment of pancreatic acinar cells for 30 min with 10 μM jasplakinolide once SMCE had been activated
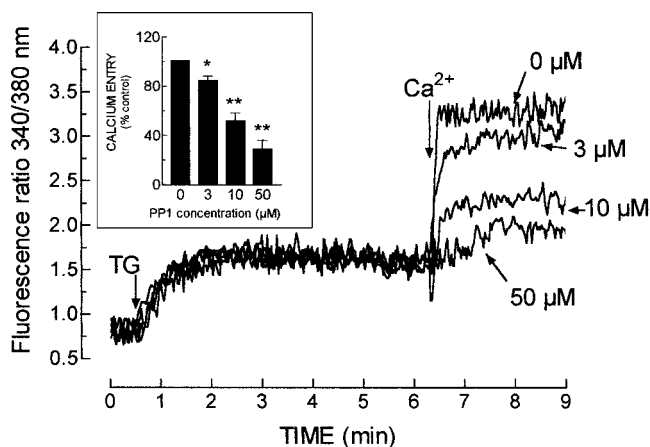
Figure 5 **Role of Src tyrosine kinases on the activation and maintenance of SMCE in pancreatic acinar cells**

Fura 2-loaded pancreatic acinar cells were incubated at 37 °C for 30 min in the presence of increasing concentrations of PP1 (3–50 μM) or the vehicle (0). At the time of the experiment, 250 μM EGTA was added. Cells were then stimulated with thapsigargin (TG; 1 μM) and 6 min later CaCl$_2$ (final concentration 2 mM) was added to the medium to initiate Ca^{2+} entry. Changes in [Ca^{2+}]$_i$ were monitored as described in the Materials and methods section. Traces shown are representative of 4–7 independent experiments. Inset: histograms indicate the percentage of Ca^{2+} entry in the presence of different concentrations of PP1 relative to the control, estimated as described in the Materials and methods section. $*P < 0.05$ and $**P < 0.01$ compared with control.

did not modify either thapsigargin- or CCK-8- (Figure 4E) induced Ca^{2+} entry (94.5$\pm$5.4 and 96.7$\pm$7.2% of control for thapsigargin- or CCK-8-induced Ca^{2+} entry respectively; $n = 3$–5). Consistent with this, the initial rate of [Ca^{2+}]$_i$ elevation was not modified. This observation provides further evidence indicating that stabilization of the cortical actin filaments inhibits the activation, but not the maintenance, of SMCE and is consistent with a secretion-like coupling model for the activation of SMCE in these cells. In addition, these findings indicate, in agreement with previous studies [4,5], that jasplakinolide is not a Ca^{2+} chelator or a Ca^{2+} channel blocker.

Role of Src proteins in Ca^{2+} entry and actin polymerization in pancreatic acinar cells

To investigate the involvement of the Src family of protein-tyrosine kinases in SMCE, PP1, a potent and selective inhibitor of these proteins [31], was used. As shown in Figure 5, treatment of pancreatic acinar cells for 30 min at 37 °C with PP1 resulted in a concentration-dependent inhibition of thapsigargin-stimulated SMCE. Treatment with PP1 significantly decreased Ca^{2+} entry by 16$\pm$4, 48$\pm$6 and 71$\pm$7% at 3, 10 and 50 μM respectively (Figure 5, inset; $P < 0.05$; $n = 4$–7). In addition, the initial slope was decreased in a concentration-dependent manner (0.0785$\pm$0.0021 in control cells versus 0.0674$\pm$0.0062, 0.0249$\pm$0.0045 and 0.0180$\pm$0.0052 in cells treated with 3, 10 and 50 μM PP1 respectively; $P < 0.05$; $n = 4$–7). It is also worth noting that preincubation with PP1 at any concentration did not modify the resting [Ca^{2+}]$_i$ or thapsigargin-induced Ca^{2+} release from the intracellular stores, indicating that accumulation of Ca^{2+} in the stores was unaffected by treatment with this inhibitor (Figure 5). In addition, the effect of PP1 on SMCE was not due to the activation of Ca^{2+} extrusion, since the decay constant for [Ca^{2+}]$_i$ recovery after treatment with thapsigargin was

0.0069$\pm$0.0002 in control and 0.0066$\pm$0.0004 in cells treated with 50 μM PP1 ($n = 5$).

