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Tyrosine phosphatase and cytochrome P450 activity are critical in regulating store-operated calcium channels in human fibroblasts

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Abbreviations: BAPTA-AM, bis-(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra-acetoxymethyl ester; BK, Lys-bradykinin; $[Ca^{2+}]_i$, intracellular free calcium concentration; EET, epoxyeicosatrienoic acids; HSWP, human foreskin fibroblast cells; Ins $(1,3,4,5)P_4$, inositol 1,3,4,5-tetrakisphosphates; Ins$(1,4,5)P_3$, inositol 1,4,5-trisphosphates; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphates

Abstract

Diverse signaling pathways have been proposed to regulate store-operated calcium entry (SOCE) in a wide variety of cell types. However, it still needs to be determined if all of these known pathways operate in a single cell type. In this study, we examined involvement of various signaling molecules in SOCE using human fibroblast cells (HSWP). Bradykinin (BK)-stimulated Ca^{2+} entry, previously shown to be *via* SOCE, is enhanced by the addition of vanadate, an inhibitor of tyrosine phosphatases. Furthermore, SOCE is regulated by cytochrome P-450, as demonstrated by the fact that the products of cytochrome P-450 activity (14,15 EET) stimulated SOCE while econazole, an inhibitor of cytochrome P450, suppressed BK-stimulated Ca^{2+} entry. In contrast, Ca^{2+} entry was unaffected by the guanylate cyclase inhibitor LY83583, or the membrane permeant cyclic GMP analog 8-bromo-cyclic GMP (8-Br-cGMP). Neither nitric oxide donors nor phorbol esters affected BK-stimulated Ca^{2+} entry. SOCE in HSWP cells is primarily regulated by tyrosine phosphorylation and the cytochrome P-450 pathway, but not by cyclic GMP, nitric oxide, or protein kinase C. Thus, multiple pathways do operate in a single cell type leading to the activation of Ca^{2+} entry and some of these signaling pathways are more prominently involved in regulating calcium entry in different cell types.

Keywords: bradykinin; calcium channels; protein-tyrosine kinases; protein-tyrosine phosphatases

Introduction

One of the early events following stimulation of many G protein coupled receptors is an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Bird *et al.*, 2004), which initiates a cascade of signal transduction events often leading to DNA synthesis and cell proliferation (Parekh and Putney, 2005). Typically, the Ca^{2+} response evoked by agonists is a biphasic one that includes a peak and a sustained plateau. The peak is due to the release of intracellular Ca^{2+} from Ins$(1,4,5)P_3$ -sensitive internal stores and the sustained plateau is due to Ca^{2+} entry from the extracellular space. The relationship between the release of internal Ca^{2+} stores and activation of Ca^{2+} entry has been examined in depth and the prevailing hypothesis is that the two events are functionally coupled. Jim Putney first defined "capacitative calcium entry", also called "store-operated calcium entry (SOCE)", as Ca^{2+} entry initiated by a signal generated when Ca^{2+} stores are emptied (Putney and McKay, 1999). But what is the signal that is produced by a decrease in the Ca^{2+} loading state of the internal store that, in turn, communicates with Ca^{2+} channels in the plasma membrane? A direct interaction between store-operated channels and IP_3 receptors, the insertion of sub-membrane vesicles containing store-operated channels into the plasma membrane, or a secretion-like coupling may be involved in regulation of SOCE (Parekh and Putney,

2005b). One can not ignore the wealth of evidence arguing for the involvement of various signal transduction pathways in the regulation of SOCE.

Irvine proposed a model that suggested the involvement of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 receptors in activating Ca^{2+} entry following depletion of Ca^{2+} stores (1991). In addition to inositol phosphates, several other signaling molecules have been reported to play a role in Ca^{2+} entry following internal Ca^{2+} store depletion. Cyclic GMP has been implicated in the regulation of SOCE in pancreatic acinar cells (Bahnson *et al.*, 1993), in vascular endothelial cells (Kwan *et al.*, 2000) and platelets (Rosado *et al.*, 2001). A role for cytochrome P450 enzymes has been suggested for the regulation of SOCE in thymocytes (Alvarez *et al.*, 1991), platelets (Alonso *et al.*, 1991), and human neutrophils (Montero *et al.*, 1991). In addition, small molecular weight G proteins have been shown to regulate SOCE in mouse lacrimal acinar cells (Bird and Putney, 1993) and HL-60 granulocytes (Jaconi *et al.*, 1993). In Jurkat T cells, a small molecule named Ca^{2+}-influx factor (CIF), generated during stimulation of cells, was reported to activate Ca^{2+} influx in several cell lines (Randriamampita and Tsien, 1993). The nitric oxide pathway (Favre *et al.*, 1998) and epoxyecosatrienoic acids (EETs) (Graier *et al.*, 1995; Rzigalinski *et al.*, 1999) also have been suggested for regulation of SOCE.

In addition to the pathways described above, we have provided evidence for the role of tyrosine kinases in the regulation of SOCE in human fibroblast cells (Lee *et al.*, 1993). A number of subsequent reports have supported our findings that tyrosine kinases are involved in regulating SOCE in many cell types (Marhaba *et al.*, 1996; Davis and Sharma, 1997; Krutetskaia *et al.*, 1997; Taketo *et al.*, 1997). In addition, studies of platelets indicate that depletion of Ca^{2+} from intracellular stores leads to the tyrosine phosphorylation of a 130 kD protein, which led to speculation that this protein is involved in the regulation of Ca^{2+} entry (Vostal *et al.*, 1991).

Since the studies defining the role of these numerous signaling pathways in regulating SOCE involve a wide range of cell types, the question arises whether the evidence for multiple signaling pathways simply reflects variations in the method of regulation between different cell and tissue types, or whether these multiple signaling pathways are all involved in regulating SOCE in a single cell type. That is, do all of these signaling pathways converge on SOCs in most cell types or are there differences between cell types in how they regulate SOCE? To answer this question, we have stimulated human fibroblast (HSWP) cells with bradykinin or thapsigargin, both agents capable of inducing SOCE (Lee *et al.*, 1993) and examined the role of various signaling pathways. Our results indicate that Ca^{2+} entry into HSWP cells is dynamically regulated by tyrosine kinases and phosphatases and is independent of cyclic GMP, nitric oxide, and diacylglycerol acting *via* a classic PKC pathway.

Materials and Methods

Cell culture

Human foreskin fibroblast cells (HSWP) were obtained from J. Regan, Oak Ridge National Laboratory. Cells between the 15th and 25th passage were plated in 100 mm dishes in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum. DDT1MF-2 cells were provided by Donald Gill, University of Maryland School of Medicine, and were grown in DMEM + 10% FBS. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. For single cell image analysis, cells were subcultured on 25 mm round glass coverslips.

Immunoblotting of whole cell lysates with monoclonal anti-phosphotyrosine antibodies

HSWP cells were serum deprived in EMEM for 6 h at 37°C. Following stimulation, cells were washed with isotonic ice-cold Tris buffered saline (TBS, 10 mM Tris, 75 mM NaCl, 75 mM KCl, pH 7.4) and extracted in isoelectrofocusing stop solution (IEF, 8M urea, 0.5% SDS, 50 mM 2-mercaptoethanol). A fraction (10 μg of protein) of each sample was solubilized by boiling in an equal volume of 2 × Laemmli buffer (62 mM Tris, 1% SDS, 0.001% pyronin Y, 10% glycerol, 5% 2-mercaptoethanol) and separated by SDS-PAGE on 7.5% polyacrylamide minigel. Proteins were transferred to nitrocellulose membranes and nonspecific binding sites were blocked by incubating the membranes in blocking buffer (150 mM NaCl, 10 mM sodium phosphates, pH 7.4, 2 mM EDTA, 0.2% NP-40) containing 5 mg/ml of BSA for 2 h at room temperature. Monoclonal anti-phosphotyrosine antibodies at a concentration of 0.5 μg /ml were added to the blot and incubated overnight at 4°C. The blot was washed 5 times with blocking buffer. Then, anti-mouse IgG F(ab')$_2$ conjugated to horseradish peroxidase (2 μg /ml in blocking buffer) was added to the blot and incubated for 1 h at room temperature. The blot was washed 5 times and subjected to enhanced chemiluminescence (ECL).

