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Protein Kinase C Potentiates Capacitative Ca^{2+} Entry That Links to Steroidogenesis in Bovine Adrenocortical Cells

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ABSTRACT—I investigated the role of protein kinase C (PKC) in regulation of the capacitative Ca^{2+} entry and steroidogenesis in bovine adrenocortical (BA) cells. Thapsigargin (TG)-treatment depleted intracellular Ca^{2+} stores followed by induction of Ca^{2+} influx from the extracellular pool and also increasing of Mn^{2+} influx as an indicator of divalent cation influx in BA cells. Calphostin C, a PKC inhibitor, inhibited the TG-induced $[Ca^{2+}]_i$ elevation dose-dependently ($0.1-1\,\mu M$) and attenuated Mn^{2+} entry. Phorbol 12-myristate 13-acetate (PMA), an activator of PKC, potentiated the elevation of $[Ca^{2+}]_i$ and enhanced Mn^{2+} entry by TG treatment. These results suggest that PKC may modulate capacitative Ca^{2+} entry in BA cells. In the presence of extracellular Ca^{2+}, TG enhanced cortisol production in BA cells. Calphostin C attenuated the TG-induced steroidogenesis dose-dependently ($0.25-1\,\mu M$). PMA enhanced the steroidogenesis dose-dependently (1 – 100 nM). These results suggested that PKC may have a modulatory effect on the capacitative Ca^{2+} entry that links to steroidogenesis in BA cells.

Keywords: Capacitative Ca^{2+} entry, Protein kinase C, Adrenocortical cell

In various types of cells, depletion of the intracellular Ca^{2+} stores induces Ca^{2+} entry across the plasma membrane. This Ca^{2+} entry is termed capacitative Ca^{2+} entry (1, 2). However, the regulatory mechanisms of this Ca^{2+} entry are still unclear. The considered hypotheses are direct protein-protein interactions (3, 4) (conformational coupling mechanism) and indirect gating through diffusable messengers (cGMP (5), Ca^{2+} influx factor (6)) or kinase/phosphatase (calmodulin kinase II (7), tyrosine kinase (8), protein kinase C (PKC) (9), protein phosphatase (10)) generated by the storage organelles. Current experiments have led to the suggestion that the capacitative Ca^{2+} entry is regulated by phosphorylation. Indeed, there are many reports that the capacitative Ca^{2+} entry is regulated by serine/threonine kinase, especially PKC (9, 11 – 13). However, in some cell types, PKC activation facilitates (9) or inhibits the capacitative Ca^{2+} entry (12, 13).

As regards to the physiological function of the capacitative Ca^{2+} entry, it has not become clear in several cell types. In bovine adrenocortical (BA) cells, the elevation of cytosolic Ca^{2+} induced steroid synthesis (14).

In the present study, I investigated the role of regulating the capacitative Ca^{2+} entry by utilizing reagents that modulate PKC activity. Furthermore, I studied a correlation between the regulation of capacitative Ca^{2+} by phosphorylation and steroidogenesis in BA cells.

MATERIALS AND METHODS

Cell culture

Bovine adrenal glands were purchased from Shibaura slaughter house in Tokyo. BA cells were prepared and cultured primarily as previously described (15). Briefly, BA cells were isolated aseptically from minced bovine adrenal cortex by collagenase-DNase dissolved in Krebs-Ringer bicarbonate glucose albumin buffer (KRBGA). The treatment was performed by 1-h incubation at 37°C under 95% O_2 – 5% CO_2 mixture as a gas phase. The isolated cells were cultured in Ham's F-10 medium containing 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum and antibiotics (100 IU/ml penicillin G, 100 μg/ml streptomycin and 50 μg/ml gentamicin). The isolated cells were plated on glass coverslips for measurement of $[Ca^{2+}]_i$ and seeded in 12-well plates (Linbro; each well, measuring 3.8 cm^2 in size) at a cell density of approximately 40×10^4 cells/well. The 3-day primary cultured BA cells were used for the experiments.