To assess whether Src proteins are also required for the maintenance of SMCE, PP1 was added to the cell suspension once the Ca^{2+} stores had been depleted, as reported above. Treatment of pancreatic acinar cells for 30 min with 50 μM PP1 after SMCE had been activated by the addition of thapsigargin or the physiological agonist CCK-8 had negligible effects on Ca^{2+} entry (111.4$\pm$8.5 and 98.6$\pm$6.8% of control respectively; $n = $ 3–5), suggesting that, as shown in platelets [26], the Src family of tyrosine kinases are important for the activation, but not the maintenance, of SMCE in pancreatic acinar cells. In addition, this finding indicates that the inhibitory effect of PP1 on the activation of SMCE is not mediated by Ca^{2+} channel blockade or Ca^{2+} chelation.

Activation and cytoskeletal association of pp60^{src} after depletion of intracellular Ca^{2+} stores

We have reported previously [26] that the tyrosine kinase of the Src family pp60^{src} associates with the platelet cytoskeleton by depletion of the intracellular Ca^{2+} stores, a process that is important for the interaction with their substrates. Hence we have investigated whether this protein associates with the actin cytoskeleton in pancreatic acinar cells by Western-immunoblot analysis performed on the cytoskeletal fraction of resting and Ca^{2+}-store-depleted cells which had been treated with thapsigargin. As reported in platelets [26], treatment of pancreatic acinar cells with 1 μM thapsigargin for 6 min induced association of pp60^{src} with the cytoskeletal fraction (Figure 6A, $n = 3$).

In addition, we have analysed the possible activation of pp60^{src} upon depletion of the Ca^{2+} stores in pancreatic acinar cells by Western blotting using a specific anti-(phospho-c-Src Tyr^{416}) antibody, which specifically detects pp60^{src} phosphorylated at Tyr^{416}, a process indicative of pp60^{src} activation [24]. In a Ca^{2+}-free medium (250 μM EGTA was added), treatment of pancreatic acinar cells with 1 μM thapsigargin for 6 min to fully deplete the intracellular Ca^{2+} stores increased the activation of pp60^{src} by 415$\pm$32% of control (non-treated cells; Figure 6B, lanes 1 and 2; $n = 3$). In order to investigate whether Ca^{2+} entry is important for pp60^{src} activation, we performed a series of experiments stimulating cells with thapsigargin in a medium containing 2 mM Ca^{2+}. As shown in Figure 6B (lanes 3 and 4), under this condition, thapsigargin induced a slightly higher pp60^{src} activation (458$\pm$45% of control), suggesting that Ca^{2+} entry is not essential for the activation of pp60^{src} in pancreatic acinar cells. Finally, the activation of pp60^{src} was confirmed with the physiological agonist CCK-8, which has been reported to induce pp60^{src} activation in these cells [32]. Treatment of pancreatic acinar cells in a medium containing 2 mM Ca^{2+} for 6 min with 1 nM CCK-8 increased pp60^{src} activation by 525$\pm$48% of control (Figure 6B, lanes 5 and 6; $n = 3$).