Measurements of $[Ca^{2+}]_i$ by image analysis

Image analysis was carried out as previously described (Byron and Villereal, 1989; Lee *et al.*, 1993). All image analysis was conducted at room temperature. Coverslips were washed twice with control medium (135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM glucose, 11 mM Hepes, pH 7.4) and then incubated in the same medium with 2 μM fura-2 AM, 0.5% bovine serum albumin and 0.02% Pluronic F127 detergent (Poenie *et al.* 1986) for 90-120 min at room temperature in the dark. After loading, the cells were washed twice and incubated in the dark in control medium for 1-2 h prior to the image analysis. Fura-2 fluorescence was then measured in cell populations with a Perkin-Elmer LS50B fluorescence spectrophotometer. This instrument was equipped with a rotating filter wheel that was used to alternate between 340 and 380 nm excitation, and the emitted fluorescence (at 510 nm) was determined. A coverslip was mounted vertically at a 30 deg angle to the light path in a cuvette that was continuously perfused with media. A four-way valve mounted just above the cuvette allowed rapid switching of solutions. Replacement of the medium bathing the cells took approximately 10s. The excitation light illuminated an area of approximately 30 mm^2 on the coverslip for recording of fluorescence from several thousand cells. The ratio of fluorescence at 340 nm to that at 380 nm was calculated and calibrated in terms of $[Ca^{2+}]_i$.

Materials

Fura-2 free acid, Fura-2 AM, and BAPTA-AM were purchased from Molecular Probes. Lys-Bradykinin and EGF were purchased from Peninsula Laboratories. Monoclonal anti-phosphotyrosine antibodies were purchased from UBI. Peroxidase conjugated anti-mouse IgG $F(ab')_2$ and ECL kits were purchased from Amersham. Econazole, 8-bromo cyclic GMP, and sodium orthovanadate were purchased from Sigma. LY83583 was purchased from Calbiochem. 14,15 EET was synthesized by Dr. J.R. Falck (University of Texas Southwestern Medical Center, Dallas, TX). GEA3162 was purchased from Alexis Corp (San Diego, CA).

Statistics

Results are expressed as means ± S.E. Where appropriate, results were compared using Student's *t*-test.

Results

Vanadate enhanced tyrosine phosphorylation and Ca^{2+} entry stimulated by BK

We showed previously that the amount of SOCE stimulated by either BK or thapsigargin is decreased by tyrosine kinase inhibitors (Lee *et al.*, 1993). Since the level of tyrosine phosphorylation is determined by the activity of tyrosine kinases and tyrosine phosphatases, we investigated whether modulation of cellular tyrosine phosphatase activity affects the Ca^{2+} entry induced by BK, as well as the level of tyrosine phosphorylation. Figure 1 shows the time course for protein tyrosine phosphorylation following stimulation of HSWP cells with BK, as analyzed by Western blots with anti-phosphotyrosine antibodies. BK-induced protein tyrosine phosphorylation peaked at 2 min and gradually declined in 10 min to a level that was still substantially higher than that of the unstimulated control. Two abundant substrates for BK-stimulated tyrosine phosphorylation are proteins of approximately 130 kDa and 70 kDa (Figure 1). In addition to these proteins, minor proteins in the 205-220 kDa, 190 kDa, 100-110 kDa, and 80 kDa ranges, which also undergo tyrosine phosphorylation by BK, were detected upon longer exposure of the blots. The increase in tyrosine phosphorylation could be the result of stimulation of tyrosine kinase activity and/or inhibition of tyrosine phosphatase activity.

Figure 2A shows the effect of vanadate, a well known inhibitor of protein tyrosine phosphatases (Swarup *et al.*, 1982), on basal or BK-stimulated tyrosine phosphorylation. Incubation of cells with 200 μM vanadate resulted in elevation of phosphotyrosine levels in both the 130 kDa and 70 kDa proteins. The onset of action for vanadate was very fast, since elevation of tyrosine phosphorylation was detected within 1 min of incubation (Figure 2A, lane 2). BK further increased the phosphotyrosine levels of the 130 and 70 kDa proteins in cells preincubated

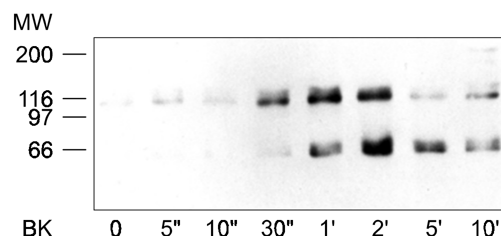

Figure 1. BK stimulates tyrosine phosphorylation in a time-dependent manner. HSWP cells were stimulated with 100 ng/ml of BK for the indicated time and analyzed for tyrosine-phosphorylated proteins by immunoblotting as described under Materials and Methods. " and ' represent seconds and min, respectively. The results shown are representative of 5 different experiments.

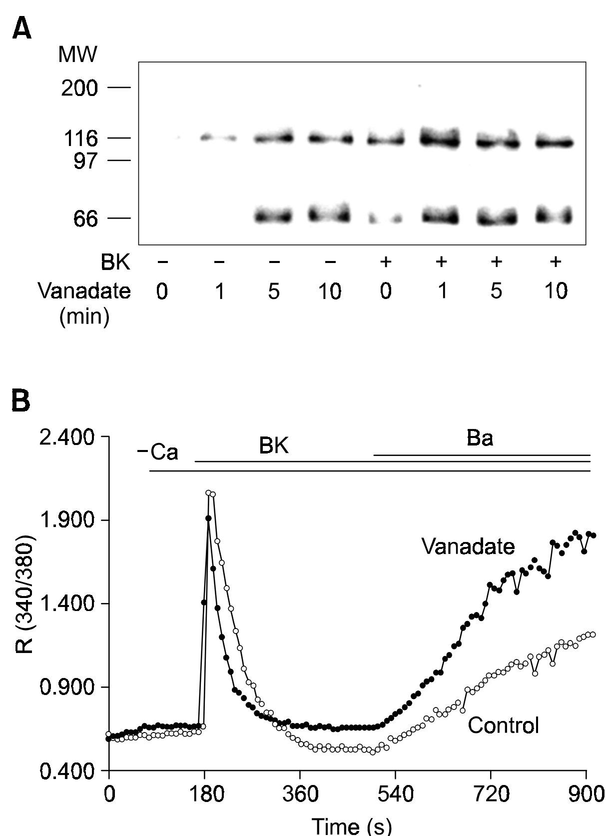

Figure 2. Effects of vanadate on tyrosine phosphoryaltion and Ba^{2+} influx induced by BK. (A) Cells were incubated with 200 μM vanadate (lane 1-4) for the time indicated. In lanes 5-8, cells were preincubated with 200 μM vanadate for the time indicated and stimulated with BK for 1 min. Subsequently tyrosine-phosphorylated proteins were analyzed by immunoblotting as described under Materials and Methods. The data shown represents the results from two identical experiments. (B) Each trace is from an individual cell in a field of cells and represents the typical response. Cells were perfused with Hepes-HBSS and then switched to the medium shown at the time indicated by the bar. For the vanadate-treated cells, 200 μM vanadate was included during the time BK was present. The concentration for BK was 100 ng/ml. The rate of Ba^{2+} entry, calculated from the slope of Ba^{2+}-containing phase, was $15.5 \pm 7.5 \times 10^{-4}$ (mean ± S.E.; *n* = 17) in the control and $26.4 \pm 4.6 \times 10^{-4}$ (mean ± S.E.; *n* = 19) in the group treated with vanadate. These values were statistically different ($P < 0.0001$).

with vanadate (Figure 2A, lane 6-8). The additive effect of vanadate and BK on tyrosine phosphorylation supports our previous findings that BK-stimulated tyrosine phosphorylation is mediated by activation of tyrosine kinases rather than by modulation of tyrosine phosphatases. Tyrosine phosphorylation of the 130 kDa range proteins was detected prior to that of the 70 kDa range proteins in cells stimulated by vanadate (Figure 2A, lane 2).

