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Protein Kinase C Activates Store-operated Ca^{2+} Channels in Human Glomerular Mesangial Cells*

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Store-operated Ca^{2+} channels (SOC) are expressed in cultured human mesangial cells and activated by epidermal growth factor through a pathway involving protein kinase C (PKC). We used fura-2 fluorescence and patch clamp experiments to determine the role of PKC in mediating the activation of SOC after depletion of internal stores by thapsigargin. The measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) revealed that the thapsigargin-induced Ca^{2+} entry pathway was abolished by calphostin C, a protein kinase C inhibitor. The PKC activator, phorbol 12-myristate 13-acetate (PMA), promoted a Ca^{2+} influx that was significantly attenuated by calphostin C and La^{3+} but not by diltiazem. Neither PMA nor calphostin C altered the thapsigargin-induced initial transient rise in $[Ca^{2+}]_i$. In cell-attached patch clamp experiments, the thapsigargin-induced activation of SOC was potentiated by PMA and abolished by both calphostin C and staurosporine. However, SOC was unaffected by thapsigargin when clamping $[Ca^{2+}]_i$ with 1,2-bis (o-Aminophenoxy)ethane-N,N,N′,N′tetraacetic acid tetra(acetoxymethyl)ester. In the absence of thapsigargin, PMA and phorbol 12, 13-didecanoate evoked a significant increase in NP_O of SOC, whereas calphostin C did not affect base-line channel activity. In inside-out patches, SOC activity ran down immediately upon excision but was reactivated significantly after adding the catalytic subunit of 0.1 unit/ml of PKC plus 100 μM ATP. Neither ATP alone nor ATP with heat-inactivated PKC rescued a rundown of SOC. Metavanadate, a general protein phosphatase inhibitor, also enhanced SOC activity in inside-out patches. Bath $[Ca^{2+}]$ did not significantly affect the channel activity in inside-out patch. These results indicate that the depletion of Ca^{2+} stores activates SOC by PKC-mediated phosphorylation of the channel proteins or a membrane-associated complex.

Store-operated Ca^{2+} channels (SOC),[1] present in the plasma membrane of most nonexcitable cells (1–4) as well as some excitable cells (5–7), are activated by decreasing concentrations of Ca^{2+} in the lumen of the endoplasmic reticulum (2, 3, 8, 9). The physiological pathway for activating SOC is initiated by an agonist binding to its receptor with a subsequent generation of inositol 1,4,5-trisphosphate and release of internal stores of Ca^{2+}. Thus, the major role for SOC is to replenish intracellular stores of Ca^{2+} (10, 11). However, the mechanism by which the release of Ca^{2+} stores causes activation of SOC is not completely understood.

Protein kinase C (PKC) has a significant role in signal transduction of several biologically active substances that regulate a variety of cellular functions including proliferation, migration, and vasoconstriction (9, 12–15). Many of these PKC effects are achieved by modulating ion channels. PKC inhibits Ca^{2+}-activated K^+ channels and ATP-sensitive K^+ channels (16, 17) and modulates voltage-gated K^+ channels (18, 19), voltage-gated Ca^{2+} channels (20), and Cl^- channels (21). Phosphorylation of multiple sites on channel proteins has been proposed to account for the varied actions of PKC (18, 19).

Depending on the specifically tested cell and the experimental conditions (8, 22–24), variable results have been reported on the modulation of SOC by PKC. For example, PKC decreases Ca^{2+} influx through SOC in *Xenopus oocytes* (8) and thyroid FRTL-5 cells (24) but activates SOC in RINm5F cells (22), mouse pancreatic acinar cells (23), bovine adrenocortical cells (25), and NIH 3T3 cells with submaximal depletion of internal stores (26). In many of these studies, Ca^{2+}-sensitive fluorescence was used to evaluate Ca^{2+} influx in response to depleting internal Ca^{2+} stores. However, the cytosolic Ca^{2+} concentration measured by this technique does not distinguish between enhanced Ca^{2+} influx and decreased Ca^{2+} efflux.

Recently, single channel currents of SOC were recorded in cultured human mesangial cells (HMCs) (5). It was found subsequently that epidermal growth factor activated SOC through a PKC pathway (27), suggesting that PKC may directly regulate SOC. In this study, we have attempted to determine a specific pathway involving PKC regulation of SOC. The effects of PKC on the overall maintenance of cytosolic Ca^{2+} were determined with the fluorescent Ca^{2+} indicator dye fura-2. To complement these observations, the influence of PKC on single SOC currents was determined using conventional cell-attached and inside-out patch configurations. The results suggest that PKC stimulates Ca^{2+} entry by direct phosphorylation of SOC proteins or an associated membrane complex.

EXPERIMENTAL PROCEDURES

Preparation of Cultures of Mesangial Cells—The procedures and methods for culturing HMC were described previously (28). HMCs were

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[1] The abbreviations used are: SOC, store-operated Ca^{2+} channels; PKC, protein kinase C; HMC, human mesangial cells; PDD, phorbol 12, 13-didecanoate; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance; *NP*o, open probability of channels; AM, acetoxymethyl; BAPTA, 1,2-bis(o-Aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid.

cultured in Dulbecco's modified Eagle's medium from Sigma supplemented with 10 mM HEPES, 2.0 mM glutamine, 0.66 unit/ml of insulin, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 units/ml of penicillin, 100 μg/ml streptomycin, and 20% fetal bovine serum, pH 7.2–7.4. Only subpassages less than eleven generations were used. Upon achieving confluency, cells were passed onto 22 × 22 1-mm cover glasses (Fisher) and studied within 56 h. The cover glasses served as the floor of a perfusion chamber (Warner RC-2OH, 23 °C) used in both fura-2 and patch clamp experiments.