Recent studies [26,33] have reported that actin polymerization is a step required for the association of pp60^{src} with the cytoskeletal fraction, a process essential for pp60^{src} to exert its function. To test whether the activation of pp60^{src} depends on the actin filament polymerization, cells were treated with cyto-chalasin D prior to addition of thapsigargin. Treatment of pancreatic acinar cells with 10 μM cytochalasin D for 1 h decreased thapsigargin-induced pp60^{src} activation by 95$\pm$3% compared with untreated thapsigargin-stimulated cells (Figure 6C, lanes 5 and 6 compared with lanes 1 and 2; $P < 0.01$; $n = 3$). The ability of cytochalasin D to inhibit activation of pp60^{src} after depletion of the intracellular Ca^{2+} stores suggests that this
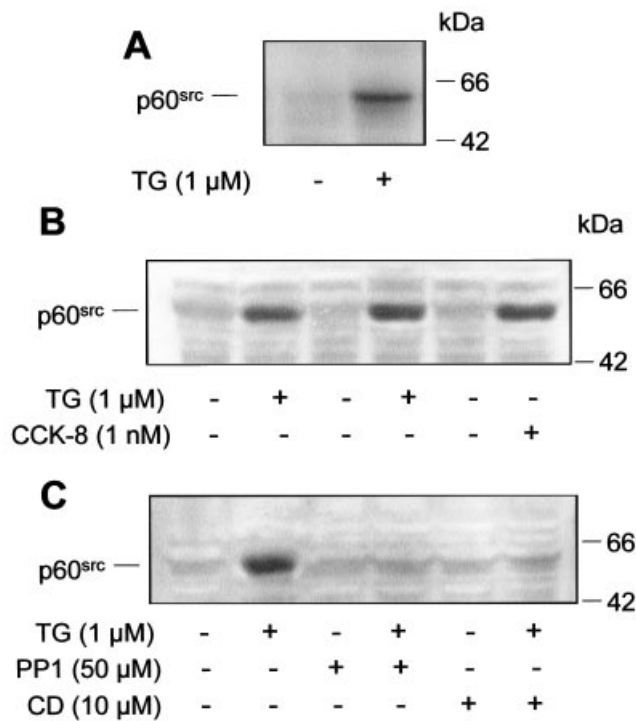
Figure 6   Thapsigargin-induced pp60^{src} activation is prevented by disruption of the actin filament network

(**A**) Pancreatic acinar cells were treated with either 1 μM thapsigargin (TG) or the vehicle for 6 min in the absence of external Ca²⁺ (250 μM EGTA was added) as indicated and then lysed. Samples were subjected to SDS/PAGE and Western blotting with the specific anti-pp60^{src} monoclonal antibody GD11 antibody, as described in the Materials and methods section. (**B**) Pancreatic acinar cells were treated with either 1 μM thapsigargin (TG) or the vehicle (—) for 6 min in the absence (lanes 1 and 2; 250 μM EGTA was added) or presence of 2 mM extracellular Ca²⁺ (lanes 3 and 4), or 1 nM CCK-8 for 6 min in the presence of 2 mM external Ca²⁺ (lanes 5 and 6) as indicated. Cells were lysed and subjected to SDS/PAGE and Western blotting as in (**A**). (**C**) Pancreatic acinar cells were preincubated in the absence (lanes 1 and 2) or presence of 50 μM PP1 (lanes 3 and 4) and 10 μM cytochalasin D (CD; lanes 5 and 6), treated with either 1 μM thapsigargin (TG) or the vehicle (—) for 6 min in the absence of external Ca²⁺ (250 μM EGTA was added), as indicated, and then lysed. Samples were subjected to SDS/PAGE and Western blotting with the specific anti-(phospho-c-Src Tyr⁴¹⁶) antibody as described in the Materials and methods section. Bands were revealed using chemiluminescence. The panels show results from one experiment representative of two others. Molecular-mass markers (in kDa) are shown on the right.

kinase might associate with the actin cytoskeleton in pancreatic acinar cells, a process that has been shown to be important for phosphorylation of their substrates [33,34]. Furthermore, we investigated whether pp60^{src} was also required for actin polymerization. Our results, in agreement with previous studies in platelets [26], indicated that exposure of pancreatic acinar cells to 50 μM PP1 for 30 min did not modify the F-actin content in resting or thapsigargin-stimulated cells ($n = 4$; results not shown). Finally, we confirmed further that treatment of pancreatic acinar cells with 50 μM PP1 induced complete inactivation of pp60^{src} (Figure 6C, lanes 3 and 4; $n = 3$).

DISCUSSION

The actin cytoskeleton is a highly dynamic structure, organized into two major structures: a cytoplasmic actin network and a membrane-associated cytoskeleton. In pancreatic acinar cells, the actin cytoskeleton modulates a large number of cellular processes, such as secretion [21], G-protein signalling [35] or

activation of focal adhesion plaque-associated proteins [36]. In response to different physiological agonists, the actin cytoskeleton is dramatically reorganized in these cells [37,38]

Although the study of the mechanism underlying the activation of SMCE has produced conflicting results, several studies in different non-excitable cells have provided compelling evidence supporting a secretion-like conformational coupling mechanism for the activation of SMCE [4–6,39,40]. This model suggests that Ca²⁺ store-depletion leads the trafficking of portions of the ER towards the PM to facilitate the coupling between proteins in both membranes, where the actin cytoskeleton plays a regulatory role. In support of this coupling, recent studies [41–43] have presented the interaction between Ca²⁺ channels in the PM and Ins(1,4,5)P_3 receptors in the ER.