Since vanadate enhances tyrosine phosphorylation in BK-stimulated cells, we next examined whether an additive effect of vanadate can also be observed on BK-stimulated Ca^{2+} entry. For this purpose, we performed image analysis to monitor intracellular Ca^{2+} concentration in individual HSWP cells using fura-2. In order to look at Ca^{2+} influx pathways more closely, we applied a protocol that has been described previously (Lee *et al.*, 1993). Specifically, cells were stimulated with BK in the absence of extracellular Ca^{2+}, so that the BK-sensitive internal Ca^{2+} stores were depleted. Subsequently, Ba^{2+} (2 mM $BaCl_2$) was added to the medium and the rate of Ba^{2+} entry was monitored. Since Ba^{2+} can enter the cell as Ca^{2+} does, but cannot be pumped out of the cell or into the internal Ca^{2+} stores, Ba^{2+} is trapped in the cytoplasm (Schilling *et al.*, 1989). Therefore, increases in Ba^{2+} concentration in the cells reflect Ba^{2+} influx across the plasma membrane, which is indicative of activation of Ca^{2+} entry pathways. Since Ba^{2+} shifts the Ca^{2+} calibration of fura-2 (Schilling *et al.*, 1989), the data are presented as a ratio of the 340 and 380 nm signals, designated as R (340/380). Similar to the effects of vanadate on BK-stimulated tyrosine phosphorylation, vanadate caused enhancement of BK-stimulated Ba^{2+} influx [Figure 2B, $15.5 \pm 1.81 \times 10^{-4}$ (mean ± S.E.; *n* = 17) in the control and $26.4 \pm 1.05 \times 10^{-4}$ (mean ± S.E.; *n* = 19) in the vanadate-treated group]. The rate of Ba^{2+} entry in the vanadate-treated group was statistically different from that observed in the control group ($P < 0.001$). Vanadate treatment did not change the level of the Ca^{2+} peak, suggesting that the Ca^{2+} release caused by BK was not significantly affected by vanadate. These results indicate that SOCE caused by BK stimulation is regulated by vanadate and this effect correlates with the inhibitory action of vanadate on the tyrosine phosphatase activity.

Role of cyclic GMP in the BK-stimulation of Ca^{2+} entry and protein tyrosine phosphorylation

Previous observations in pancreatic acinar cells and platelets suggest that cyclic GMP can regulate SOCE. Since BK has been shown to activate guanylate cyclase and generate cyclic GMP (Snider and Richelson, 1984), we examined the possibility of cyclic GMP being a modulator of SOCE in HSWP cells. To address this question, we first examined the effect of LY83583, an inhibitor of guanylate cyclase (Mulsch *et al.*, 1988), on the Ca^{2+} response stimulated by BK. In contrast to reports concerning pancreatic acinar cells, neither the acute addition of LY83583 nor preincubation for 10 min prior to BK stimulation had any effect on the intracellular Ca^{2+} responses produced by BK (Figure 3A and B). These results were further verified by application of

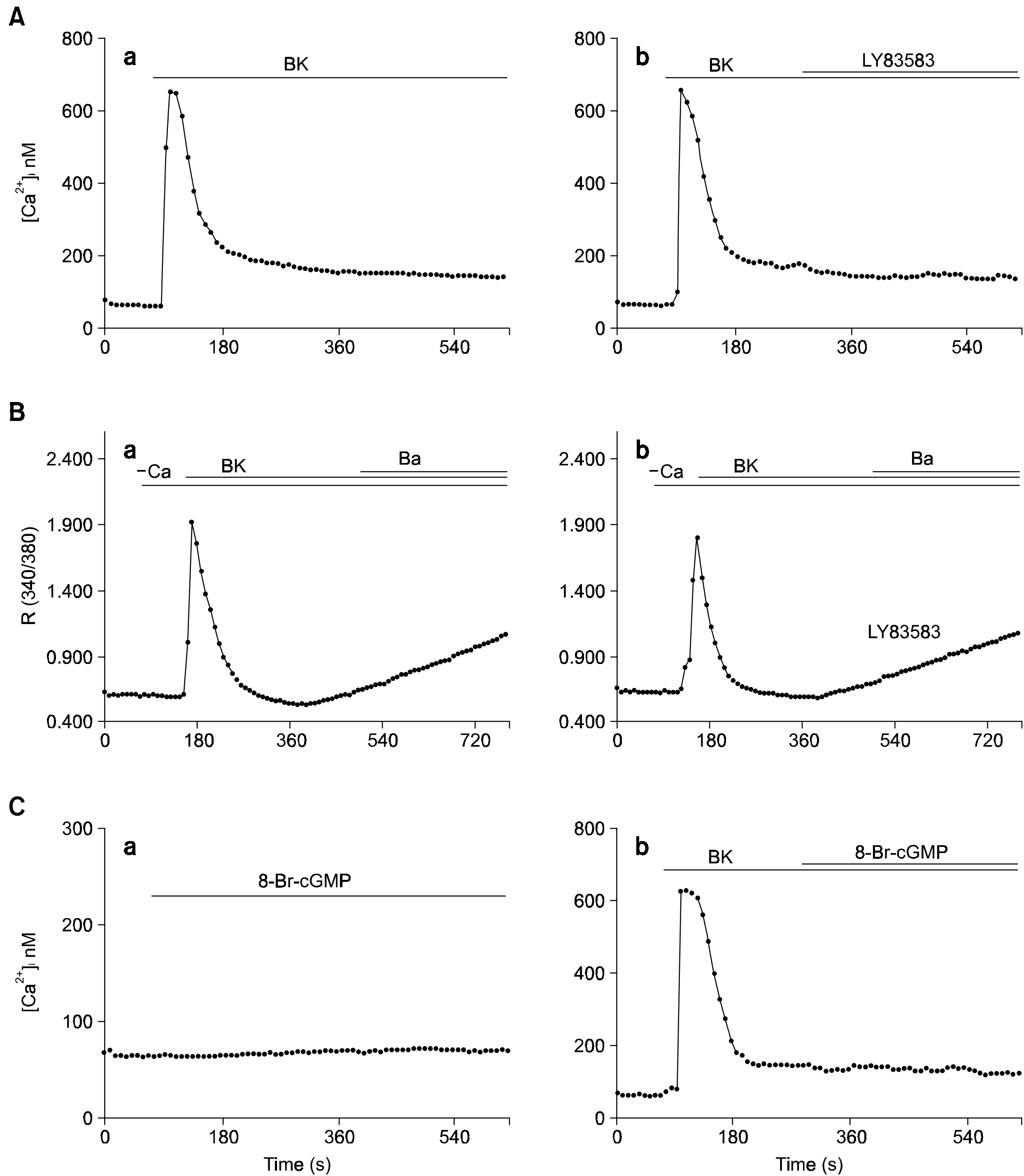

Figure 3. Effects of LY83583 and 8-Br-cGMP on the BK-stimulated Ca^{2+} entry. Each trace shown is from an individual cell in a field of cells and represents a typical response of 45 cells from 3 individual trials. (A) Cells were initially perfused with Hepes-HBSS, then at the time indicated by the bar, the perfusion was switched to the same medium containing BK (a); BK followed by BK + 20 μM LY83583 (b). (B) Cells were preincubated with (b) or without (a) 20 μM LY83583 for 10 min, and then image analysis was performed. Cells were initially perfused with Hepes-HBSS, and then switched to Ca^{2+}-free Hepes-HBSS, Ca^{2+}-free Hepes-HBSS with BK, and subsequently Ca^{2+}-free Hepes-HBSS with BK + 2 mM $BaCl_2$. (C) Cells were perfused with Hepes-HBSS and subsequently 1mM 8-Br-cGMP was added at the time indicated by the bar (a) or the perfusion was changed to the same medium containing BK and subsequently BK + 1 mM 8-Br-cGMP (b). The concentration of BK was 100 ng/ml.

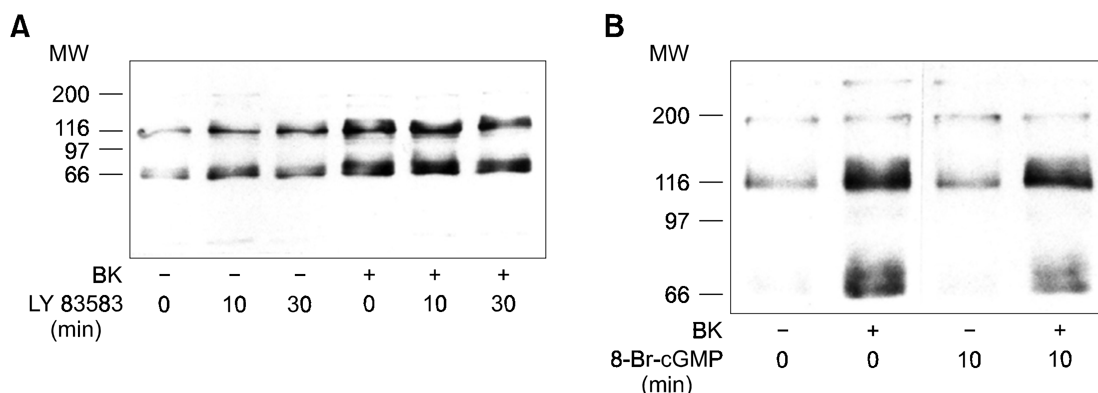

Figure 4. Effects of LY83583 and 8-Br-cGMP on the BK-stimulated tyrosine phosphorylation. (A) Cells were either treated with 20 μM LY83583 alone (lanes 2 and 3) or in combination with BK (100 ng/ml, 1 min, lanes 5 and 6). Subsequently, cells were lysed and subjected to Western blotting. (B) Cells were preincubated with 1mM 8-Br-cGMP and incubated in the presence or absence of 100 ng/ml of BK for 1 min (lanes 3 and 4). Lanes 1 and 2 were from cells that had not been treated with 8-Br-cGMP. Subsequently, cells were lysed and analyzed for tyrosine-phosphorylated proteins as described under Materials and Methods. The results shown are representative of at least 2 experiments,

8-Br-cGMP to either naive cells (Figure 3C, a) or BK-stimulated cells (Figure 3C, b). Addition of 8-Br-cGMP did not change either the basal or the BK-stimulated $[Ca^{2+}]_i$.