KRBGA: 123.4 mM NaCl, 5.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25.3 mM $NaHCO_3$, 0.01 mM EGTA, 2 mg/ml glucose, 0.3 mg/ml bovine serum albumin (BSA) (pH 7.4).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was monitored as described by Matsui (15). Briefly the 3-day primary cultured cells, which had been grown on coverslips, were used for $[Ca^{2+}]_i$ measurements. The monolayer cells on coverslips were loaded with 5 μM fura-2/acetoxymethyl ester (fura-2/AM) in Krebs-Ringer HEPES buffer (123.4 mM NaCl, 5.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.01 mM EGTA, 2 mg/ml glucose, 1.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4). The fura-2 loaded cells were placed diagonally in a quartz cuvette containing the Krebs-Ringer HEPES buffer. For Ca^{2+}-free experiments, I used the medium in which Ca^{2+} was removed from the above buffer. The fluorescence was monitored by an Hitachi F-2000 spectro-fluorometer (Hitachi Industry, Tokyo) at an emission wavelength of 510 nm, while the excitation wavelength was between 340 and 380 nm. The increase in $[Ca^{2+}]_i$ was expressed as the 340/380-nm fluorescence intensity ratio (I_{340}/I_{380}).

Ca^{2+} influx was assessed by Mn^{2+}-induced quenching of fura-2 that is directly used to determine bivalent cation influx into cells (16). After addition of Mn^{2+}, the initial drop of fluorescence that is insensitive to changes in Ca^{2+} is the quenching of the extracellular dye. Excitation and emission wavelengths were 360 and 510 nm, respectively. Ca^{2+} free medium was used to investigate Mn^{2+} influx, and thapsigargin (TG) was added at 50 s before Mn^{2+} addition.

Assay of steroidogenesis

The 3-day cultured cells (40×10^4 cells/well) were washed twice with 0.5 ml Ca^{2+}-free phosphate-buffered saline (pH 7.4) containing 0.5 mM EGTA and then washed once with 0.5 ml Krebs-Ringer HEPES buffer. The cells were incubated with Krebs-Ringer HEPES buffer and various reagents at 37°C for 1 h under 95% O_2 – 5% CO_2 mixture as the gas phase. Final incubation volume was 1 ml. After the incubation, 0.5 ml of the medium was taken for the corticoid assay. Corticosteroid was determined fluorometrically using cortisol as the standard (17).

Materials

Fura-2/AM was purchased from Dojindo Laboratories (Kumamoto). Collagenase (type I) was purchased from Cooper Biomedicals (Malvern, PA, USA) and BSA was from Nacalai Tesque (Kyoto). Phorbol 12-myristate 13-acetate (PMA), calphostin C, TG were obtained from Sigma (St. Louis, MO, USA). SK&F 96365 was purchased from Biomol Research Laboratories (Plymouth, PA, USA).

Statistics

Statistical significance of differences was assessed by Student's *t*-test.

RESULTS

Effect of TG on $[Ca^{2+}]_i$

Figure 1A shows the effect of external Ca^{2+} in the presence of TG in BA cells. In the absence of extracellular Ca^{2+}, TG induced a transient increase of $[Ca^{2+}]_i$. It was due to release of Ca^{2+} from intracellular Ca^{2+} stores. The addition of 2.4 mM $CaCl_2$ caused a second rapid increase in $[Ca^{2+}]_i$, followed by a sustained $[Ca^{2+}]_i$ rise. Substantial Ca^{2+} entry was seen only after intracellular Ca^{2+} stores