The secretion-like coupling mechanism can be divided into three components: (i) an early component, which corresponds with the activation of SMCE, (ii) the maintenance of SMCE and (iii) a late component, which leads to the end of the event once the Ca²⁺ stores had been refilled. In the present study, we have focused on the involvement of the actin cytoskeleton in the first two mechanisms, namely activation and maintenance of SMCE.

In pancreatic acinar cells, latrunculin A and cytochalasin D, two widely used inhibitors of actin polymerization, disrupted the actin filament network, so that little actin filament staining was detected in these cells. These findings show that 'treadmilling' in pancreatic acinar cells is extremely active, since both agents are inhibitors solely of actin assembly and filament destruction occurs naturally from its 'minus' end. Interestingly, disorganization of the actin cytoskeleton resulted in a partial reduction of SMCE in pancreatic acinar cells.

Our observations are in agreement with previous studies in astrocytes and vascular endothelial cells [9,10] and our recent studies in human platelets or the HepG2 cell line [5,11], where the integrity of the actin filament network has been shown to be required for a normal Ca²⁺ entry. In contrast, Pedrosa-Ribeiro et al. [12] and Patterson et al. [4] have reported basically opposite results in 3T3 and muscle cells. The cause of these discrepancies is still unclear. In pancreatic acinar cells, the actin cytoskeleton is greatly reorganized in the apical area during exocytosis, so that secretory granules engaging in exocytosis become coated with actin filaments. This process is essential for the transport of granules to the apical membrane [44]. A similar reorganization of the subplasmalemmal actin network has been reported in platelets and vascular endothelial cells upon activation [45,46]. Therefore actin reorganization might be essential in allowing coupling between the ER and PM in these cells, but not in others with a more distributed cytoskeletal architecture, thus explaining the discrepancies in the effect of cytoskeletal disruption in SMCE in different cell types.

In a secretion-based coupling model, cortical F-actin should prevent constitutive SMCE activation, as it does in exocytosis, where the apical cytoskeleton blocks the approach and docking of secretory granules with the PM [21]. In agreement with this observation, jasplakinolide, a peptide that induces polymerization and reorganization of actin filaments exclusively near the PM [30], prevented thapsigargin- and CCK-8-induced SMCE without affecting Ca²⁺ release from the intracellular stores. Jasplakinolide inhibits SMCE by formation of a cortical actin barrier at the PM that excludes organelles from this area, thus preventing the interaction between the PM and internal organelles, as shown previously by its negative effect in secretion [47]. The negative role of stabilization of a cortical filament barrier in SMCE is a common feature shown in many different cell types, such as muscle cells, platelets, endothelial cells or lymphocytes [4,5,7,8].

Our present findings suggest that it is unlikely that an indirect coupling model, on the basis of the release of a diffusible messenger from the ER to gate a PM Ca^{2+} channel, mediates SMCE in pancreatic acinar cells. Such a factor could reach the PM through the cortical actin barrier, since $Ins(1,4,5)P_3$ generated by CCK-8 was able to release Ca^{2+} from the ER under these conditions. In contrast, SMCE was inhibited by the peripheral actin barrier induced by jasplakinolide. Therefore our results are in favour of a model based on a physical interaction between the ER and PM.