Measurement of $[Ca^{2+}]_I$ and Mn^{2+} Quench—The intracellular Ca^{2+} concentration was monitored in HMC by using fura-2 and dual excitation wavelength fluorescence microscopy as described previously (29, 30). Cells were loaded with fura-2 by incubation for 60 min (20 °C) in physiological saline solution containing 7 μM fura-2 AM, 0.09 g/dl of Me_2SO, and 0.018 g/dl of Pluronic F-127 (Molecular Probes, Eugene, OR). After loading with fura-2, HMCs were placed in a perfusion chamber mounted on the stage of a Nikon Diaphot-300 inverted microscope. With light provided by a DeltaScan dual monochromator system (Photon Technology International, Monmouth Junction, NJ), the cells were illuminated alternately at excitation wavelengths of 340 and 380 nm (bandwith = 3 nm). To limit the detection of emitted fluorescence (510 nm, 20-nm bandpass) to that emitted from a single cell, an adjustable optical sampling window was positioned within the light path upstream from the photon-counting photomultiplier. Background-corrected data were collected at a rate of 5 points/s, stored, and analyzed using the FeliX software package (Photon Technologies). Calibration of the fura-2 signal was performed according to established methods described previously (29, 30). Calcium influx was assessed by Mn^{2+}-induced quenching of fura-2 using buffer containing 0.5 mM Mn^{2+} in the bathing solution. Fluorescence quenching was measured at an excitation wavelength of 360 nm and an emission wavelength of 510 nm. To minimize the influence of other factors such as cell volume on the results, 10 μM ionomycin was applied at the end of each experiment to maximize fluorescence quenching by Mn^{2+}. Mn^{2+} influx was quantified by measuring the normalized rate (slope) of fura-2 quenching.

Patch Clamp Procedures—Patch clamp experiments were performed either with the pipette attached to the membrane (cell-attached) or excised from the cell (inside-out). Plain glass pipettes (Fisher) were prepared with a pipette puller and polisher (PP-830 and MF-830, respectively, Narishige, Tokyo, Japan). The diameter of the pipette tip was about 1 μm.

Single channel currents were recorded and analyzed using standard patch clamp techniques (28, 31). The patch pipette, partially filled with 90 mM $BaCl_2$ solution, was in contact with a Ag-AgCl wire on a polycarbonate holder connected to the head stage of the patch clamp (PC-501A, Warner Instrument Corp., Hamden, CT). The pipettes were lowered onto the cell membrane, and suction was applied to obtain a high resistance (>10 giga ohm) seal. All experiments were conducted at room temperature (22–23 °C). Data were digitized for single channel analysis using an analog-to-digital interface (Axon Instruments, Foster City, CA) and recorded by a computer system. Low pass filter was set at 500 Hz.

Single Channel Analysis—The unitary current (i) defined as zero for the closed state (C) was determined as the mean of the best fit Gaussian distribution of the amplitude histograms. Channels were considered open (O) when the total current (I) was $>(n - 0.5)$I and $<(n + 0.5)$I, where n is the maximum number of current levels observed. The open probability (P_O) was defined as the time spent in an open state divided by the total time of the analyzed record. The channel activity was calculated as $NP_O = \mathrm{En}P_n$, where P_n is the probability of finding n channels open. Therefore, NP_O could be calculated without making assumptions about the total number of channels in a patch or the open probability of individual single channels. The Axoscope acquisition program and pClamp program set 6.02 (Axon Instruments) were used to record and analyze currents.

Solutions and Chemicals—For all fura-2 and cell-attached patch experiments, the initial extracellular physiological saline solution contained 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, and 1 mM $CaCl_2$. For inside-out patches, the bathing solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 μM $CaCl_2$, and 10 mM HEPES. The pipette solution for all patch experiments contained 90 mM $BaCl_2$ plus 10 mM HEPES. In fura-2 experiments, the free Ca^{2+} concentration of the bath was adjusted to <10 nM by buffering physiological saline solution with 1.08 mM EGTA according to the calcium concentration program by MTK software. The pH in all solutions was adjusted to 7.4. Thapsigargin, calphostin C, staurosporine, phorbol 12-myristate 13-acetate (PMA), phorbol 12, 13-didecanoate (PDD), BAPTA/AM, ATP, and catalytic subunit of PKC (PKCc) were purchased from Calbiochem. Sodium metavanadate, lanthanum chloride, and diltiazem were obtained from Sigma.

Statistical Analysis—In patch clamp experiments, all NP_O values were calculated from at least 10 s of single channel recording. Whenever possible, experiments were conducted in a paired fashion with each patch acting as its own control. In these cases, the average change in NP_O for a group of experiments compared before and after experimental manipulation was analyzed using a paired t test. One-way ANOVA and one-way repeated measures ANOVA followed by Student-Newman-Keuls tests were used for comparisons among multiple groups and sequential treatments in one group, respectively. Data are reported as means ± S.E., where n is the number of cells. Significance was $p < 0.05$. Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

Fura-2 Experiments

The Effects of Calphostin C and PMA on Thapsigargin-induced Activation of SOC—Thapsigargin, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (32), activates SOC in a variety of cell types (33–35). Using fura-2 fluorescence measurements, the $[Ca^{2+}]_i$ response to thapsigargin was monitored with and without altering PKC activity. Fig. 1*A* shows a typical profile of the change in $[Ca^{2+}]_i$ induced by thapsigargin and subsequent manipulation of bath calcium concentration ($[Ca^{2+}]_o$). The application of 1 μM thapsigargin in the presence of 1 mM $[Ca^{2+}]_o$ evoked an instant increase in $[Ca^{2+}]_i$ to 180 nM, resulting from the inhibition of Ca^{2+} uptake into the endoplasmic reticulum. The $[Ca^{2+}]_i$ began to decline from a peak value of 180 nM ~100 s after the addition of thapsigargin, and then it was maintained elevated at 80 nM during a plateau phase. The removal of bath Ca^{2+} reduced the $[Ca^{2+}]_i$ from the plateau phase to 10 nM. Subsequent readmission of Ca^{2+} to the bath induced an immediate increase in $[Ca^{2+}]_i$ to 185 nM. This peak change in $[Ca^{2+}]_i$ defined as $\Delta[Ca^{2+}]_i$, is the result of Ca^{2+} entering the cell through SOC (5). Pretreatment with 1 μM calphostin C, a specific inhibitor of PKC, did not affect the thapsigargin-induced transient response (peak $[Ca^{2+}]_i$ = 130 nM) but nearly abolished the SOC pathway ($\Delta[Ca^{2+}]_i$ = 10 nM) (Fig. 1*B*). In the presence of the phorbol ester PMA, the initial thapsigargin-induced spike was 230 nM, and the $\Delta[Ca^{2+}]_i$ was 170 nM (Fig. 1*C*). As shown in Fig. 1*C*, PMA (5 μM) caused a slow and progressive elevation of $[Ca^{2+}]_i$ from 48 nM to 55 nM within 500 s.

