Modulatory effect of protein kinase C on thapsigargin-induced calcium entry in thyroid FRTL-5 cells

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The aim of the present study was to investigate the regulation of calcium influx in thyroid FRTL-5 cells. Stimulating Fura 2-loaded cells with thapsigargin rapidly increased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which then stabilized at a new elevated plateau level. The initial increase in $[Ca^{2+}]_i$ consisted mainly of the release of sequestered Ca^{2+}. The plateau phase was totally dependent on extracellular Ca^{2+}. The influx of Ca^{2+} was blocked by Ni^{2+} and was decreased in depolarized cells. The importance of protein kinase C in regulating influx of Ca^{2+} was then evaluated. Addition of the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate prior to thapsigargin significantly decreased the influx of extracellular Ca^{2+}. Studies with bisoxonol to measure membrane potential showed that TPA depolarized the plasma membrane in FRTL-5 cells. In cells where protein kinase C was downregulated or was inhibited by staurosporine, the thapsigargin-induced influx of Ca^{2+} was enhanced. The results indicate that emptying intracellular Ca^{2+} pools is sufficient to induce influx of Ca^{2+} in FRTL-5 cells, and that protein kinase C has a modulatory effect on this process.

INTRODUCTION

In electrically non-excitable cells, changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) may be mediated via two distinct pathways. One pathway is mediated by agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and the release of sequestered intracellular Ca^{2+} [1]. The other pathway involves influx of extracellular Ca^{2+} mediated by activation of putative receptor-operated Ca^{2+} channels or second-messenger-operated Ca^{2+} channels in the plasma membrane [2–5]. Recently much attention has been focused on the relationship between agonist-induced release of sequestered Ca^{2+} and the influx of extracellular Ca^{2+}. Substantial evidence indicates that as a result of the release of sequestered Ca^{2+}, influx of extracellular Ca^{2+} is activated, followed by refilling of the intracellular Ca^{2+} stores [4,6]. Furthermore, the refill status of the intracellular Ca^{2+} store *per se* may be sufficient to determine membrane Ca^{2+} permeability [7,8].

Investigations in our laboratory with thyroid follicular FRTL-5 cells have shown that stimulation of these cells with the purinergic agonist ATP induces a rapid release of sequestered Ca^{2+}, followed by an immediate influx of extracellular Ca^{2+} [9–11]. The release of sequestered Ca^{2+} is mediated by stimulation of the IP_3 pathway [12]. The mechanism for the induced influx of extracellular Ca^{2+} is, however, not known at present. In the present study in FRTL-5 cells, the interrelationships between emptying of intracellular Ca^{2+} pools and influx of extracellular Ca^{2+} were investigated. We show that release of sequestered Ca^{2+} using thapsigargin, a sesquiterpene lactone tumour promotor, is accompanied by a substantial influx of extracellular Ca^{2+}. The results suggest that emptying intracellular Ca^{2+} pools in FRTL-5 cells is sufficient to activate influx of extracellular Ca^{2+}. Furthermore, protein kinase C (PKC) has a regulatory function on influx of calcium in FRTL-5 cells.

Part of this study has been previously presented in abstract form [13].

MATERIALS AND METHODS

Materials

Culture medium, serum and hormones for cell culture were purchased from Gibco (Grand Island, NY, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). Culture dishes were obtained from Falcon Plastics (Oxnard, CA, U.S.A.). ATP, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and staurosporine were purchased from Sigma. Fura 2-AM and bisoxonol were purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.), and thapsigargin was from LC Services Corp. (Woburn, MA, U.S.A.). All other chemicals used were of reagent grade.

Cell culture

Rat thyroid FRTL-5 cells were a gift from Dr Leonard D. Kohn (NIH, Bethesda, MD, U.S.A.). The cells were grown in Coon's modified Ham's F 12 medium, supplemented with 5% calf serum and six hormones (insulin, 10 μg/ml; transferrin, 5 μg/ml; cortisol, 10 nM; the tripeptide Gly-L-His-L-Lys, 10 ng/ml; thyrotropin, 1 munit/ml; somatostatin, 10 ng/ml) [14]. The cells were grown in a water-saturated atmosphere of 5% CO_2 and 95% air at 37 °C. Before an experiment, cells from one donor culture dish were harvested with a 0.25% trypsin solution and plated on to 100 mm culture dishes. The cells were grown for 7–8 days before an experiment, with two to three changes of the culture medium. Fresh medium was always added 24 h prior to an experiment.