Next, we examined the effects of LY83583 and 8-Br-cGMP on the BK-stimulated protein tyrosine phosphorylation (Figure 4). Neither the basal nor the BK-stimulated tyrosine phosphorylation was affected significantly by incubation with either LY83583 (Figure 4A) or 8-Br-cGMP (Figure 4B). These results suggest that, at least in HSWP cells stimulated by BK, cyclic GMP does not have a significant role in regulating either the tyrosine kinase pathway or the BK-stimulated SOCE.

Inhibition of BK-stimulated Ca^{2+} entry and BK-stimulated tyrosine phosphorylation by econazole, an inhibitor of cytochrome P450

Cytochrome P450 has been implicated in the regulation of SOCE based on data showing that various inhibitors of cytochrome P450 enzymes, such as the imidazole antifungal agents, also inhibited SOCE (Alonso *et al*., 1991; Alvarez *et al*., 1991; Montero *et al*., 1991). We investigated whether inhibition of cytochrome P450 activity by econazole, a cytochrome P-450 inhibitor, causes any change in the BK-stimulated Ca^{2+} responses in HSWP cells. As seen in Figure 5, econazole did not seem to affect the BK-stimulated Ca^{2+} release since there was no significant difference in the level of the Ca^{2+} peak between the econazole-treated and untreated groups [R value; 1.56 ± 0.01 (mean ± S.E.; n = 42) in the control and 1.55 ± 0.02 (mean ± S.E; n = 23) in the econazole-treated group].

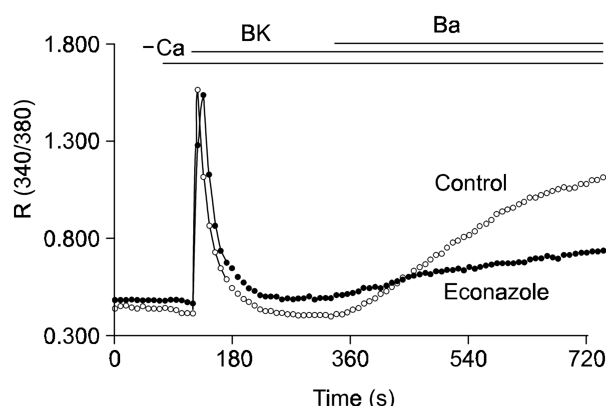

Figure 5. Effects of econazole on the BK-stimulated Ba^{2+} influx. Each trace shown is from an individual cell in a field of cells (control; n = 42, econazole; n = 23) and represents a typical response. Essentially as in Figure 2B, cells were perfused with Hepes-HBSS, and then switched to Ca^{2+}-free medium, Ca^{2+}-free medium containing 100 ng/ml of BK, and subsequently Ca^{2+}-free medium containing BK + 2 mM $BaCl_2$. For the econazole treated group (filled circle), 10 μM econazole was included during the time BK was present. The control trace is plotted as an open circle.

However, the BK-stimulated Ca^{2+} entry into the cells, measured as Ba^{2+} entry, was markedly decreased by econazole treatment. The mean rate of Ba^{2+} entry was $7.1 \pm 1.10 \times 10^{-4}$ (mean ± S.E.; n = 42) in the control and $1.97 \pm 0.32 \times 10^{-4}$ (mean ± S.E.; n = 23) in the econazole-treated group. These values were statistically different with a P value < 0.001 (Student t). These results are comparable to those previously reported (Alonso *et al*., 1991; Alvarez *et*

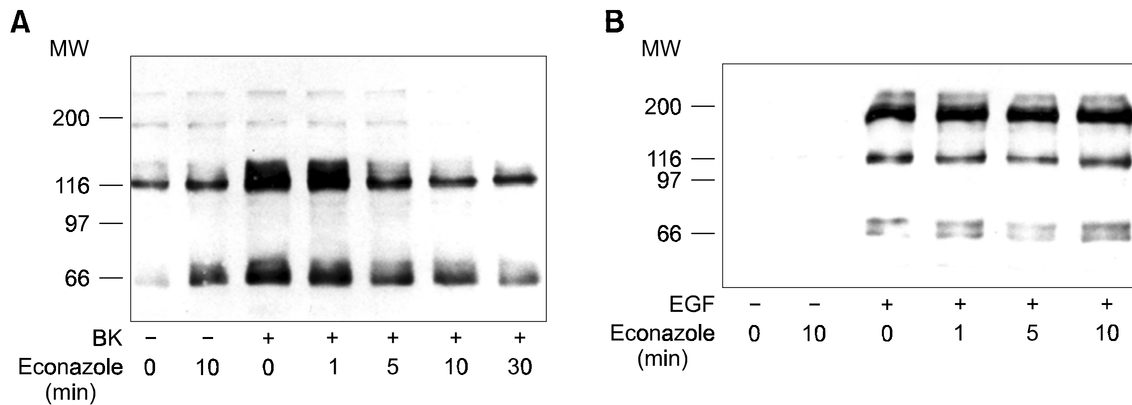

Figure 6. Effects of econazole on the BK-stimulated and EGF-stimulated tyrosine phosphorylation. HSWP cells were analyzed for tyrosine-phosphorylated proteins as described under Materials and Methods. Cells were preincubated with 10 μM econazole for the indicated time and stimulated with either 100 ng/ml of BK for 1 min (A) or 100 ng/ml of EGF for 5 min (B). The data shown represent typical results from 3 different experiments.

al., 1991; Moore *et al.*, 1998) and indicate that cytochrome P450 activity may be involved in regulating the BK-stimulated Ca^{2+} influx.

Since tyrosine kinases also appear to be involved in regulating SOCE (Lee *et al.*, 1993), we tried to define how these two pathways interact along the signaling cascade to regulate plasma membrane Ca^{2+} entry. One way to address this question was to examine the effect of econazole on tyrosine phosphorylation induced by BK. We found that econazole markedly inhibited tyrosine phosphorylation induced by BK in a time dependent manner, with maximal inhibition occurring following 10 min preincubation prior to BK stimulation (Figure 6A). There was no inhibitory effect on protein tyrosine phosphorylation by econazole under the unstimulated conditions (Figure 6A, lane 2). We next addressed the specificity of the inhibitory effects of econazole on BK-induced tyrosine phosphorylation. One way to approach this question was to examine the effect of econazole on the tyrosine phosphorylation that is exerted by epidermal growth factor (EGF), which signals *via* activation of receptor tyrosine kinases. If inhibition of tyrosine phosphorylation by econazole occurred as a result of general activation of tyrosine phosphatases, econazole would be expected to have a similar inhibitory effect on EGF-stimulated tyrosine phosphorylation. However, econazole preincubation did not affect the tyrosine phosphorylation produced by EGF (Figure 6B). This result indicates that the action of econazole does not seem to involve a general effect on the tyrosine phosphatase activity or nonspecific effects on tyrosine kinases. It is more likely that econazole exhibits its inhibitory effect *via* a mechanism involving the tyrosine kinase activity activated by Ca^{2+} store depletion.