The summary data in Fig. 2 illustrate the influence of calphostin C and PMA on (*a*) the thapsigargin-induced initial transient response and (*b*) the capacitative Ca^{2+} influx ($\Delta[Ca^{2+}]_i$). Neither calphostin C nor PMA affected the initial spike induced by thapsigargin (Fig. 2*A*). The peak Ca^{2+} response was 131.1 ± 33.5 nM in control (thapsigargin-treated group) and was not significantly affected by calphostin C (88.7 ± 10.5 nM) or by PMA (84.2 ± 20.6 nM). As shown in Fig. 2*B*, thapsigargin significantly enhanced Ca^{2+} entry ($\Delta[Ca^{2+}]_i$) from 3.8 ± 0.5 nM of control group (in the absence of thapsigargin) to 131.6 ± 25.7 nM (thapsigargin-treated group). This thapsigargin-induced response was reduced by calphostin C from 131.6 ± 25.7 to 9.3 ± 1.2 nM, however, $\Delta[Ca^{2+}]_i$ was not affected by PMA (95.0 ± 12.6 nM, $p > 0.05$ compared with the thapsigargin-treated group, Fig. 2*B*).

We employed Mn^{2+} quenching of the fura-2 dye to rule out potential changes in $[Ca^{2+}]_i$ because of Ca^{2+} efflux. Mn^{2+} penetrates the cell membrane through the same channels employed by Ca^{2+}, rapidly quenching fura-2 fluorescence by irreversible binding (6). As shown in Fig. 3*A*, the addition of 0.5 mM $MnCl_2$ to cells equilibrated in Ca^{2+}-free solution resulted in a slow progressive reduction of the fluorescence emission at 360 nm wavelength. Stimulation of the cells with 1 μM thapsigargin rapidly increased Mn^{2+} quenching of fura-2 fluorescence. The thapsigargin-induced response was greatly depressed by pre-

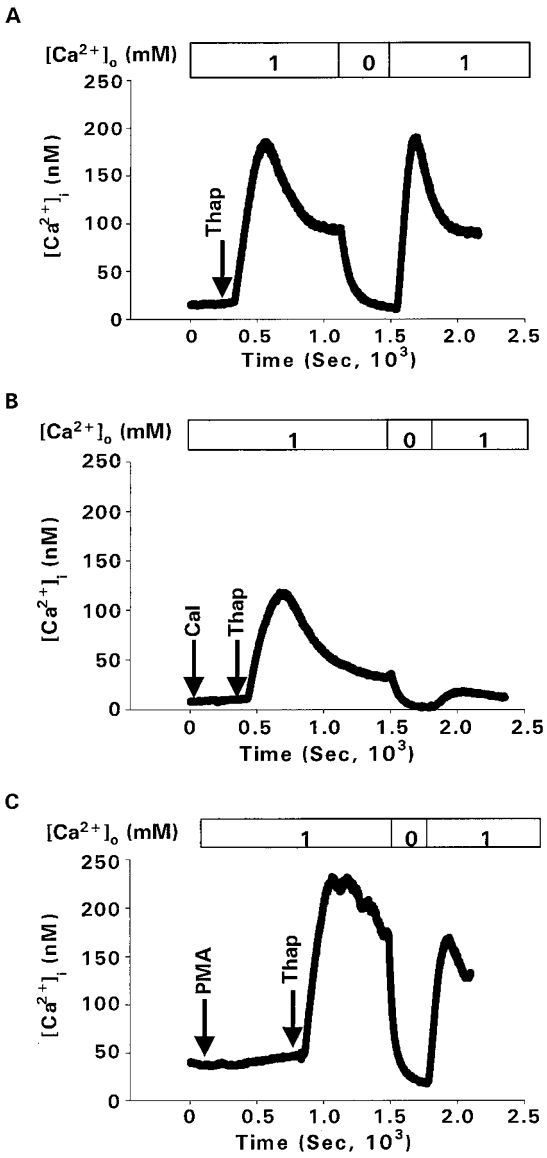

FIG. 1. **Tracings of $[Ca^{2+}]_i$ in HMC shows the effects of calphostin C (*Cal*) and PMA on thapsigargin-induced capacitative Ca^{2+} entry.** *A,* typical $[Ca^{2+}]_i$ response to 1 μM thapsigargin (*Thap*) and subsequent elevation of $[Ca^{2+}]_o$ from 10 nM to 1 mM in the continued presence of thapsigargin. The increase in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$ = 180 nM) is a measure of SOC. *B,* pretreatment with calphostin C (1 μM) attenuated $\Delta[Ca^{2+}]_i$ to 10 nM. *C,* Pretreatment with PMA (5 μM) did not affect $\Delta[Ca^{2+}]_i$.