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

The cells were washed once with buffered saline solution (BSS) containing 0.02% EDTA and no $CaCl_2$. The cells were then harvested with BSS containing 0.02% EDTA and 0.1% trypsin. After washing the cells three times by pelleting (centrifugation at 1300 rev./min and resuspension in BSS), the cells were incubated with 1 μM Fura 2-AM for 35 min at 37 °C. Following the

Abbreviations used: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; BSS, buffered saline solution (NaCl, 118 mM, KCl, 4.6 mM; glucose, 10 mM; $CaCl_2$, 1.0 mM; Hepes, 20 mM; pH 7.2).

loading period, the cells were washed twice with BSS buffer, incubated for at least 10 min at room temperature and washed once again. The cells were added to a quartz cuvette, kept at 37 °C and stirred throughout the experiment. Fluorescence was measured with a Hitachi F2000 fluorimeter. The excitation wavelengths were 340 nm and 380 nm and emission was measured at 510 nm. The signal was calibrated by addition of 1 mM $CaCl_2$ and digitonin to obtain $F_{max.}$ and by chelating extracellular Ca^{2+} with 5 mM EGTA. The addition of Tris base was used to elevate the pH above 8.3, to obtain $F_{min.}$. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. [15], using a computer program designed for the fluorimeter, with a K_d value of 224 nM for Fura 2.

Measurement of membrane potential

The cells were grown and harvested as for the $[Ca^{2+}]_i$ experiments. After the final wash the cells were added to a quartz cuvette, 100 nM bisoxonol (final concentration) was added and the cells allowed to stabilize for at least 10 min prior to an experiment. An excitation wavelength of 540 nm was used and emission was measured at 580 nm [16]. Each experiment was calibrated by addition of 50 mM K^+, to make comparison between different experiments possible.

Statistics

The results are expressed as the mean ± S.E.M. The Figures show representative traces of each experiment. Statistical analysis was made using Student's *t*-test for paired observations. When three or more means were tested, analysis of variance was used.

RESULTS

Effect of activation of PKC on thapsigargin-induced influx of extracellular Ca^{2+} in FRTL-5 cells

As in several other cell types, thapsigargin depletes intracellular calcium stores and activates influx of extracellular calcium in FRTL-5 cells (Table 1). The influx is dependent on extracellular calcium and the membrane potential and is blocked by Ni^{2+}. Thus the thapsigargin-induced calcium entry is very similar to that induced by ATP in these cells [10,11]. Activators of PKC may either enhance or decrease Ca^{2+} fluxes in cells [17,18]. Addition of 200 nM TPA to cells stimulated with thapsigargin decreased the final steady-state level of $[Ca^{2+}]_i$ by 42 ± 7 nM (Figure 1). Addition of the inactive phorbol ester 4α-phorbol had no effect on $[Ca^{2+}]_i$ (Figure 1). When TPA was added to the cell suspension prior to the addition of thapsigargin, the initial transient increase in $[Ca^{2+}]_i$ was 136 ± 16 nM compared with 181 ± 10 nM in control cells ($P < 0.05$). Furthermore, the plateau phase was only 55 ± 11 nM, compared with 130 ± 14 nM in control cells ($P < 0.05$, Figure 1). Addition of 4α-phorbol had no effect on the thapsigargin-induced increase in $[Ca^{2+}]_i$ (Figure 1). The effect of TPA on the thapsigargin-induced changes in $[Ca^{2+}]_i$ was not the result of increased efflux of Ca^{2+}, as no difference in thapsigargin-induced efflux of $^{45}Ca^{2+}$ was seen between cells treated with TPA and control cells (results not shown). The results thus suggest that activation of PKC decreases influx of extracellular Ca^{2+} in FRTL-5 cells.