One possibility for the inhibitory effect by econazole on tyrosine phosphorylation is that tyrosine phosphorylation is secondary to a rise in the cytosolic Ca^{2+} concentration and is, therefore, inhibited because Ca^{2+} entry from the external medium is blocked by econazole. To address this question, we tested whether we could still detect the inhibitory effect of econazole when intracellular Ca^{2+} is chelated so that Ca^{2+} entry during BK stimulation can not contribute to changes in cytosolic Ca^{2+}. To test for this possibility, we incubated cells with the membrane permeable, highly selective Ca^{2+} chelator BAPTA-AM, which is hydrolyzed into the active Ca^{2+} chelator BAPTA upon entering the cell. In BAPTA-loaded cells, we observed that BK-stimulated Ca^{2+} responses were completely inhibited (Figure 7A). BK-stimulated tyrosine phosphorylation of both the 130 kDa and 70 kDa proteins was inhibited by approximately 40% in BAPTA-loaded cells (Figure 7B, lane 4). These data suggest that a large component of the tyrosine phosphorylation is independent of the rise in the cytosolic Ca^{2+} concentration. Econazole further decreased BK-stimulated phosphotyrosine levels in BAPTA-loaded cells (Figure 7B, lane 7). Lanes 5 and 6 represent typical results for econazole or econazole and BK, which were observed under the same conditions as for Figure 6A. These results suggest that a substantial component of the inhibitory effects of econazole on BK-stimulated tyrosine phosphorylation does not result from perturbation of Ca^{2+} responses, but rather is mediated by another mechanism, presumably by inhibition of the pathway leading to activation of tyrosine kinases. Therefore, the results from these studies indicate that the inhibitory action of econazol on Ca^{2+} entry probably results from blocking the step

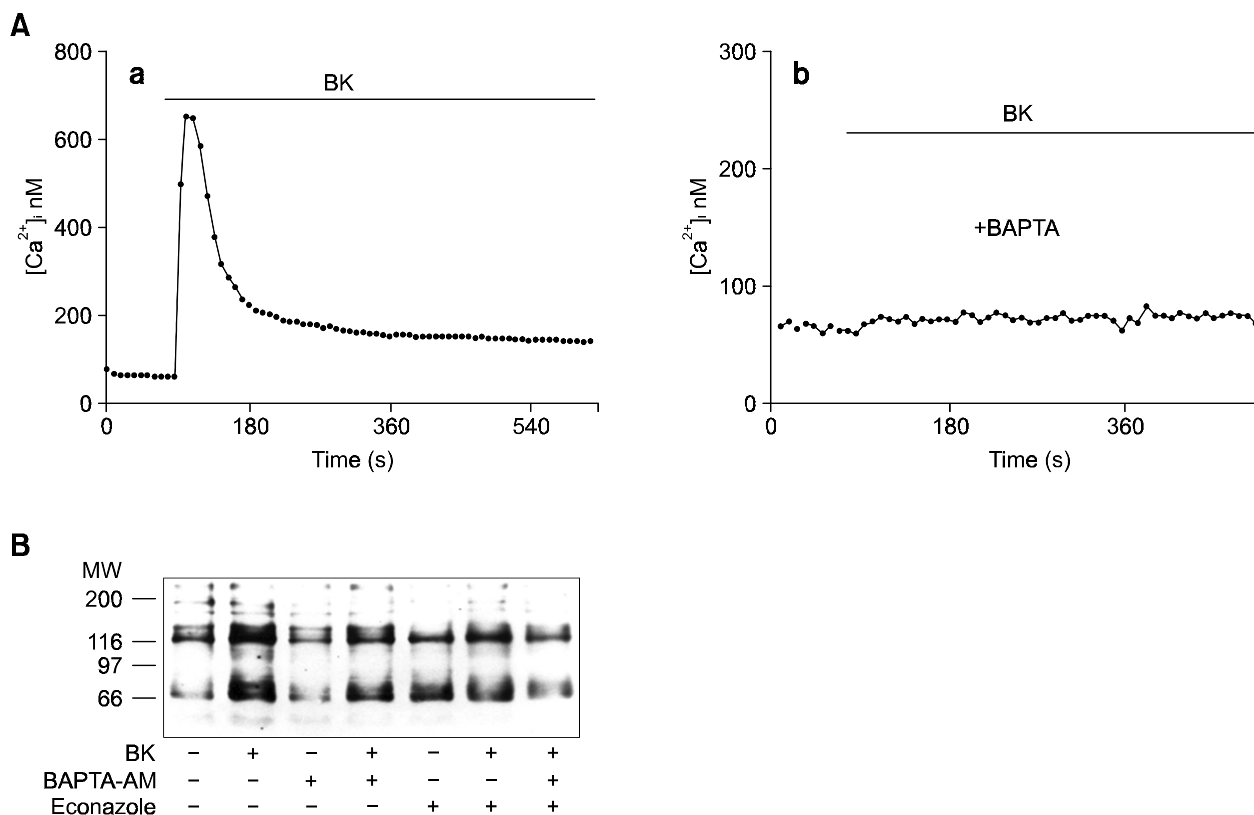

Figure 7. Effects of BAPTA-AM on the Ca^{2+} response and tyrosine phosphorylation induced by BK. (A) Intracellular Ca^{2+} responses were monitored by image analysis using fura-2, essentially as in Figure 3A. (A-a) BK (100 ng/ml) causes biphasic calcium responses. (A-b) cells were preincubated with 25 μM BAPTA-AM for 30 min, and incubated for additional 90 min in fresh medium at 37°C prior to BK addition. The trace shown is from an individual cell and represents a typical response of cells from 3 different experiments (total 35 cells). (B) Where indicated, cells were treated with BAPTA-AM in a manner similar to A (lanes 3,4,7) and followed by stimulation with 100 ng/ml of BK for 1 min (lane 4,7). For the econazole-treated condition, 10 μM econazole was included for 10 min prior to BK stimulation (lanes 5,7). Cells were then lysed and subjected to Western blotting with anti-phosphotyrosine antibodies, as described in Materials and Methods. The results are representative of three identical experiments.

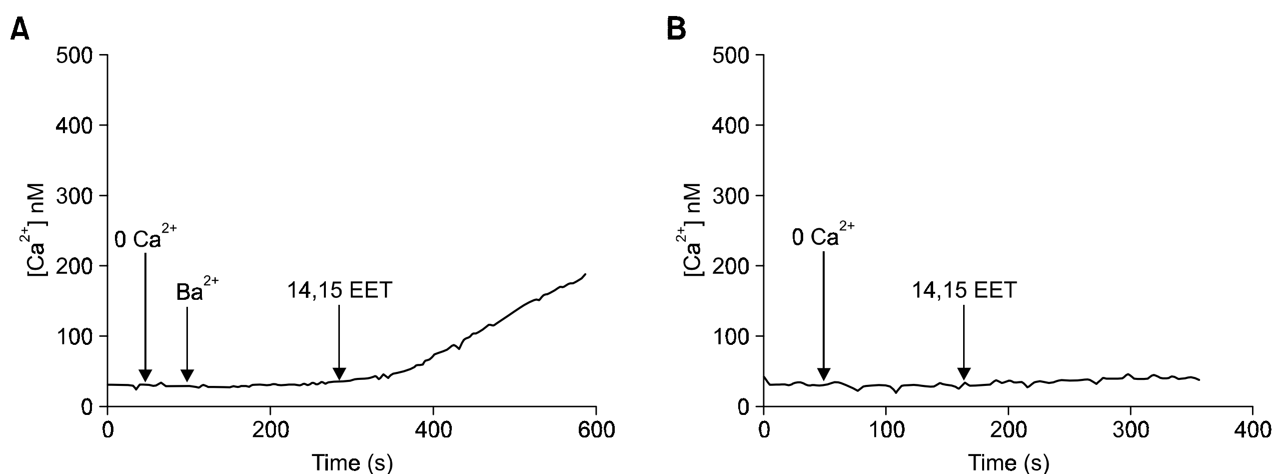

Figure 8. Effect of 14,15 EET on Ca^{2+} entry in HSWP cells. (A) Cells were perfused with HBSS followed by Ca^{2+}-free HBSS. Then, Ca^{2+}-free HBSS containing 2 mM Ba^{2+} was added to establish the basal Ba^{2+} entry. The perfusion was halted with the cells bathed in 2 mM Ba^{2+} and 14,15 EET was added to the chamber to a final dose of 20 μM. (B) Cells were perfused with HBSS followed by Ca^{2+}-free HBSS. The perfusion was halted with the cells bathed in Ca^{2+} -free medium and 14,15 EET was added to the chamber to a final dose of 60 μM.

leading to activation of tyrosine kinases.

Effect of EETs on Ca^{2+} entry

If econazole inhibits a pathway leading to the activation of a tyrosine kinase by Ca^{2+} store depletion, then this implies that a product of cytochrome P450 metabolism of arachidonic acid is stimulating tyrosine kinase activity, and in turn activating SOCE. Therefore, we tested whether various isoforms of EET activate Ca^{2+} entry in HSWP cells. We examined the effect of the 14, 15 isoform of EET since it has been shown to activate tyrosine kinase activity in renal epithelial cells (Chen *et al.*, 1998).