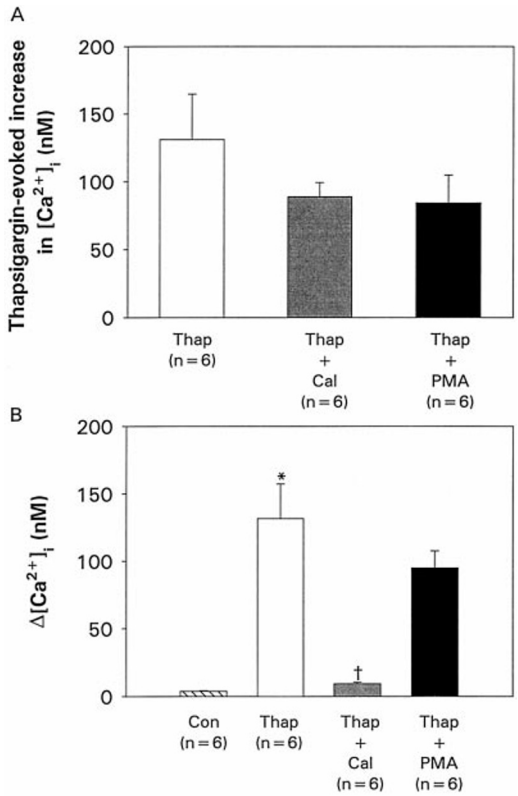

FIG. 2. *A, bar plots* illustrating the effects of calphostin C and PMA on the thapsigargin-induced initial transient increase in $[Ca^{2+}]_i$. Neither calphostin C nor PMA significantly affected the initial phase of the thapsigargin-induced response. *B, bar plots* illustrating the change in $[Ca^{2+}]_i$ evoked by increasing bath $[Ca^{2+}]$ from <10 nM to 1 mM ($\Delta[Ca^{2+}]_i$) in the absence of thapsigargin control (*Con*), in the presence of thapsigargin (*Thap*), and thapsigargin plus Cal (*Thap + Cal*) or PMA (*Thap + PMA*). Thapsigargin evoked a significant capacitative Ca^{2+} entry ($\Delta[Ca^{2+}]_i$), which was significantly abolished by pretreatment with 1 μM calphostin C but unaffected by 5 μM PMA. *, $p < 0.05$, Thap *versus* Con; †, $p < 0.01$, Thap + Cal *versus* Thap using one-way ANOVA followed by Student-Newman-Keuls tests.

treatment with calphostin C. Ionomycin evoked immediate and potent quenching of fluorescence under both conditions. The quantification of Mn^{2+} influx was assessed by measuring the slope of fluorescence quenching relating to the entire range of alteration in fluorescence (the difference in fluorescence between base line and after ionomycin). As shown in the summarized plots of Fig. 3*B*, thapsigargin-induced fluorescence quenching was significantly inhibited by calphostin C.

Effects of PMA on SOC—As described above, although PMA did not affect the thapsigargin-induced increase in SOC, it did cause a mild slow increase in $[Ca^{2+}]_i$, indicating a possible influence of PKC on Ca^{2+} mobilization (influx or efflux) that is independent of depleting internal Ca^{2+} stores. To further investigate the effects of PMA, the response of $[Ca^{2+}]_i$ to PMA was determined in the absence of thapsigargin. Fig. 4*A* is a representative tracing showing a PMA-evoked slow and progressive increase in $[Ca^{2+}]_i$ (from 40.2 nM to 56.1 nM) with 1 mM Ca^{2+} in the bathing solution. Removing Ca^{2+} from the bath resulted in an immediate decrease in $[Ca^{2+}]_i$ (from 56.1 nM to 22.2 nM). The re-addition of 1 mM Ca^{2+} to the bath evoked a sharp and dramatic rise in $[Ca^{2+}]_i$ (from 22.2 nM to 148 nM). Because the $\Delta[Ca^{2+}]_i$ was attributed to Ca^{2+} entry through the plasma membrane, the properties of the PMA-induced $\Delta[Ca^{2+}]_i$ was characterized pharmacologically. In the first experiment, pretreatment with calphostin C (5 μM for 10 min) nearly abolished PMA-induced Ca^{2+} entry (from 84.2 ± 25.7 nM to 12.7 ± 1.7 nM**,** Fig. 4, *B* and *C*). Furthermore, the PMA-induced response was significantly reduced to 10.5 ± 2.2 nM by La^{3+} (2 μM), a relatively specific blocker of SOC at this concentration (5, 8, 23) (Fig. 4, *B* and *C*). Although diltiazem (5 μM), a voltage-gated Ca^{2+} channel blocker, attenuated the $\Delta[Ca^{2+}]_i$ to 42.3 ± 6.6 (Fig. 4*C*), this value was not significantly different from the PMA-induced value.

Patch Clamp Experiments

Regulation of Single SOC Currents by PKC—Single SOC currents have been recorded previously in cultured HMC, and the pharmacological and biophysical properties have been described previously (5). In this study, the cell-attached configuration was used to examine the SOC response to depletion of internal stores in the absence and presence of calphostin C or PMA. Fig. 5*A* shows SOC currents under four different conditions. Consistent with previous studies (5), unstimulated SOCs have spontaneous openings in cell-attached patches (Fig. 5*A*, *top tracing*, NP_O = 0.62). The number of observed channels in

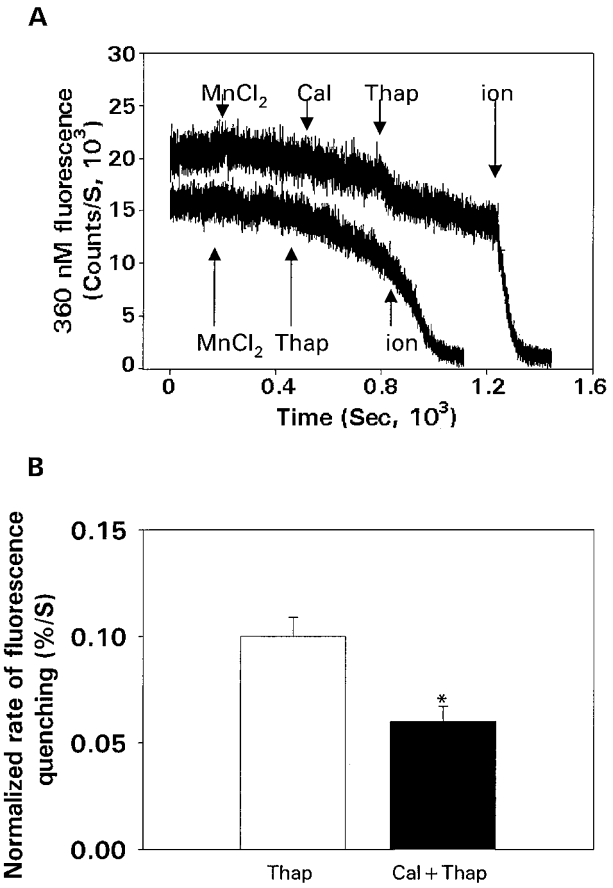

FIG. 3. **Effects of calphostin C on the thapsigargin-induced fluorescence quenching by Mn^{2+}.** *A,* representative tracings showing that thapsigargin evoked rapid and dramatic fluorescence quenching. Ionomycin (10 μM) was applied at the end of the experiments to maximize Mn^{2+} influx. *Ion*, ionomycin. *B,* a summary graph showing the rate of fluorescence quenching normalized to the range of fluorescence from base line to minimal level after treatment of ionomycin. *, $p < 0.05$, Student's *t* test.