To evaluate further whether PKC may modulate Ca^{2+} fluxes, PKC was downregulated by incubating the cells for 24 h with 200 nM TPA [19]. Stimulating these cells with thapsigargin enhanced both the initial increase in $[Ca^{2+}]_i$ (230 ± 13 nM; $P < 0.05$) and the plateau level of $[Ca^{2+}]_i$ (187 ± 12 nM; $P < 0.05$), compared with control cells (Figure 2). Furthermore, if 100 μM of the PKC inhibitor staurosporine was added to the cell suspension prior to thapsigargin, both the initial increase in $[Ca^{2+}]_i$ (221 ± 9 nM) and the plateau phase (195 ± 4 nM) were increased ($P < 0.05$) compared with control cells (Figure 2). The enhanced increase in $[Ca^{2+}]_i$ appeared to be due to an enhanced influx of extracellular Ca^{2+}, as no significant difference in the thapsigargin-induced increase in $[Ca^{2+}]_i$ was observed between PKC-downregulated cells (139 ± 8 nM), cells pretreated with staurosporine (129 ± 10 nM) and control cells (144 ± 16 nM) when the experiments were repeated in a Ca^{2+}-free buffer (not shown). In addition, TPA had no effect on the thapsigargin-induced increase in $[Ca^{2+}]_i$ (117 ± 15 nM) in cells in a Ca^{2+}-free buffer. Downregulation of PKC had no effect on the thapsigargin-induced efflux of $^{45}Ca^{2+}$ in FRTL-5 cells (results not shown). The data thus suggest that activation of PKC is of importance in regulating membrane permeability to calcium.

An interesting observation was that, in the presence of staurosporine, TPA was still able to blunt the thapsigargin-

Table 1 Characterization of the thapsigargin-induced $[Ca^{2+}]_i$ response in FRTL-5 cells

Before an experiment the cells were harvested and loaded with Fura 2 as described in the Materials and methods section. The cells were then resuspended in Ca^{2+}-containing buffer (control), in the presence of Ni^{2+} (1 mM final concentration), in the presence of a depolarizing buffer (55 mM K^+) or in a nominally Ca^{2+}-free buffer containing 100 μM EGTA. The cells were then stimulated with 2 μM thapsigargin and the increase in $[Ca^{2+}]_i$ was measured. Each increase gives the mean ± S.E.M. of five to twelve determinations. – Denotes that no response was observed. * Significantly different ($P < 0.05$) from control.

	Increase in $[Ca^{2+}]_i$ (nM)	
Treatment	Initial increase	Plateau phase
Control	167 ± 7	127 ± 3
Ni^{2+} (1 mM)	157 ± 6	–
K^+ (55 mM)	127 ± 7*	85 ± 4*
Ca^{2+}-free	119 ± 6*	–

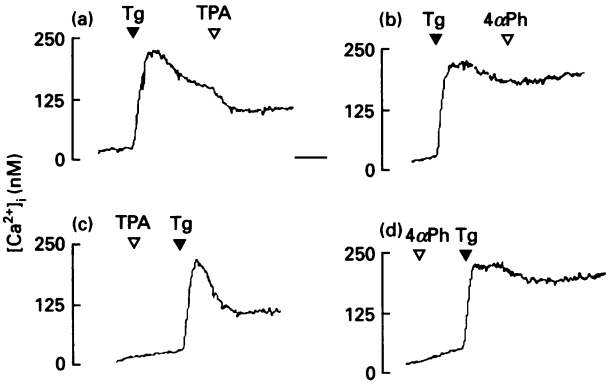

Figure 1 Effect of TPA on thapsigargin-induced changes in $[Ca^{2+}]_i$

The cells were harvested and loaded with Fura 2 as described in Materials and methods. (**a**) Addition of 200 nM TPA to cells stimulated with 2 μM thapsigargin. (**b**) Addition of 200 nM 4α-phorbol to cells stimulated with 2 μM thapsigargin. (**c**) Addition of 200 nM TPA prior to stimulating cells with 2 μM thapsigargin. (**d**) Addition of 200 nM 4α-phorbol prior to stimulating cells with 2 μM thapsigargin. The horizontal bar denotes 1 min. Abbreviation used: Tg: thapsigargin; 4αPh, 4α-phorbol.

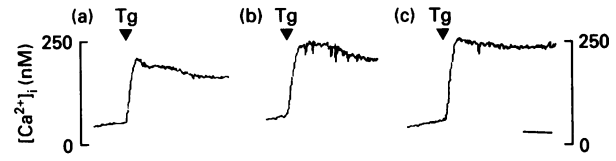

Figure 2 Effect of inhibiting protein kinase C on thapsigargin-induced changes in $[Ca^{2+}]_i$

The cells were harvested and loaded with Fura 2 as described in Materials and methods. The cells were stimulated with 2 μM thapsigargin (Tg) (**a**), after down-regulating protein kinase C with 200 nM TPA for 24 h (**b**) or after incubating the cells with 100 μM staurosporine for 2 min (**c**). The horizontal bar denotes 1 min.