14, 15 EET stimulated Ca^{2+} entry as detected by a significant rise in the intracellular uptake of Ba^{2+} (Figure 8A). This increase in Ba^{2+} entry due to EET was not secondary to depletion of the intracellular Ca^{2+} store, since EET did not stimulate Ca^{2+} release in HSWP cells (Figure 8B). Therefore, a product of cytochrome P450 is capable of stimulating the Ca^{2+} entry channels used by SOCE, even in the absence of Ca^{2+} store depletion.

Effect of nitric oxide donors on Ca^{2+} entry in HSWP cells

Since store-operated Ca^{2+} channels have been suggested to be regulated by S-nitrosylation in the presence of nitric oxide donors, such as sodium nitroprusside and GEA3162 (Favre *et al.*, 1998), we tested these agents for their effects on Ca^{2+} entry in HSWP cells. The addition of 100 μM GEA3162 to HSWP cells resulted in only a modest change in the cytosolic Ca^{2+} levels (Figure 9). Although difficult to see on the scale of Figure 9, there is usually a rise from the basal level of 50 nM to approximately 70-80 nM. A number of experiments with sodium nitroprusside (SNP) used at 3 times the dose used by Favre *et al.* (1998), resulted in approximately the same level of response (data not shown). However, even these small changes in Ca^{2+} levels in response to GEA3162 or SNP do not have the characteristics one would expect in response to activation of surface Ca^{2+} channels. For example, when cells are incubated with the NO donors in Ca^{2+}-free medium for 5 min, and then Ca^{2+} is added back to the cells, only approximately 10% of the cells in the field respond, and those that do, respond asynchronously over the next five min (data not shown). Also, the responses are not maintained as expected for an influx. A few cells increase in the first minute and return to the base line by the third minute, at which time a few more cells will begin a transient response. All of these observations are in sharp contrast to the results we obtained using DDT_1MF-2 cells, which were used in the original study by Favre *et al.* (1998). As shown in Figure 9, after a brief lag, there is a robust rise in the Ca^{2+} concentration in DDT_1MF-2 cells that peaks in the 600-800 nM range, and then declines over time. These data suggest that, in contrast to results in DDT_1MF-2 cells, S-nitrosylation due to GEA3162 is not a major regulator of Ca^{2+} entry in SOCE of HSWP cells.

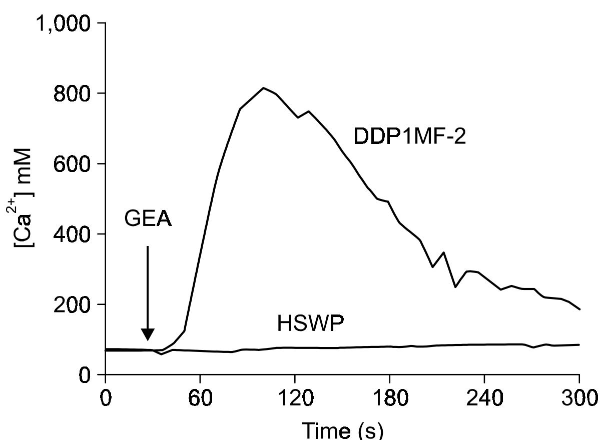

Figure 9. Effect of GEA3162 on Ca^{2+} levels in HSWP and DDT1MF-2 cells. HSWP cells and DDT1MF-2 cells were loaded with fura-2 and the Ca^{2+} concentration was monitored by image analysis. Cells were perfused with HBSS and then 100 μM GEA3162 was added at the time indicated by the arrow.

Phorbol 12-myristate 13-acetate (PMA) inhibits the Ca^{2+} plateau that is induced by thapsigargin by a mechanism involving Ca^{2+} extrusion

BK has also been shown to stimulate protein kinase C activity in HSWP cells *via* generation of diacylglycerol, which is an activator of protein kinase C (Hardie, 2003). Therefore, we next examined whether protein kinase C activity is involved in regulating the SOCE. A similar approach was used as that described above except that thapsigargin was used as a stimulus. One of the reasons to use thapsigargin in this set of experiments was that it produces a more homogeneous Ca^{2+} response than BK in terms of the level of Ca^{2+} plateaus. Thapsigargin has been shown to mobilize intracellular Ca^{2+} by inhibiting microsomal Ca^{2+}-ATPases (Thastrup *et al.*, 1994) and, thereby, activates Ca^{2+} entry (Takemura *et al.*, 1989). Thapsigargin stimulates tyrosine phosphorylation in HSWP cells and thapsigargin-stimulated Ca^{2+} entry is inhibited by tyrosine kinase inhibitors (Lee *et al.*, 1993), suggesting that thapsigargin stimulates Ca^{2+} entry *via* a mechanism similar to that induced by BK. When we applied 200 nM

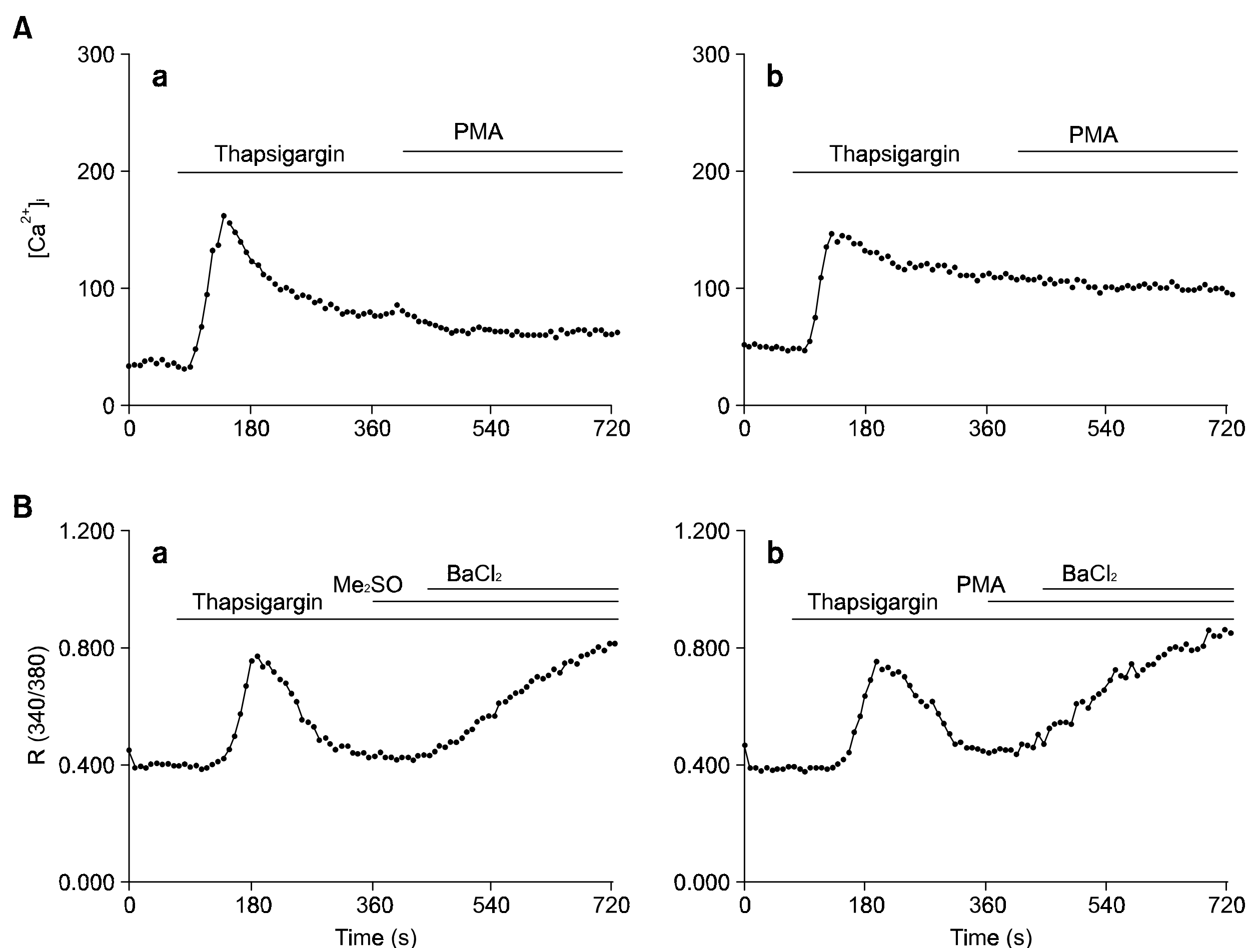

Figure 10. Effects of PMA on the thapsigargin-simulated Ca^{2+} response. (A) Cells were either preincubated with (b) or without (a) 200 nM PMA for 24 h prior to experiments. Subsequently, cells were loaded with fura-2 and image analysis was carried out as described under Materials and Methods. Initially, cells were perfused with Hepes-HBSS and, at the time indicated by the bar, the perfusion was changed to the same medium containing 1 μg/ml of thapsigargin, and subsequently 1 μg/ml of thapsigargin + 200 nM PMA. (B) Cells were initially perfused with Ca^{2+}-free Hepes-HBSS and, at the time indicated by the bar, the perfusion was switched to the same medium containing 1 μg/ml of thapsigargin, 1 μg/ml of thapsigargin + 0.1% Me_2SO (a) or 1 μg/ml of thapsigargin + 200 nM PMA (b), and subsequently 1 μg/ml of thapsigargin + 0.1% Me_2SO + 2mM $BaCl_2$ (a) or 1 μg/ml of thapsigargin + 200 nM PMA + 2 mM $BaCl_2$ (b).