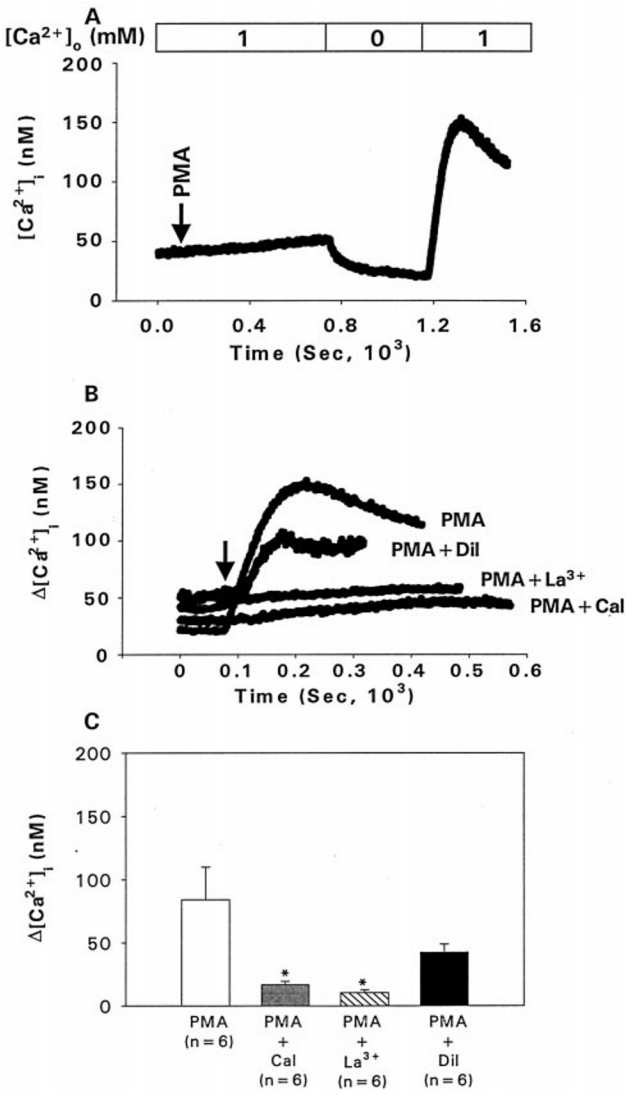

FIG. 4. Effects of 5 μM PMA on $[Ca^{2+}]_i$ and $\Delta[Ca^{2+}]_i$ in (*A*) control and (*B*) the presence of 1 μM calphostin C (*PMA + Cal*), 5 μM diltiazem (*PMA + Dil*), or 2 μM La^{3+} (*PMA + La^{3+}*). *Arrow* indicates the time of increasing $[Ca^{2+}]_o$ from <10 nM to 1 mM. *C, Bar graph* summarizing the effects of calphostin C, La^{3+}, and diltiazem on the PMA-induced $\Delta[Ca^{2+}]_i$. *, $p < 0.05$, compared with PMA using one-way ANOVA plus Student-Newman-Keuls tests.

a single patch varied from 1 to 3, and the amplitude of the single channel current was ~0.2–0.3 pico ampere at a holding potential ($-V_p$) of −80 mV. The estimated single channel conductance was 2.1 pico siemen. In most cases, SOC revealed characteristically long open and closed states in the basal condition. The activity (NP_O) of SOC was enhanced by the addition of thapsigargin (1 μM) to the bath (Fig. 5*A*, *middle tracing*, NP_O = 0.89). However, in the presence of 1 μM calphostin C, thapsigargin failed to activate SOC (Fig. 5*A*, *bottom tracing*, NP_O = 0.41). As illustrated by the plots of the summarized data (Fig. 5*B*), thapsigargin significantly increased the NP_O of SOC (by 24.5 ± 5.8%). This activation of SOC was significantly abolished by calphostin C (−15.7 ± 16.8%) and enhanced by PMA (45.1 ± 6.8%).

To determine if the thapsigargin-induced activation of SOC was secondary to a rise in $[Ca^{2+}]_I$, $[Ca^{2+}]_i$ was clamped with BAPTA/AM while monitoring SOC activity in the cell attached configuration. Fura-2 fluorescence measurements of $[Ca^{2+}]_i$ confirmed that 10 μM BAPTA/AM abolished the thapsigargin-induced increase in $[Ca^{2+}]_i$ (data not shown). Pretreatment of cells with 10 μM BAPTA/AM for 30 min did not significantly affect the thapsigargin-evoked increase in NP_O of SOC (Fig. 5*B*).

To further confirm the specificity of PKC in the mediation of thapsigargin-induced SOC activation, the effects of another (albeit less specific) PKC inhibitor, staurosporine, were examined in cell-attached patches. Similar to calphostin C, 10 nM staurosporine completely abolished the thapsigargin-induced response (−5.6 ± 20.6%, Fig. 5*B*).

Effects of Calphostin C and PMA on Basal SOC Activity—To investigate whether PKC regulates SOC activity independent of the depletion of internal Ca^{2+} stores, the response of SOC was examined in the cell-attached configuration. Calphostin C (1 μM) did not affect spontaneous SOC activity (NP_O = from 0.99 ± 0.21 to 1.09 ± 0.29 before and after calphostin C, $p > 0.05$, paired *t* test). However, the administration of PMA increased the NP_O of SOC from 1.12 ± 0.06 to 1.52 ± 0.07 ($p < 0.05$, paired *t* test). Similarly, another phorbol ester, PDD (500 nM), significantly increased SOC activity by 38.3 ± 18.3% ($p < 0.05$, paired *t* test).