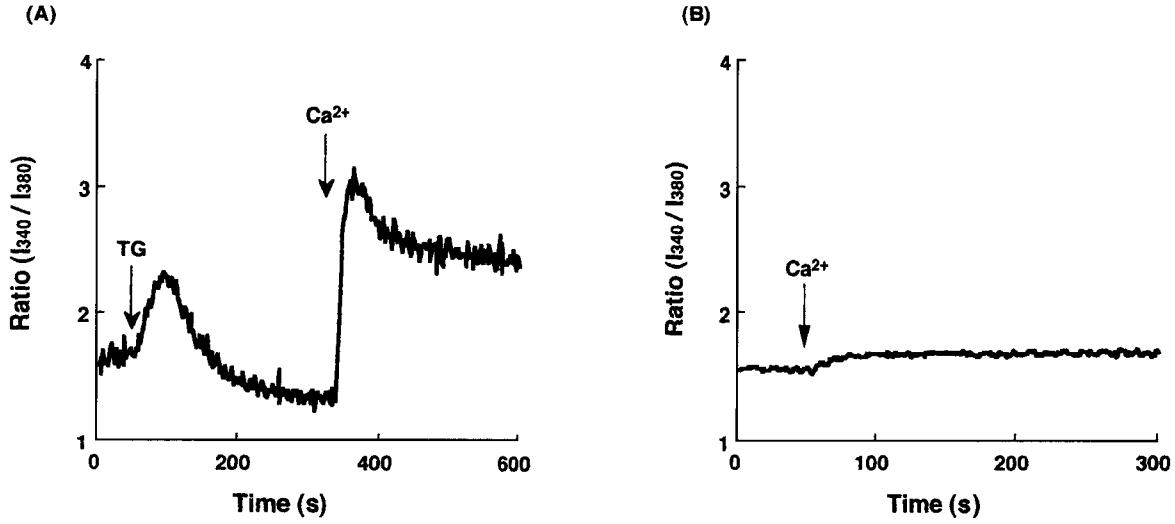

Fig. 1. Effect of external 2.4 mM Ca^{2+} in the presence (A) and absence (B) of 2 μM thapsigargin (TG) in BA cells. The cells were loaded with Fura-2 in Ca^{2+}-free Krebs-Ringer HEPES buffer. Incubation were carried out in the absence of extracellular Ca^{2+}. The ratio was calculated as described in "Materials and Methods". The experiments are representative of three similar ones.

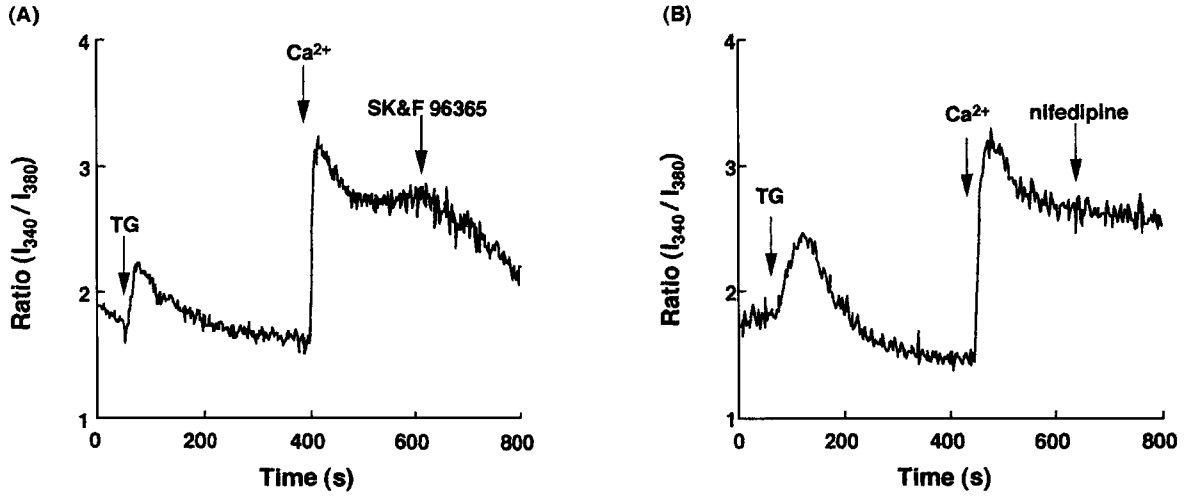

Fig. 2. Effects of SK&F 96365 and nifedipine on the Ca^{2+} entry in thapsigargin (TG)-treated BA cells in the absence of extracellular Ca^{2+}. In the presence of 2 μM TG, 2.4 mM Ca^{2+} followed by 10 μM SK&F 96365 (A) or 10 μM nifedipine (B) was added, as shown by the arrows. The experiments are representative of four similar ones.

were depleted with TG. Following the transient increase of $[Ca^{2+}]_i$, ionomycin (cells were permeabilized by ionomycin) was added, and I assessed intracellular Ca^{2+} store depletion by the inability to increase $[Ca^{2+}]_i$ (data not shown). The sustained $[Ca^{2+}]_i$ increase is dependent on the presence of extracellular Ca^{2+} and due to Ca^{2+} entry from the extracellular Ca^{2+} pool into the cytosol. In the absence of extracellular Ca^{2+}, addition of 2.4 mM $CaCl_2$ without TG pretreatment increased in $[Ca^{2+}]_i$ only slightly (Fig. 1B).

To determine whether the sustained $[Ca^{2+}]_i$ increase is induced by capacitative Ca^{2+} entry, I investigated the effects of the inhibitors on the TG-induced Ca^{2+} elevation in BA cells. SK&F 96365, an inhibitor of capacitative Ca^{2+} entry, inhibited the TG-induced Ca^{2+} elevation (Fig. 2A). However, 10 μM nifedipine, an inhibitor of voltage-operated Ca^{2+} channels (VOC), did not affect the TG-induced Ca^{2+} elevation (Fig. 2B). Moreover, SK&F 96365 but not nifedipine treatment before TG also attenuated the TG-induced Ca^{2+} elevation (data not shown).