PMA, which rapidly stimulates protein kinase C activity, to the Ca^{2+} plateau phase (Figure 10A-a), a slight decrease in the Ca^{2+} level was consistently detected. The mean (± S.D.) plateau $[Ca^{2+}]_i$ was 112.3 ± 4.05 nM before PMA addition whereas that obtained after PMA addition was 71.4 ± 11.74 nM (n = 39). The difference between the untreated group and the PMA-treated group was statistically significant ($P < 0.0001$). This result indicates that PMA-sensitive protein kinase C may be involved in either inhibiting Ca^{2+} entry or activating Ca^{2+} extrusion. Since PMA-sensitive protein kinase C activity can be depleted by prolonged treatment of cells with PMA (Etscheid *et al.*, 1991; Simonson and Dunn, 1991), we examined whether the absence of this activity affected the Ca^{2+} entry induced by thapsigargin. The results shown in Figure 10A-b indicate that down regulation of PMA-sensitive protein kinase C did not markedly affect the Ca^{2+} release stimulated by thapsigargin, since there was no significant difference in the level of the Ca^{2+} peaks between the control [a, 163 ± 17.1 nM (n = 33)] and protein kinase C-depleted cells [b, 157 ± 13.2 nM (n = 35)]. In agreement with protein kinase C downregulation, no effect was observed on the thapsigargin-induced Ca^{2+} plateaus after acute addition of PMA in PMA-treated cells (b). This effect was further investigated where the effect of PMA on the Ca^{2+} influx was directly examined using the Ba^{2+} protocol described above (Figure 10B). The mean rate of Ba^{2+} entry, measured from the slope of the Ba^{2+}-containing phase stimulated by thapsigargin

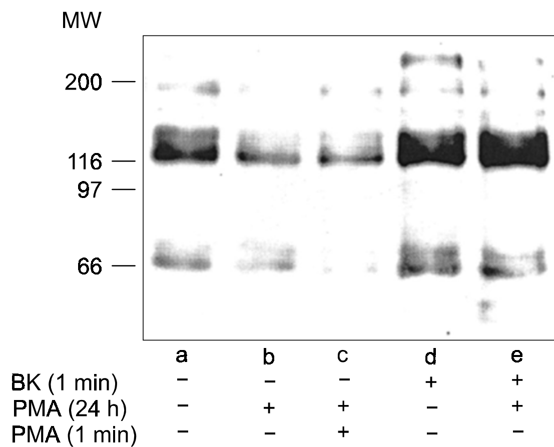

Figure 11. Effects of PMA on the BK-stimulated tyrosine phosphorylation. HSWP cells were preincubated with 200 nM PMA for 24 h (b,c,e) and subsequently stimulated with either 200 nM PMA (c) or 100 ng/ml of BK for 1 min (e). a and d represent, respectively, the basal level and the BK-stimulated level of tyrosine phosphorylation in the absence of PMA treatment. Cells were then lysed and Western blotting was performed as described under Materials and Methods.

was 12.51×10^{-4} (n = 38) in the absence of PMA, and 13.41×10^{-4} (n = 38) in the presence of PMA. The difference between these groups was not statistically significant. These results indicate that PMA-sensitive protein kinase C may regulate Ca^{2+} responses through a mechanism involving Ca^{2+} extrusion from the cytosol, but does not directly regulate Ca^{2+} entry.

We next examined whether PMA-sensitive protein kinase C regulates protein tyrosine phosphorylation. As shown in Figure 11, BK-stimulated tyrosine phosphorylation in the protein kinase C-down regulated cells (e) was comparable to that observed in the untreated cells (d). It was noticed that a long treatment with PMA caused a slight decrease in the phosphotyrosine level of 130 kDa proteins (b), which may indicate that PMA-sensitive protein kinase C plays a role in regulating tyrosine phosphorylation under basal, nonstimulated conditions. Thus, these results demonstrate that the activation of protein kinase C does not affect the activation of tyrosine kinases or tyrosine phosphorylation stimulated by BK. In addition, PMA-sensitive protein kinase C is probably involved in Ca^{2+} homeostasis by activating Ca^{2+} extrusion mechanisms.

Discussion

We have examined the role of intracellular signaling molecules in regulating SOCE and protein tyrosine phosphorylation in human fibroblast cells. The data presented here suggest that SOCE is regulated by either tyrosine kinases or tyrosine phosphorylation (Figure 2), but not by cyclic GMP (Figure 3), nitric oxide (Figure 9), or diacylglycerol acting *via* a PKC pathway (Figure 10). The involvement of cytochrome P450 in regulating Ca^{2+} entry is somewhat complicated since econazole, which inhibits cytochrome P450 activity, also inhibits tyrosine phosphorylation of a number of proteins in HSWP cells (Figure 6). This observation confirms a previous report that econazole reduces agonist induced tyrosine phosphorylation (Sargeant *et al.*, 1994a) and extends the report by demonstrating that the inhibitory effect of econazol on tyrosine phosphorylation is not secondary to perturbation of Ca^{2+} entry (Figure 7B) and is not the result of a general inhibition of tyrosine phosphorylation since it has no effect on the EGF induced tyrosine phosphorylation (Figure 6B). Therefore, the remaining possibilities are either that econazole is a nonspecific inhibitor of tyrosine kinases or that econazole blocks cytochrome P450 activity that acts upstream of tyrosine kinases in the signaling cascade leading to activation of Ca^{2+} entry. Alternatively, we cannot rule out the possibility that the effects of econazole seen on both tyrosine phosphorylation and Ca^{2+} entry are not related and that econazole nonspecifically inhibits both events. For example, other reports indicate that the inhibitory action of econazol on Ca^{2+} entry may be accomplished by a mechanism that does not involve cytochrome P450 activity (Vostal and Fratantoni, 1993; Koch *et al.*, 1994). These published results indicate that cytochrome P450 activity may not be critical for regulating SOCE and an alternative explanation may be that econazole exerts its inhibitory effect by an alternative mechanism, perhaps by inhibiting tyrosine kinase activity.

We did not observe any modulatory effect of cyclic GMP on SOCE, consistent with previous reports involving several cell types (Fasolato *et al.*, 1993; Randriamampita and Tsien, 1993), but contrary to reports involving pancreatic acinar cells where the role of cyclic GMP in stimulating SOCE is well supported (Pandol and Schoeffield-Payne, 1990; Bahnson *et al.*, 1993; Xu *et al.*, 1994), or in platelets where the role of cGMP in inhibiting SOCE has been reported (Rosado *et al.*, 2001). Xu *et al.* (1994) hypothesized that nitric-oxide synthase and guanylate cyclase operate in series to activate Ca^{2+} entry controlled by Ca^{2+} store depletion and showed that cyclic GMP has a dual effect on Ca^{2+} entry. Increasing the cyclic GMP concentration up to 10-fold above control levels activated Ca^{2+} entry while further increases up to 80-fold above control levels inhibited Ca^{2+} entry. The absence of the effects seen by other investigators might be due, the authors guessed, to application of high and inhibitory con-

centrations of cyclic GMP to the cells. One could argue that this could explain the lack of effect for the membrane permeable cyclic GMP analogue 8-Br-cGMP on basal or BK-stimulated Ca^{2+} entry (Figure 3C). However, the finding that the guanylate cyclase inhibitor LY83583 did not affect BK-stimulated Ca^{2+} entry favors the conclusion that cyclic GMP does not play a significant role in regulating Ca^{2+} entry. Our observations in human fibroblasts do not call into question the role of cyclic GMP in the stimulation of Ca^{2+} entry in pancreatic acinar cells or its role in inhibition of SOCE in platelets (Rosado *et al.*, 2001) or vascular endothelial cells (Kwan *et al.*, 2000). Rather, our results suggest that there may be a variety of methods by which different cells achieve activation (or modulation) of Ca^{2+} entry pathways.