Influence of PKC on SOC in Inside-out Patches—To determine whether PKC is a direct regulator of SOC, the effects of the catalytic subunit of PKC (PKCc), which is active in the absence of phospholipids and Ca^{2+}, on single SOC currents were determined in inside-out patches. In these experiments, a spontaneous decrease in SOC activity (rundown) was routinely observed (Fig. 6*A*). After base-line activity was stabilized (~1 min post-excision), PKCc (0.1 unit/ml) and Mg-ATP (100 μM)

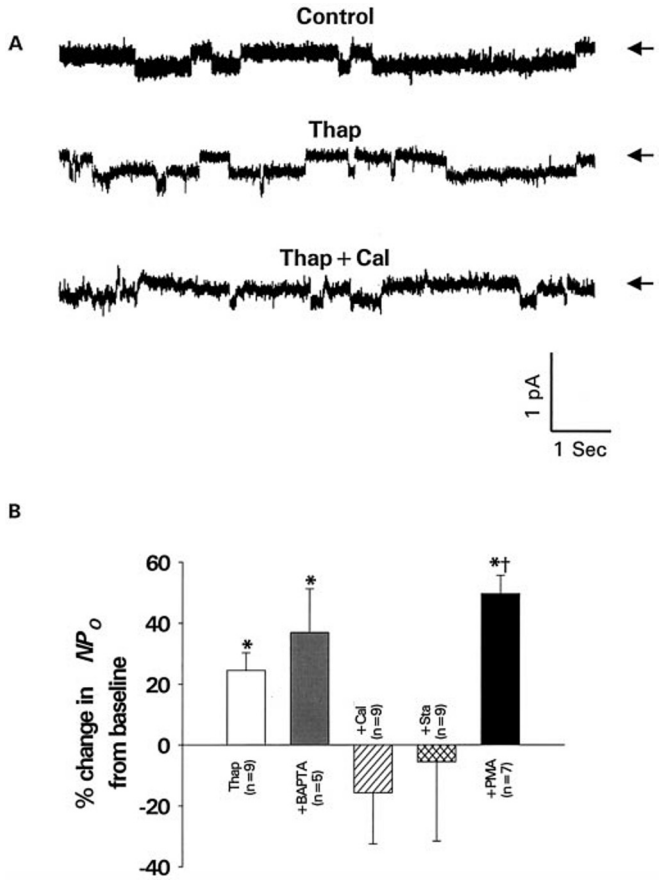

FIG. 5. **Effects of calphostin C and PMA on thapsigargin (1 μM)-induced activation of SOC in cell-attached patches.** *A,* representative tracings of single SOC currents in control (*top*), after application of thapsigargin (*middle*), and thapsigargin plus 1 μM calphostin C (*bottom*) at a holding potential of $-V_p = -80$ mV. *Arrows* indicate closed state. Inward currents are downward. *B,* a summary graph showing the effects of BAPTA/AM, calphostin C, staurosporine (*sta*) and PMA on the thapsigargin-induced activation of SOC. *, $p < 0.05$, compared with base-line SOC activity using paired *t* test; †, $p < 0.05$, compared with the thapsigargin-treated group using unpaired *t* test.

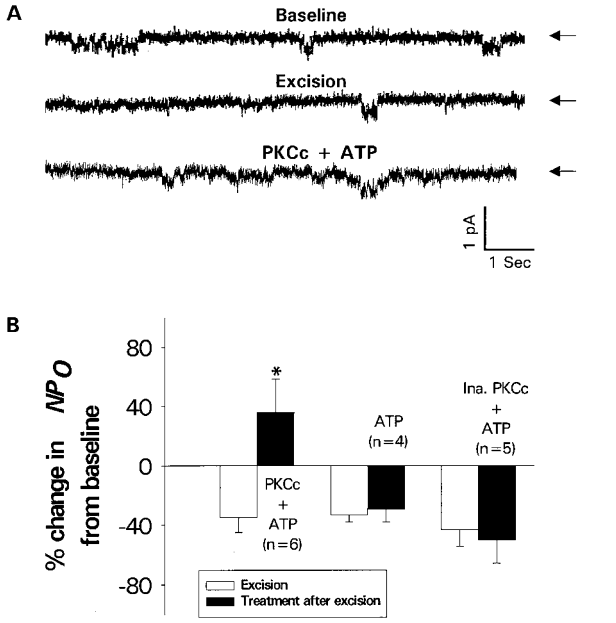

FIG. 6. **Effects of PKCc on SOC in inside-out patches ($-V_p = -80$ mV).** *A,* representative tracings showing the base-line SOC activity (*top*) after excision (*middle*) and reactivation (*bottom*) on the addition of PKCc (0.1 unit/ml) plus ATP (100 μM). *B, bar graph* illustrating reactivation of SOC by PKCc plus ATP after excision rundown. *Blank bars* represent post-excision values. *Filled bars* represent treatment values. Neither ATP alone nor heat-inactivated PKCc (*Ina PKCc*) restored SOC activity after excision-induced rundown. *, $p < 0.05$, compared with corresponding post-excision value.

were added to the bathing solution. Representative tracings of single SOC currents ($-V_p = -80$ mV) under control conditions after excision and subsequent application of PKCc are shown in Fig. 6*A*. The NP_O of SOC declined after excision but was reactivated by the application of PKCc combined with Mg-ATP. A summary data of six observations (Fig. 6*B*) revealed a significant post-excision reduction in NP_O (by $-34.9\% \pm 9.9$) and a complete recovery ($35.9\% \pm 22.5$) of SOC activity by PKCc plus Mg-ATP.

Experiments were performed to determine whether the effects of PKCc on single SOC currents were the specific results of the enzymatic activity of PKCc. The effects on SOC of Mg-ATP alone and Mg-ATP plus inactivated PKCc (heating at 60 °C for 30 min) were tested under the same conditions as those for active PKCc. Neither Mg^{2+}-ATP alone ($-29.2\% \pm 8.8$) nor heat-inactivated PKCc (0.1 unit/ml) plus Mg-ATP ($-50.1\% \pm 15.5$) significantly affected the post-excision rundown of SOC ($-33.1\% \pm 4.8$ and $-43.2\% \pm 11.3$, ATP alone and heat-inactivated PKCc, respectively). In the absence of Mg-ATP, a post-excision rundown ($-42.0\% \pm 23.9$) was unaffected by active PKCc ($-46.0\% \pm 19.3$) from post-excision.