Effect of phosphorylation on the TG-induced $[Ca^{2+}]_i$

To know the participation of protein kinases on the TG-induced Ca^{2+} entry, we examined the effects of protein kinase inhibitors on the TG-induced Ca^{2+} elevation in BA cells. Figure 3 shows the effects of a PKC agonist and antagonist on the TG-induced Ca^{2+} elevation. The cell were treated with calphostin C for 1 h before the addition of TG. Calphostin C (1 μM) inhibited the TG-induced Ca^{2+} elevation (Fig. 3A). The inhibitory effect of calphostin C (100 nM – 1 μM) was concentration-dependent (Fig. 3C). The approximate IC_{50} value of calphostin C on the TG-induced Ca^{2+} elevation was 0.65 ± 0.04 μM (mean ± S.E.M.). Furthermore, the pretreatment with PKC-stimulating PMA (100 nM) for 1 h increased the TG-induced Ca^{2+} elevation (Fig. 3B). Calphostin C and PMA did not affect the filling state of the store because the transient increase of $[Ca^{2+}]_i$ induced by TG (increase of ratio $= 1.12 \pm 0.28$) was unaffected by pretreatment with calphostin C and PMA (increase of ratio $= 1.48 \pm 0.45$ and 1.07 ± 0.35, respectively). H-89, a protein kinase A inhibitor; genistein, a phosphatase inhibitor; and trifluoperazine, a calmodulin inhibitor, had no effect on the TG-induced Ca^{2+} elevation (data not shown).

Effect of PKC on the TG-induced Mn^{2+} entry

To investigate whether the effect of PKC in modulating $[Ca^{2+}]_i$ results from Ca^{2+} entry across the plasma membrane, Ca^{2+} entry was assessed directly by adding Mn^{2+} to fura-2 loaded cells as a surrogate for Ca^{2+} influx (16). Quenching of the fluorescence by Mn^{2+} reflects Ca^{2+} influx. Figure 4 shows the effects of PKC activation or inhibition on the TG-induced Mn^{2+} entry. TG caused an increase in the rate of quenching of the fluorescence signal, Mn^{2+} entry. Pretreatment with PMA for 1 h before the addition of TG induced more acceleration of the Mn^{2+} entry observed on the addition of TG alone. In contrast, pretreatment of calphostin C for 1 h before the addition of TG reduced the Mn^{2+} entry. These findings suggest that PKC modulated Ca^{2+} influx across the plasma membrane induced by the Ca^{2+} store-depletion.

Effect of PKC on the TG-induced steroidogenesis

The increase of $[Ca^{2+}]_i$ evoked steroidogenesis in BA cells. TG induced the steroidogenesis in the presence of