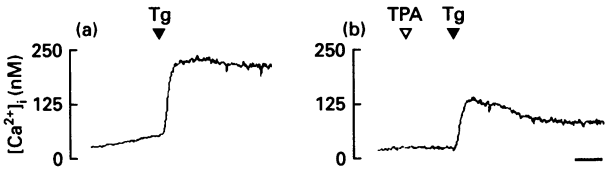

Figure 3 Inhibitory effect of TPA on thapsigargin-induced changes in $[Ca^{2+}]_i$ in FRTL-5 cells incubated with staurosporine

The cells were harvested and loaded with Fura 2 as described in Materials and methods. The cells were incubated with 100 μM staurosporine for 2 min and then stimulated with 2 μM thapsigargin (Tg) (**a**). In (**b**) the cells were incubated with staurosporine and then 200 nM TPA was added prior to stimulating the cells with 2 μM thapsigargin. The horizontal bar denotes 1 min.

induced response in $[Ca^{2+}]_i$: the initial increase in $[Ca^{2+}]_i$ was 133 ± 19 nM ($P < 0.05$) and the plateau level was 71 ± 14 nM ($P < 0.05$) (Figure 3).

In the next series of experiments, the cells were stimulated with thapsigargin in a Ca^{2+}-free buffer and Ca^{2+} was added after the termination of the thapsigargin response. This resulted in a rapid increase in $[Ca^{2+}]_i$ which was dependent on membrane potential and influx of extracellular calcium (Table 2). If 200 nM TPA was

Table 2 Effect of re-addition of Ca^{2+} on $[Ca^{2+}]_i$ in FRTL-5 cells stimulated with thapsigargin in Ca^{2+}-free buffer

Before an experiment the cells were harvested and loaded with Fura 2 as described in Materials and methods. The cells were then resuspended in a nominally Ca^{2+}-free buffer containing 100 μM EGTA (control), in the presence of Ni^{2+} (1 mM final concentration) or in the presence of a depolarizing buffer (55 mM K^+). The cells were then stimulated with 2 μM thapsigargin. After 3 min, Ca^{2+} (1 mM final concentration) was added and the increase in $[Ca^{2+}]_i$ was measured. Each value is the mean $\pm$ S.E.M. of five to eight determinations. * Significantly different ($P < 0.05$) from control.

	Increase in $[Ca^{2+}]_i$ (nM)	
Treatment	Initial increase	Plateau phase
Control	362 ± 9	204 ± 10
Ni^{2+} (1 mM)	47 ± 11*	47 ± 11*
K^+ (55 mM)	221 ± 7*	150 ± 13*

added prior to calcium, the Ca^{2+}-induced transient increase in $[Ca^{2+}]_i$ (261 ± 50 nM) was no different from that in control cells (382 ± 30 nM). However, the plateau in $[Ca^{2+}]_i$ was decreased (117 ± 21 nM, $P < 0.05$) compared with control cells (222 ± 20 nM) (Figure 4). If PKC was downregulated with 200 nM TPA for 24 h, or if 100 μM staurosporine was added to the cell suspension, addition of Ca^{2+} resulted in an enhanced ($P < 0.05$) transient increase in $[Ca^{2+}]_i$ (566 ± 55 nM and 607 ± 20 nM) and an elevated ($P < 0.05$) plateau level of $[Ca^{2+}]_i$ (330 ± 21 and 359 ± 14 nM) compared with control cells (Figure 4). An interesting observation was that addition of TPA to PKC-downregulated cells prior to calcium inhibited ($P < 0.05$) the enhanced transient increase in $[Ca^{2+}]_i$ (329 ± 68 nM) and plateau (176 ± 36 nM) level of $[Ca^{2+}]_i$ (Figure 4). However, the decrease was not of the same magnitude as that seen in control cells (compare Figure 4f with Figure 4b). Taken together, the results indicate further that activation of PKC is important in regulating influx of extracellular Ca^{2+} in FRTL-5 cells and that some