Inhibition of SOC by Dephosphorylation—The inside-out experiments suggested a direct phosphorylation of SOC by PKC. To determine whether SOC rundown was the result of dephosphorylation, the effects of metavanadate, a protein phosphatase inhibitor, was determined after the post-excision rundown of SOC in inside-out patches. In a representative tracing shown in Fig. 7*A*, SOC activity declined dramatically after excision, consistent with rundown (see Fig. 6*A*). As shown, sodium metavanadate (1 mM) reversed the post-excision rundown of SOC activity, an effect observed in all seven individual experiments (Fig. 7*B*). On average, the NP_O of SOC declined from 0.76 ± 0.10 to 0.44 ± 0.08 after excision but reversed significantly to 0.99 ± 0.1 by metavanadate.

Ca^{2+} Sensitivity of SOC—Because SOC was activated concomitantly with an elevation of $[Ca^{2+}]_i$ upon the release of stores, it was possible that Ca^{2+} directly regulated SOC. To determine whether the channels are sensitive to direct Ca^{2+} ligand interaction, the influence of Ca^{2+} concentration was examined in inside-out patch experiments. Consistent with the inside-out experiments described above, SOC activity ran down rapidly after excision even though the Ca^{2+} concentration of 1 μM in the bathing solution was much greater than in the mesangial cytosol (~50–100 nM). Either decreasing the bath Ca^{2+} concentration to 100 nM or increasing it to 100 μM failed to significantly affect channel activity (-11.2 ± 27.3 and 1.1 ± 22.5, respectively, $p > 0.05$).

DISCUSSION

Store-operated Ca^{2+} channels are ubiquitous channels identifiable by their marked sensitivity to thapsigargin-evoked depletion of intracellular stores of Ca^{2+}. This study showed that thapsigargin-evoked activation of SOC was inhibited by calphostin C, a relatively specific blocker of PKC. Moreover, the PKC activator PMA mimicked the activation of SOC by thapsigargin. Reversal of SOC rundown in inside-out patches by PKC and metavanadate suggested direct phosphorylation of SOC or a closely associated regulator. These results are seemingly in conflict with some previous studies. However, the role of PKC can be more clearly defined by fluorescent measurements of intracellular concentration of Ca^{2+} and Ca^{2+} influx in combination with single SOC currents.

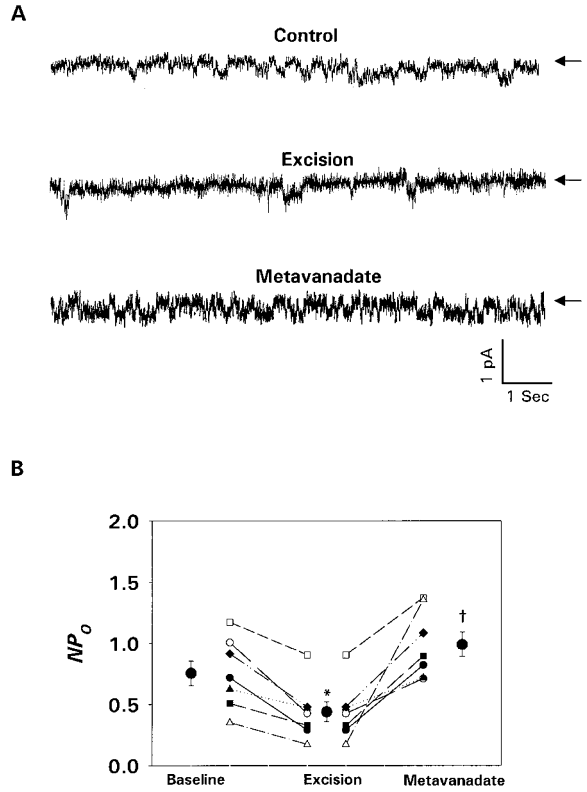

FIG. 7. **Effects of metavanadate on SOC activity in an inside-out patch ($-Vp = -80$ mV).** *A,* tracings demonstrate rundown of SOC after patch excision and reactivation after adding metavanadate (1 mM) to the bathing solution. *B,* individual NP_O plots of SOC before excision, after excision, and subsequent addition of metavanadate. The means and standard errors are indicated. *, $p < 0.05$, compared with base line. †, $p < 0.05$, compared with value after excision (one-way ANOVA for repeated measurements).

Measurements of $[Ca^{2+}]_i$ and Fluorescence Quenching by Mn^{2+}—As previously demonstrated by this laboratory, SOC are identified with fura-2 fluorescence microscopy as a large elevation in $[Ca^{2+}]_i$ when raising the extracellular Ca^{2+} concentration from <10 nM to 1 mM in the presence of thapsigargin. This activation of SOC nearly was completely prevented by pretreatment with calphostin C. In addition, thapsigargin-induced fluorescence quenching by Mn^{2+} was significantly inhibited by calphostin C. Moreover, the addition of PMA to the bathing solution enhanced La^{3+}-sensitive but not diltiazem-sensitive Ca^{2+} entry. These effects of calphostin C and PMA on $[Ca^{2+}]_i$ are strong evidence that PKC is an endogenous regulator of SOC.

Because the PKC inhibitor effectively prevented the thapsigargin-evoked activation of SOC, it was expected that PMA would enhance thapsigargin-evoked activation of SOC. However, the addition of PMA before thapsigargin application did not have a significant effect on the SOC pathway. There are at least two possible explanations for this result. First, the store release by thapsigargin may have maximally stimulated PKC. In this case, PMA would not additionally affect SOC. Second, PKC may have multiple complex actions on Ca^{2+} mobilization. Low intracellular concentrations of PKC may predominately stimulate Ca^{2+} influx. However, higher concentrations elicited by PMA may stimulate Ca^{2+} efflux by activating the plasmalemmal Ca^{2+}-ATPase (36, 37). Recently, Peterson and Berridge (34) reported that PKC biphasically regulated $[Ca^{2+}]_i$ depending on the level of intracellular PKC activity.

Voltage-gated Ca^{2+} channels might also be involved in PMA-induced Ca^{2+} in-flow, because PKC was reported to modulate the activity of voltage-gated Ca^{2+} channels (20). Although this possibility could not be ruled out entirely, the contribution of this type of Ca^{2+} channel is slight, if any, because diltiazem, a voltage-gated Ca^{2+} channels blocker, did not significantly affect the PMA-induced Ca^{2+} influx (Fig. 3). The dose (5 μM) of diltiazem used in this study was much higher than its IC_{50} (0.4 μM) for blocking voltage-gated Ca^{2+} channels (38).