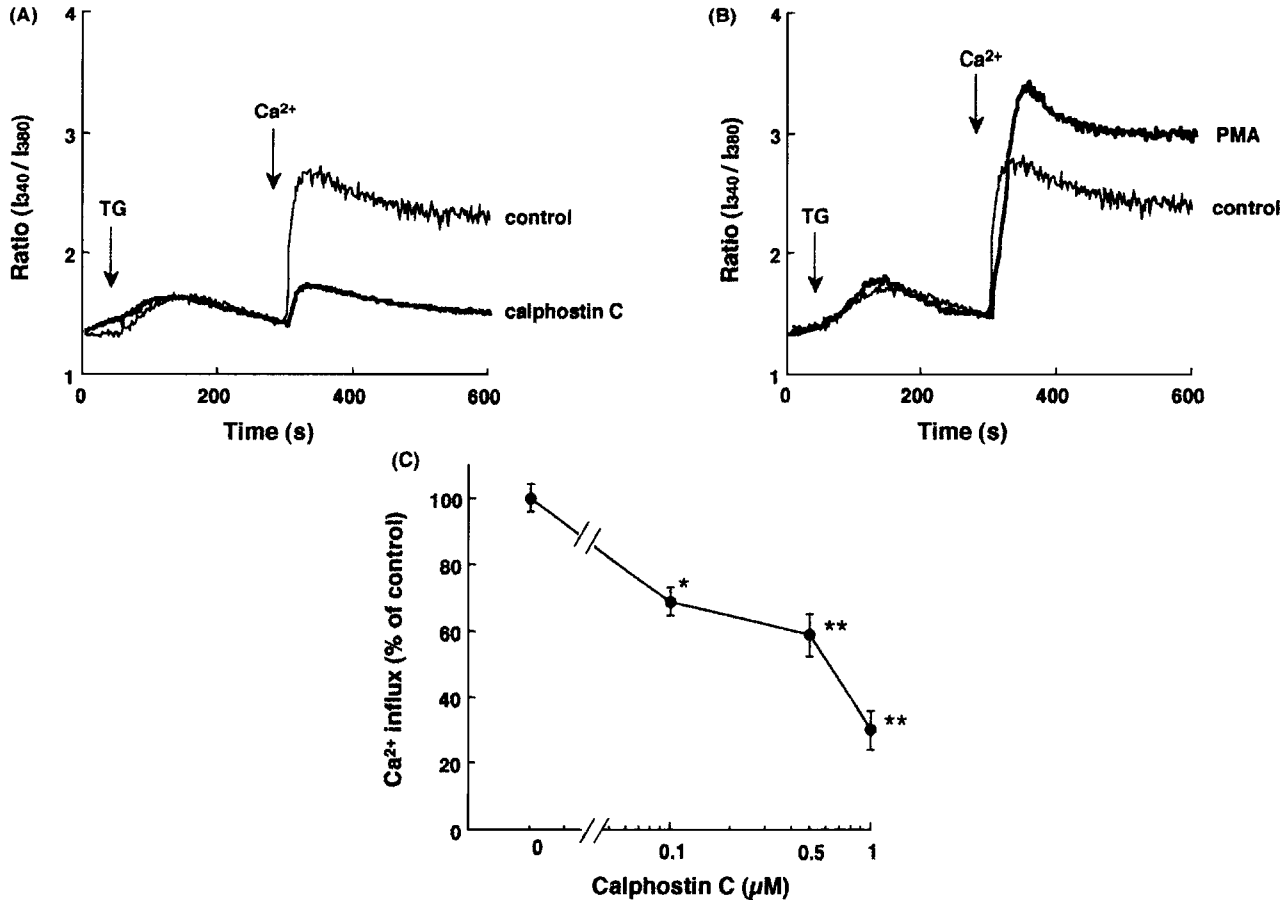

Fig. 3. Effects of calphostin C and PMA on the Ca^{2+} entry in thapsigargin (TG)-treated BA cells. In the presence of 2 μM TG, 2.4 mM Ca^{2+} was added to cells pretreated with no agent (control) or 1 μM calphostin C for 1 h (A), and no agent (control) or 100 μM PMA for 1 h (B). The experiments are representative of three similar ones. C: Dose-dependence of the effect of calphostin C on TG-induced Ca^{2+} entry. The magnitude of the maximal Ca^{2+} influx after 2.4 mM Ca^{2+} addition in the presence of 2 μM TG was taken as 100%. Values are means ± S.E.M. of four separate experiments. The Ca^{2+} influx is significantly different from the control, *P<0.05, **P<0.01.

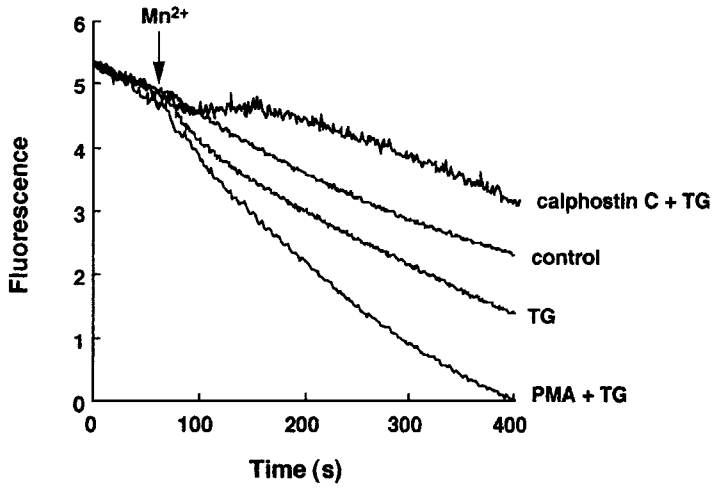

Fig. 4. Effects of the activation or inhibition of PKC on thapsigargin (TG)-induced manganese entry in BA cells. $MnCl_2$ (200 μM) was added to cells pretreated with no agent (control), 2 μM TG, 1 μM calphostin C + 2 μM TG or 100 nM PMA + 2 μM TG for 1 h (TG was added at 50 s before Mn^{2+} addition). Mn^{2+} entry was monitored from the quenching of fura-2 fluorescence excited at 360 nm. The experiments are representative of four similar ones.