The observations described above suggest that the actin cytoskeleton plays a negative regulatory role in SMCE, but also an organized filament network is required for the activation of this process, since Ca^{2+} entry is reduced by disruption of the actin cytoskeleton. There are at least two possible explanations for the requirement of the actin cytoskeleton in SMCE. First, an actin-dependent vesicle trafficking of portions of the ER towards the PM is required to facilitate the coupling mechanism, and secondly, the interaction between the ER and PM needs an actin-provided support. To investigate the last possibility, we examined the effect of the actin network disruption once SMCE had been activated. If the actin cytoskeleton is required for the maintenance of SMCE, one would postulate that disruption of the actin-provided scaffold would prevent the interaction between ER and PM before store depletion, which would explain the effect of latrunculin A and cytochalasin D in the activation of SMCE, and also once the stores had been depleted, which would interrupt SMCE. We have found that addition of latrunculin A or cytochalasin D after store depletion reversed Ca^{2+} entry activated by thapsigargin. This suggests that the interaction between the ER and PM that underlies SMCE requires a physical support provided by the actin cytoskeleton. Consistent with this, jasplakinolide, which stabilises the actin cytoskeleton and therefore the actin-provided support, did not modify Ca^{2+} entry once activated by thapsigargin or the physiological agonist CCK-8 as expected, in agreement with our previous studies in human platelets [5].

It has been shown recently [26,48,49] that Src kinases are involved in Ca^{2+} signalling, including Ca^{2+} entry, in different excitable and non-excitable cells. Activation of pancreatic acinar cells with physiological agonists, such as CCK-8, results in a transient activation of pp60[src] [32]. To investigate whether pp60[src] is important for the activation of SMCE in pancreatic acinar cells, we studied the effect of PP1, an inhibitor of the Src family of tyrosine kinases that potently inhibits pp60[src] [31]. Experiments using PP1 indicate that the activity of Src proteins is required for the activation, but not for the maintenance of SMCE using either thapsigargin or CCK-8 to deplete the intracellular Ca^{2+} stores. These observations are consistent with previous studies [26,48] and support a role for tyrosine kinases in the activation of SMCE in pancreatic acini and other cells [16,17,50].

To explore further the involvement of pp60[src] in SMCE, we examined the association with the cytoskeleton and activation of this protein upon depletion of the intracellular Ca^{2+} stores using thapsigargin. Consistent with the effect reported above, store depletion induced pp60[src] association with the cytoskeleton and activation. The degree of activation of this protein was similar in the absence or presence of external Ca^{2+}, suggesting that Ca^{2+} influx is not required for pp60[src] activation. To our knowledge this is the first time that pp60[src] has been shown to associate with the cytoskeleton and be activated in direct response to the depletion of the internal Ca^{2+} stores. Our results indicate that pp60[src] activation requires a functional actin network, suggesting that association with the cytoskeletal fraction after store depletion is required for pp60[src] activation. Consistent with this, we

have found that the activity of pp60[src] is not required for actin polymerization, which instead is essential for pp60[src] activation.

pp60[src] contains a regulatory Src homology 2 ('SH2') domain that binds tyrosine phosphorylated substrates [41]. The association of pp60[src] with the actin cytoskeleton might be important in bringing this protein into proximity with cytoskeletal-associated phosphorylated substrates, thus removing the auto-inhibition evoked by intramolecular binding of Src homology 2 domains to phosphorylated tyrosine residues. Although the mechanism by which pp60[src] is involved in SMCE activation deserves further investigation, activated pp60[src] might participate in the recruitment of signalling molecules, such as phospho-inositide 3-kinase [32], a component of the secretion-like coupling for SMCE in platelets [51], and caveolin [18], to specific sub-plasmalemmal locations, such as caveolae, where transient receptor potential channels have been found at the early stages of platelet activation [19,20]. In contrast, the activity of this protein does not seem to be essential for the maintenance of Ca^{2+} entry as reported in platelets [26].

In conclusion, we have provided evidence for a secretion-like coupling model for SMCE in pancreatic acinar cells based on a reversible interaction of the ER with the PM modulated by the actin cytoskeleton. This model, as for secretion, includes a dual role for actin filaments in the initiation and maintenance of the coupling process. The cortical actin cytoskeleton acts as a barrier preventing constitutive interaction between the ER and PM. In addition, a functional actin network is required for SMCE, since actin-cytoskeleton-disrupting agents inhibited Ca^{2+} entry. Interestingly, we show for the first time association of pp60[src] with the actin cytoskeleton and subsequent activation after depletion of the intracellular Ca^{2+} stores, independently of Ca^{2+} entry, a process that might be important for the activation of SMCE in pancreatic acinar cells.

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