Our results and previous findings (Lee *et al.*, 1993) strongly suggest involvement of either tyrosine kinases or tyrosine phosphorylation in the regulation of SOCE. SOCE is subject to regulation by tyrosine phosphatases (Figure 2), based on the effect of vanadate on tyrosine phosphorylation and BK-stimulated Ba^{2+} entry. Previously, vanadate was shown to have a number of effects other than inhibiting tyrosine phosphatase activity. Stimulation of PIP_2 hydrolysis *via* activation of a G-protein-linked phospholipase C (Paris *et al.*, 1987; Paris and Pouyssegur, 1987) and inhibition of the plasma membrane Ca^{2+} pump are such examples. One can argue that our results are due to nonspecific activation of phospholipase C and/or inhibition of the Ca^{2+} pump, since these effects can also lead to enhancement of the intracellular Ca^{2+} concentration. However, it is very unlikely that this is the case. We previously have detected Ca^{2+} mobilization by vanadate in HSWP cells (data not shown), which agrees with the previous findings that vanadate stimulates phospholipase C activity. However, the Ca^{2+} release produced by vanadate was small compared to that induced by BK (data not shown) and the Ca^{2+} peak generated by a combination of vanadate and BK was not greater than that induced by BK alone (Figure 2B), which suggests that BK and vanadate release Ca^{2+} from the same intracellular Ca^{2+} pools. This eliminates the possibility that augmentation of BK-stimulated Ca^{2+} influx by vanadate is due to additional Ca^{2+} release by vanadate. Furthermore, the effect seen on the BK-stimulated Ba^{2+} influx by vanadate was not due to inhibition of the Ca^{2+} pump since Ba^{2+} can not be pumped by Ca^{2+} ATPases. These results suggest that the effect of vanadate on Ca^{2+} entry is through regulation of protein tyrosine phosphorylation involving tyrosine phosphatase activity.

Neither the mechanism for activation of tyrosine kinases nor the identity of tyrosine kinases responsible for regulation of Ca^{2+} entry is known. While our data indicate that a rise in the cytosolic Ca^{2+} concentration following depletion of internal stores is necessary for full activation of tyrosine kinase activity (Figure 7B), it is also clear that there is a large component of the BK-stimulated tyrosine phosphorylation that is independent of a rise in the cytosolic Ca^{2+} concentration, since BAPTA-AM failed to inhibit approximately 60% of the BK-induced tyrosine phosphorylation (Figure 7B). This result indicates that there are at least two mechanisms for activation of tyrosine kinases by BK in HSWP cells; one dependent on and the other independent of a rise in the cytosolic Ca^{2+} concentration. The tyrosine phosphorylation that is independent of a rise in the cytosolic Ca^{2+} concentration may represent the activation of tyrosine kinases in direct response to a messenger generated by Ca^{2+} store depletion. This would be consistent with observations in platelets where a store-depletion-dependent increase in protein tyrosine phosphorylation is observed (Sargeant *et al.*, 1994b). In studies from other laboratories, Ca^{2+}-dependence on tyrosine phosphorylation was reported in thrombin-stimulated BC_3H1cells (Offermanns *et al.*, 1993) and angiotensin-stimulated WB cells (Huckle *et al.*, 1990). In contrast, bombesin stimulation of tyrosine phosphorylation of the two substrates $pp125^{FAK}$ and paxillin was shown by Rozengurt's group to be independent of Ca^{2+} (Sinnett-Smith *et al.*, 1993; Zachary *et al.*, 1993). The contradictory results from these reports together with our results concerning the Ca^{2+} independence of a large component of tyrosine phosphorylation suggest that distinct mechanisms are operative in activating tyrosine phosphorylation events. We also eliminated the possibility of protein kinase C being a regulator of either tyrosine phosphorylation or tyrosine kinases stimulated by BK. Further research will help characterize the mechanism for activation of tyrosine kinases responsible for Ca^{2+} entry.

Regarding the identity of tyrosine kinases involved in the regulation of Ca^{2+} entry, we have shown in a previous study that the two nonreceptor tyrosine kinases $pp60^{src}$ and $pp125^{FAK}$ are present and activated by BK in HSWP cells (Lee and Villereal, 1996). Enhanced Ca^{2+} entry in cells overexpressing $pp60^{v\text{-}src}$ (Niklinska *et al.*, 1992) and $pp59^{fyn}$ (Cooke *et al.*, 1991) has previously been reported. In addition, we previously reported a reduction of SOCE in fibroblasts isolated from src-deficient mice, as compared to fibroblasts isolated from wild-type mice (Babnigg *et al.*, 1997). These findings demonstrate that SOCE can be regulated by tyrosine kinases, and suggest that src family tyrosine kinases are involved in this process (Kawasaki *et al.*, 2006).

At present, it is not clear at which step tyrosine

kinase plays a role in BK-mediated Ca^{2+} entry pathways. Tyrosine phosphorylation in platelets was enhanced following depletion of internal Ca^{2+} stores, with the principal substrate being a 130 kDa protein (Vostal *et al.*, 1991). Although the authors did not test the hypothesis, they did speculate that one of the roles of phosphorylated 130 kDa proteins was to regulate plasma membrane Ca^{2+} permeability. Our results are consistent with the hypothesis that tyrosine kinase activity plays a role in Ca^{2+} entry pathways, although the mechanism of activation of plasma membrane Ca^{2+} channels is not known. It is possible that tyrosine kinases activate Ca^{2+} entry by directly phosphorylating Ca^{2+} channels. An alternative explanation for tyrosine kinase regulation of SOCE has been put forward in terms of the secretion-like coupling model that suggests that the cytoskeleton controls the interaction between the endoplasmic reticulum and the plasma membrane, which leads to *de novo* coupling of the IP_3 receptors and the calcium entry channels (Rosado *et al.*, 2005). Based on this model and the known role of tyrosine phosphorylations in remodeling the cytoskeleton, one can speculate that activation of tyrosine kinases in response to store depletion plays an important role in this process.

The mechanism for enhancement of tyrosine phosphorylation is not known, although it is clear from the BAPTA experiments (Figure 7) that enhanced phosphorylation can take place in the absence of a rise in the amount of cytosolic Ca^{2+}. One intriguing possibility is that, following depletion of internal Ca^{2+} stores, production of EET isoforms, *via* cytochrome P450 activity, leads to a stimulation of tyrosine kinase activity similar to that observed after addition of exogenous EET to renal epithelial cells (Chen *et al.*, 1998) and aortic endothelial cells (Hoebel *et al.*, 1998). In the aortic endothelial cells, addition of arachidonic acid also stimulated tyrosine kinase activity. This effect was blocked by inhibitors of P450 activity, indicating that the effects of econazole we see in HSWP cells may be at the level of production of EET isoforms rather than as a direct block of the tyrosine kinase. Studies in renal epithelial cells have suggested that the tyrosine phosphorylation induced by 14,15 EET is mediated by src kinase, since EET stimulated src kinase activity (Chen *et al.*, 1998), and that overexpression of C-terminal src kinase blocks 14,15 EET-induced tyrosine phosphorylation (Chen *et al.*, 2000). Therefore, it is tempting to speculate that the cytochrome P450 enzyme activated by Ca^{2+} store depletion generates EET isoforms, which then activate src-family tyrosine kinases and subsequently open store-operated calcium channels. Experiments are planned to investigate this idea.

The physiological role of tyrosine kinase-regulated Ca^{2+} entry pathways is not known. However, given the fact that tyrosine phosphorylation can serve as a mitogenic signal in cells following activation of G protein-coupled receptors (Seckl and Rozengurt, 1993) and the fact that the presence of extracellular Ca^{2+} is required for mitogenesis, activation of the Ca^{2+} influx pathway by tyrosine kinases probably potentiates or synergizes with mitogenic signals generated by growth factor receptors. Our results indicate that multiple pathways probably operate in a single cell type leading to activation of Ca^{2+} entry and that some of these signaling pathways are more prominently involved in regulating calcium entry in different cell types.

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