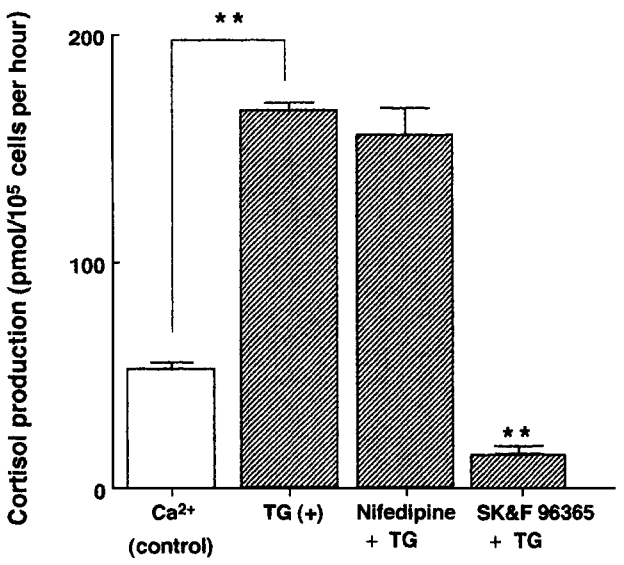

Fig. 5. Effects of nifedipine and SK&F 96365 on the thapsigargin (TG)-induced steroidogenesis in BA cells. BA cells were incubated with 1.2 mM $CaCl_2$ in the presence (▨) or absence (□) of 2 μM TG. In the presence of 2 μM TG, the cells were incubated with 1.2 mM $CaCl_2$ and 100 μM nifedipine or 10 μM SK&F 96365. Values are means ± S.E.M. of six separate experiments. The steroidogenesis is significantly different from the control, **P<0.01.

external Ca^{2+} (Fig. 5). In the absence of external Ca^{2+}, TG did not potentiate steroidogenesis (data not shown). Nifedipine had no effect on the TG-induced steroidogenesis. In contrast, SK&F 96365 inhibited the TG-induced steroidogenesis, suggesting that the TG-induced steroidogenesis results from the capacitative Ca^{2+} entry in BA cells.

Figure 6 shows the effects of calphostin C and PMA on the TG-induced steroidogenesis. The cells were pretreated with calphostin C and PMA for 1 h and washed out with Krebs-Ringer HEPES buffer, and then the cells were incubated with 1.2 mM Ca^{2+} in the presence of 2 μM TG for 1 h. Calphostin C inhibited the TG-induced steroidogenesis in a dose-dependent manner (Fig. 6A). The approximate IC_{50} value of calphostin C on TG-induced steroidogenesis was 0.48 ± 0.03 μM, which was similar to the IC_{50} value of calphostin C for inhibition of TG-induced Ca^{2+} elevation. Moreover, PMA stimulated the TG-induced steroidogenesis in a dose-dependent manner (Fig. 6B).

DISCUSSION

In BA cells, elevation of the cytosolic Ca^{2+} concentration induced by TG depends on the presence of extracellular Ca^{2+} and is not inhibited by a dihydropyridine antagonist but is inhibited by SK&F 96365. The Ca^{2+} entry is associated with increased influx of Mn^{2+}, suggesting the entry of Ca^{2+} across the plasma membrane into the cytosol. These results suggest that TG-induced elevation of the cytosolic Ca^{2+} depends on the filling state of the intracellular Ca^{2+} stores.