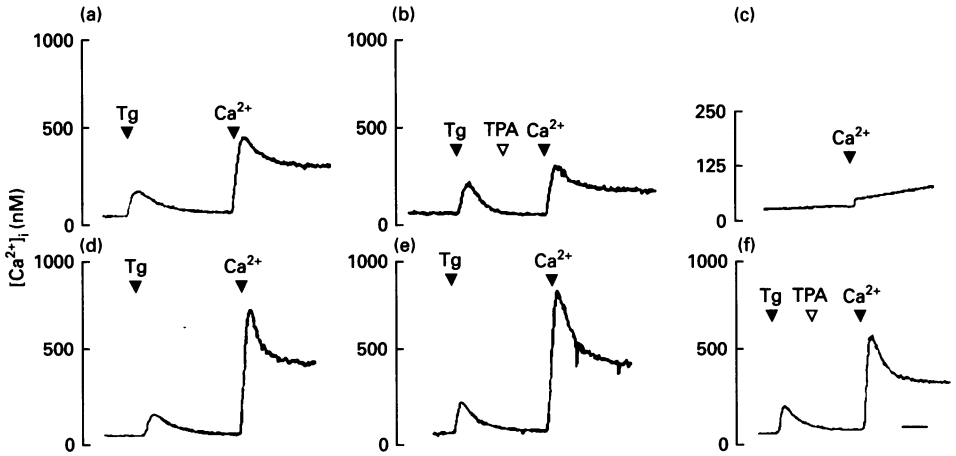

Figure 4 Modulatory effect of PKC on influx of Ca^{2+} in cells stimulated with thapsigargin in a Ca^{2+}-free buffer

The cells were harvested and loaded with Fura 2 as described in Materials and methods and then suspended in a nominally Ca^{2+}-free buffer containing 100 μM EGTA. In (**a**) the cells were stimulated with 2 μM thapsigargin (Tg), 1 mM $CaCl_2$ (final concentration) was added and the increase in $[Ca^{2+}]_i$ was measured. In (**b**) 200 nM TPA was added prior to $CaCl_2$. (**c**) shows the effect of adding Ca^{2+} only to cells in Ca^{2+}-free buffer containing 100 μM EGTA. (**d**) and (**e**) show the effect of adding Ca^{2+} to cells pretreated with 100 μM staurosporine, or cells pretreated with TPA for 24 h respectively. In (**f**) the cells were treated with 200 nM TPA for 24 h, and prior to adding Ca^{2+}, 200 nM TPA was added to the cell suspension. The horizontal bar denotes 1 min.

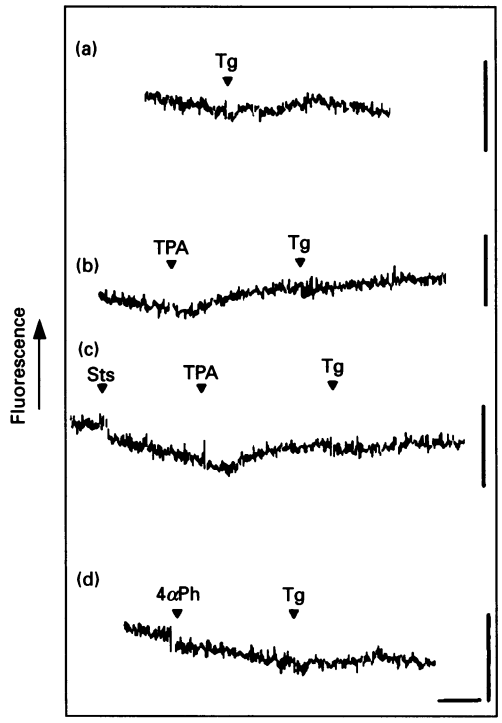

Figure 5 Action of thapsigargin and TPA on membrane potential in FRTL-5 cells

The cells were harvested and loaded with bisoxonol as described in Materials and methods. The cells were stimulated with 2 μM thapsigargin (Tg: **a**), 200 nM TPA and thapsigargin (**b**), 100 μM staurosporine (Sts), TPA and thapsigargin (**c**) or 200 nM 4α-phorbol (4αPh) and thapsigargin (**d**). The vertical bars denote the response to 50 mM K^+ and the horizontal bar denotes 1 min.

isoforms of PKC are insensitive to downregulation by long incubations with TPA.

Action of TPA on membrane potential in FRTL-5 cells

Activation of PKC may depolarize cells [20,21] and influx of calcium is decreased in depolarized FRTL-5 cells (see above) [11]. The results in Figure 5(a) show that addition of thapsigargin had almost no effect on membrane potential in FRTL-5 cells. However, addition of 200 nM TPA depolarized the cells (Figure 5b). Furthermore, the depolarizing effect of TPA was not inhibited by pretreatment of the cells with staurosporine (Figure 5c), nor was it inhibited in cells with downregulated PKC (not shown). Addition of 4α-phorbol had no effect on membrane potential (Figure 5d).