It is possible that PKC activated SOC by releasing intracellular stores of Ca^{2+}. In human neutrophils (36) and human embryonic kidney 293 cells (36), PKC affected the mobilization of Ca^{2+} between the cytosol and the endoplasmic reticulum. However, the present results do not support this notion, because neither calphostin C nor PMA affected the thapsigargin-evoked peak response of $[Ca^{2+}]_i$ (Fig. 2*A*). Moreover, Bode and GöKe (22) reported that the activation of PKC with PMA did not affect thapsigargin-evoked store depletion.

Patch Clamp Experiments—The regulation of SOC by PKC has been studied extensively using fura-2 Ca^{2+} measurements (22, 23, 33, 34), but only a few studies have used patch clamp methods (8, 35). Some general features of SOC demonstrated in this laboratory have been acknowledged by most investigators (2, 5, 39, 40). These include a low single channel conductance, voltage-independent activation, high selectivity to Ca^{2+}, and high sensitivity to blockade by La^{3+} (1–3, 5, 6, 8, 9). In the cell-attached configuration, the thapsigargin-induced activation of SOC was inhibited by the PKC inhibitors, calphostin C, and staurosporine, and enhanced by the PKC activators PMA and PDD. These results were consistent with the fura-2 experiments, suggesting an essential role for PKC in regulating SOC activity.

In the absence of thapsigargin, PMA and PDD significantly increased basal SOC activity. This result was corroborated by the fura-2 experiments, which showed a small increase in $[Ca^{2+}]_i$ on the addition of PMA. In contrast, calphostin C did not affect basal SOC activity. This result was consistent with the fura-2 experiments and the results of Yao and Tsien (8), who discovered that calphostin C did not affect base-line capacitative Ca^{2+} influx into *X. oocytes*. Thus, PKC contributes minimally to the resting SOC activity.

Inside-out patches were used to determine whether PKC could directly activate SOC or a closely associated regulator of SOC. It was discovered that PKCc, which does not require Ca^{2+} or phosphatidylserine to be in the active state (41), attenuated a rundown of SOC in the presence but not in the absence of Mg-ATP. This result suggested that the rundown of SOC activity was because of dephosphorylation on patch excision.

Additional evidence that SOC is regulated by a phosphorylation mechanism was demonstrated by the rescue from rundown by metavanadate in inside-out patches. The rescue of SOC activity by metavanadate is consistent with the notion that both PKC and a protein phosphatase are present in the inside-out patches tightly associated with SOC. Many studies have shown that kinases or phosphatases are often excised with ion channels as a tightly associated complex (42, 43). Thus, SOC in the inside-out patch is probably governed by a phosphorylation-dephosphorylation cycle. In the intact cell, protein phosphatases are probably inhibited by endogenous phosphatase inhibitors (44). An excision rundown can be attributed to a loss of phosphatase inhibitors allowing the phosphatase-dephosphorylating limb of the cycle to predominate. Thus, SOC reactivates when metavanadate, an exogenous phosphatase inhibitor, is added to the bathing solution. Further studies using specific inhibitors could reveal the specific phosphatases involved in this reaction.

The Ca^{2+} sensitivity of SOC was determined in both cell-attached and inside-out patches. When cytosolic Ca^{2+} concentration was clamped using 10 μM BAPTA, thapsigargin still

significantly increased SOC activity. In the inside-out experiments, elevating the bath [Ca^{2+}] from 1 μM to 100 μM or reducing the bath [Ca^{2+}] to 100 nM failed to influence SOC activity. Based on these experiments, we conclude that SOC is not influenced by direct interaction with Ca^{2+}.

Differential Results Regarding PKC Regulation of SOC—PKC modulates a variety of intracellular events by regulating the activity of ion channels (16–21, 25). However, the results are extremely variable with respect to the regulation of SOC by PKC (8, 22, 23, 34, 35). Several factors besides the differences in cell types could account for these large discrepancies. One recent study (8) used the whole cell configuration in *X. oocytes* and monitored a Ca^{2+}-activated Cl^- current as an indirect indicator of a store-depleted activation of Ca^{2+} current. The present experiments in which single channel Ca^{2+} currents were monitored should be a more direct assessment of PKC-modulated SOC activity. In human embryonic kidney 293 cells, PKC inhibited Ca^{2+} entry after the cells were exposed to PMA for 60 min (34). However, incubation with PMA for only 60 min could down-regulate PKC activity (41). Moreover, the multifarious isoforms of PKC and their variable functions and intracellular distribution may lead to disparate interpretations (12, 14). The concentration of PKC may also be a factor. Peterson and Berridge (34) showed that capacitative Ca^{2+} entry in *Xenopus* Oocytes was potentiated by low concentrations of PKC but inhibited by higher levels. In human embryonic kidney 293 cells, PMA increased Ca^{2+} entry at 1.6 μM but decreased Ca^{2+} entry at lower concentrations (34). Thus, different levels of PKC in different studies also might account for disparate results.

Although some isoforms of PKC are activated by Ca^{2+}, it is improbable that the Ca^{2+} released from stores is involved in activating SOC via PKC. The present and previous studies have shown that SOC is activated upon store depletion despite clamping Ca^{2+} with intracellular buffers. It is possible, however, that an unknown PKC stimulating phospholipid or protein is generated upon the depletion of internal stores. It is also possible that when the endoplasmic reticulum becomes Ca^{2+} depleted the cytoskeleton rearranges and activates PKC. It has been shown previously that the disruption of the actin cytoskeleton activates PKCα in mesenchymal cells (47). It is also reported that calponin, a cytoskeletal protein, may function in smooth muscle to regulate PKC activity by facilitating the phosphorylation of PKC (48). Activated PKC may then play a role in either translocating vesicles containing SOC to the plasma membrane or phosphorylating the SOC already existing in the membrane.

Studies intending to identify the SOC protein led to the cloning of mammalian homologues of the *Drosophila* transient receptor potential *trp* gene (49, 50). The *trp* gene family has been proposed to encode the SOC in nonexcitable cells (45–47). Interestingly, in *Drosophila*, PKC forms a supramolecular complex with *trp* and other proteins involved in Ca^{2+} mobilization (49). This complex may be a regulatory unit for Ca^{2+} influx and could convey complex regulatory signals to modulate the activity of SOC (50).

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