It was reported that VOC linked to steroidogenesis in

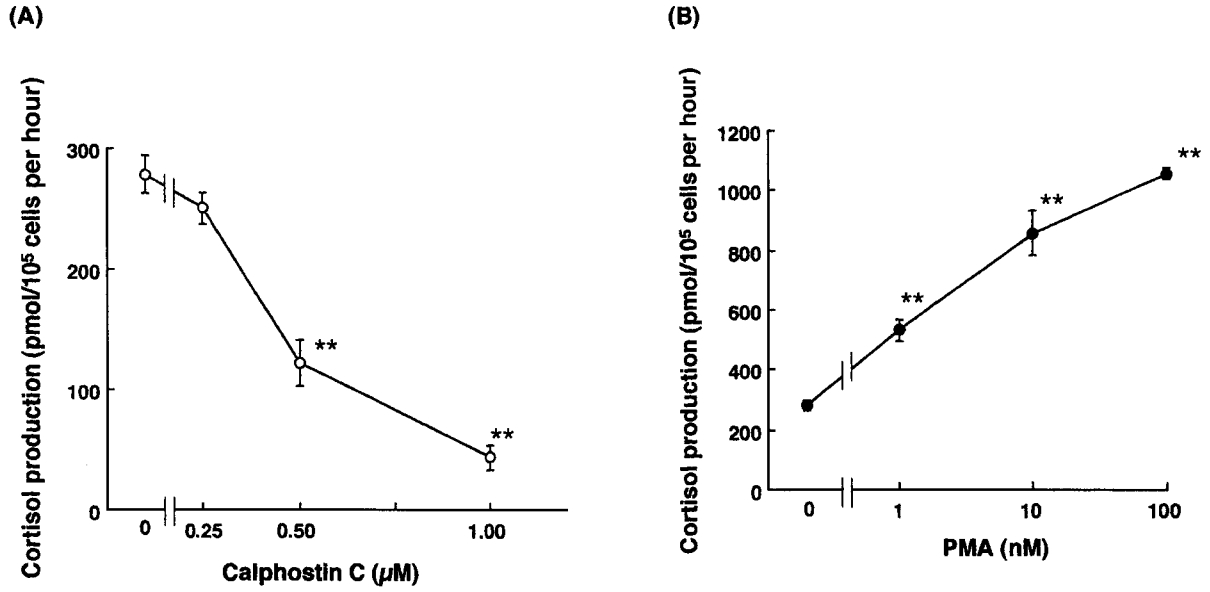

Fig. 6. Effects of calphostin C and PMA on the thapsigargin (TG)-induced steroidogenesis in BA cells. The cells were pretreated with various concentrations of calphostin C (A) or PMA (B) for 1 h. After washing the cells, they were incubated with Krebs-Ringer HEPES buffer containing 1.2 mM $CaCl_2$ in the presence of 2 μM TG for 1 h. Values are means ± S.E.M. of six separate experiments. The steroidogenesis is significantly different from the control, **P<0.01.

adrenocortical cells (18). In this study, I used the buffer that included a low concentration of K^+ (3 mM). Because high K^+ activated VOC by depolarization of BA cells, it caused increase of Ca^{2+} uptake and steroidogenesis (data not shown). I showed here that nifedipine had no effect on the TG-induced Ca^{2+} entry and steroidogenesis (Figs. 2 and 5). It is therefore likely that the TG-induced Ca^{2+} entry does not involve VOC, which is sensitive to dihydropyridines, in BA cells. It has been reported that many compounds inhibited capacitative Ca^{2+} entry (19, 20). One of the proposed inhibitors is SK&F 96365 that blocks Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) (21) and nonselective cation channels and may not be selective for capacitative Ca^{2+} entry. The lack of specific inhibitors is a major obstacle to the development of the field.

The role of PKC in regulating the capacitative Ca^{2+} entry was investigated with a PKC inhibitor, calphostin C, and a PKC-stimulating phorbol ester, PMA, in TG-treated BA cells. In this study, I have demonstrated that calphostin C suppressed the TG-induced Ca^{2+} entry, and PMA enhanced the Ca^{2+} entry. The sustained Ca^{2+} elevation mediated by TG in the presence of extracellular Ca^{2+} was regulated by PKC (Fig. 3). To check this finding, I investigated whether pretreatment with PMA down-regulated capacitative Ca^{2+} entry. Down-regulation of PKC was performed by preincubation with PMA for 48 h in BA cells. However, preexposure to PMA for 48 h did not abolish or decrease the Ca^{2+} entry (data not shown). Down-regulation is thought to be an indirect measurement of PKC activation. My results did not fit this model. Several novel PKC isoforms have been isolated in the past, and some of them, such as ε, β and δ, are more resistant to PMA-induced down-regulation than the PKCα isozyme (22). There are some reports that different PKC isozymes perform different functions (23). This suggests that the regulation of capacitative Ca^{2+} entry results from the activity of an isozyme resistant to PMA-induced down-regulation. Further study is necessary to elucidate the mechanisms through which PKC isozymes regulate capacitative Ca^{2+} entry in BA cells.

A possibile regulatory effect of PKC on TG-induced Ca^{2+} elevation may be suggested by the effect of calphostin C and PMA on the rate of efflux of Ca^{2+} in BA cells. Unlike Ca^{2+}, Mn^{2+} is not extruded from the cells, so it can be considered as a selective tracer for Ca^{2+} entry across a plasma membrane into the cytosol (24). Calphostin C and PMA also regulated TG-induced Mn^{2+} influx (Fig. 4). This would indicate that the regulation of the TG-induced $[Ca^{2+}]_i$ elevation was due to action on the influx of Ca^{2+} rather than on the rate of efflux. It would be conceivable that protein phosphorylation/dephosphorylation either directly or indirectly regulates the signal communicating the filling state of Ca^{2+} store to the capacitative Ca^{2+} channels across the plasma membrane into the cytosol.