DISCUSSION

Recent investigations in non-excitatory cells have shown that agonist-induced changes in $[Ca^{2+}]_i$ are mediated via activation of two distinct pathways: release of sequestered intracellular Ca^{2+} and activation of Ca^{2+} entry. Extensive evidence suggests that the agonist-induced release of sequestered Ca^{2+} is due to hydrolysis of PIP_2 by phospholipase C to IP_3 [1]. However, the mechanism of activation of influx of extracellular Ca^{2+} is poorly understood at present and has been subject to extensive investigations. It has been suggested that the agonist-induced emptying of Ca^{2+} pools may be the signal for activating Ca^{2+} entry [7,8,22,23].

Several investigations have shown that ATP stimulates the release of sequestered Ca^{2+} and the influx of extracellular Ca^{2+} in rat thyroid FRTL-5 cells [9–12,24]. Although ATP has been shown to stimulate the release of sequestered Ca^{2+} via activation of the IP_3 pathway [12], the mechanism activating Ca^{2+} entry is not known. The results in the present investigation suggest that the emptying of intracellular Ca^{2+} pools *per se*, without activating membrane-bound receptors and the concomitant activation of signal-transduction pathways, is sufficient to activate Ca^{2+} entry in FRTL-5 cells.

The response to thapsigargin appeared to consist of two phases: an initial fairly rapid increase in $[Ca^{2+}]_i$, primarily due to the emptying of intracellular Ca^{2+} pools, was probably mediated via inhibition of Ca^{2+} ATPase [25]. Part of the initial increase was also dependent on influx of extracellular Ca^{2+}, as the response was decreased in Ca^{2+}-free buffer and in depolarized cells. The second sustained phase consisted entirely of influx of Ca^{2+}, and showed the characteristic properties associated with agonist-induced Ca^{2+} entry: dependence on extracellular Ca^{2+}, inhibition in the presence of the non-specific cation-channel-blocker Ni^{2+} and dependence on membrane potential. The responses are very similar to those observed when the cells were stimulated with ATP [9–11].

The results in the present report also indicate an important function for PKC in calcium fluxes in FRTL-5 cells. Recent results in excitable cells, such as pituitary GH_4C_1 cells [26,27], smooth muscle cells [28], and PC12 cells [29], show that activators of PKC have an inhibitory effect on voltage-dependent calcium channels. In other cells, such as neutrophils [30] and platelets [31], activation of PKC attenuates agonist-induced changes in $[Ca^{2+}]_i$ by inhibiting influx and enhancing efflux of calcium. Furthermore, in a report by Hockberger et al., a diacylglycerol analogue was shown to inhibit calcium currents in dorsal root ganglion independent of activation of PKC [32].

In FRTL-5 cells, activation of PKC has an inhibitory effect on influx of extracellular calcium. TPA had no effect on thapsigargin-induced efflux of $^{45}Ca^{2+}$, nor on the $[Ca^{2+}]_i$ response to thapsigargin in Ca^{2+}-free buffer. Thus the effect of TPA was not due to enhanced efflux of calcium. Furthermore, none of activation of PKC with TPA, inactivation of PKC with staurosporine and downregulation of PKC had any effect on the magnitude of the thapsigargin-induced change in $[Ca^{2+}]_i$ in a calcium-free buffer. Thus activating or inhibiting PKC had no effect on the amount of sequestered calcium. However, the mechanism by which PKC can modulate calcium entry still remains obscure.

One possible mechanism of action of TPA is through depolarization of the membrane potential. Our results show that calcium influx is dependent on membrane potential and that addition of TPA depolarized the cells. A similar effect of TPA has been observed in some cells [20,21] but not in other cells [33]. We did not investigate the mechanism through which addition of TPA and activation of PKC depolarized the cells. The depolarizing effect may occur as a result of closure of K^+ channels [20,21]. Interestingly, in the presence of staurosporine or in PKC-downregulated cells, addition of TPA induced a depolarization of the membrane potential. In experiments of similar design TPA decreased the thapsigargin-induced increase in $[Ca^{2+}]_i$. The results suggest that some isoforms of PKC are insensitive to staurosporine and to downregulation by TPA. A direct calcium-channel-blocking effect of TPA (see [32]) cannot be totally excluded. Taken as a whole, the results obtained in the present study suggest that the emptying of intracellular Ca^{2+} pools is sufficient to activate entry of extracellular Ca^{2+} in FRTL-5 cells due to 'capacitative' influx of calcium, and that PKC has a modulatory effect on this process.

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