Addition of TG alone induced an acceleration of Mn^{2+} entry. Pretreatment of PMA generated more stimulation of TG-induced Mn^{2+} entry. PMA alone had no effect on Mn^{2+} entry (data not shown). In contrast, pretreatment of calphostin C not only counteracted the TG-induced Mn^{2+} entry but reduced it to less than the control level. This may be due to the inhibition of basal Mn^{2+} entry by calphostin C, because calphostin C alone had a small inhibitory effect on Mn^{2+} entry (data not shown). This also suggested that capacitative Ca^{2+} entry may be activated in the control.

In the present study, my data suggested that the capacitative Ca^{2+} entry in BA cells was regulated by PKC. These results are supported by reports that PKC can activate the capacitative Ca^{2+} entry mechanism in RINm5F cells (9), and a similar modulating action of PKC can be observed in NG115-401L neuronal cells (11). In contrast, PKC activation inhibits capacitative Ca^{2+} entry in thyroid FRTL-5 cells (12), RBL-2H3 cells (13) and HL60 cells (25). Interestingly, in *Xenopus* oocytes, a dual role for PKC on the Ca^{2+} entry is demonstrated (26). PKC has disparate effects on the capacitative Ca^{2+} entry, and this seems to be cell type specific or dependent on the recording situation.

In recent reports, a link between capacitative Ca^{2+} entry and the cell functions is demonstrated. In bovine adrenal glomerulosa cells, angiotensin II potentiates adrenocorticotrophic hormone-induced cAMP formation through capacitative Ca^{2+} entry (27). In rat mesangial cells, the fact that high glucose inhibits capacitative Ca^{2+} entry via PKC contributes to the glomerular hemodynamic change (28). Functional coupling of secretion and capacitative Ca^{2+} entry was demonstrated in PC12 cells (29). In the present study, I demonstrated that the capacitative Ca^{2+} entry system which links to steroidogenesis exists in BA cells, and PKC may have a modulatory effect on the capacitative Ca^{2+} entry. TG stimulated steroidogenesis in the presence of extracellular Ca^{2+} in BA cells. As shown in Fig. 1A, TG produced the sustained rise in $[Ca^{2+}]_i$, it appeared to result in an increase of $[Ca^{2+}]_i$ followed by steroidogenesis in BA cells. Moreover, the TG-induced steroidogenesis was inhibited by SK&F 96365, similar to the effect of the inhibitor on the TG-induced sustained rise in $[Ca^{2+}]_i$ (Figs. 2 and 5).

It is reported that extracellular ATP stimulated steroidogenesis in BA cells (30). This is an appropriate system for protecting the body against shock. It is considered that ATP-induced steroidogenesis might have a close connection to intracellular Ca^{2+} mobilization. Niitsu reported that extracellular ATP stimulated steroidogenesis extracellular Ca^{2+}-dependently via dihydropyridine-insensitive Ca^{2+} channels (31). ATP induced a biphasic response of $[Ca^{2+}]_i$, the first phase being due to Ca^{2+} release from intracellular pools via an activator of $Ins(1,4,5)P_3$, and the

second phase reflecting a sustained Ca^{2+} entry (15). These reports suggest that ATP may relate to the activation of capacitative Ca^{2+} entry. Thus, capacitative Ca^{2+} entry in BA cells might be attributed to ATP-induced steroidogenesis. Further investigations on the connection between ATP and capacitative Ca^{2+} entry should be performed in BA cells.

I have demonstrated that calphostin C attenuated the TG-induced steroidogenesis concentration-dependently and PMA stimulated the steroidogenesis concentration-dependently in BA cells. The TG-induced steroidogenesis as well as the TG-induced Ca^{2+} entry was regulated by PKC, suggesting that the capacitative Ca^{2+} entry regulated via phosphorylation of a protein(s) by PKC functionally links to the secretory response of steroidogenesis in BA cells.

In conclusion, the present results suggest that a phosphorylation-dephosphorylation by PKC has a role in the regulation of capacitative Ca^{2+} entry. Furthermore, the capacitative Ca^{2+} entry links to steroidogenesis which is modulated by PKC in BA cells. Further research is necessary to understand the mechanisms and the molecular action of PKC in regulating Ca^{2+} signaling and steroidogenesis linked functionally in BA cells.

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