0026-895X/96/061104-09$3.00/0

MOLECULAR PHARMACOLOGY, 49:1104–1112 (1996).

Thapsigargin Modulates Agonist-Stimulated Cyclic AMP Responses through Cytosolic Calcium-Dependent and -Independent Mechanisms in Rat Pinealocytes

ANTHONY K. HO, TAKAYUKI OGIWARA, and CONSTANCE L. CHIK

Departments of Physiology (A.K.H., T.O.) and Medicine (C.L.C.), Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received November 22, 1995; Accepted March 4, 1996

SUMMARY

The role of mobilization of intracellular Ca^{2+} in the adrenergic-stimulated cAMP accumulation in rat pinealocytes was investigated with thapsigargin, an agent that inhibits endoplasmic reticulum Ca^{2+}-ATPase. It was found that although thapsigargin alone had no effect on the basal cAMP accumulation, it potentiated the β-adrenergic-stimulated cAMP response by isoproterenol in a dose-dependent manner. The potentiation was abolished with ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester (EGTA-AM) but persisted in the presence of isobutylmethylxanthine, indicating that thapsigargin enhances cAMP synthesis through elevation of cytosolic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, when the pinealocytes were stimulated by norepinephrine, a mixed α_1- and β-adrenergic agonist, thapsigargin dose-dependently inhibited the cAMP response. To investigate this inhibitory effect of thapsigargin, we substituted ionomycin, a $[Ca^{2+}]_i$-elevating agent, and 4β-phorbol-12-myristate 13-acetate, an activator of protein kinase C, for the α_1-adrenergic component of the norepinephrine-stimulated response. Although thapsigargin had no effect on the potentiation of the isoproterenol-stimulated cAMP accumulation by ionomycin, it significantly inhibited the potentiation by 4β-phorbol-12-myristate 13-acetate. Furthermore, the inhibitory effect of thapsigargin was not affected by cotreatment with EGTA-AM or ionomycin, suggesting that this effect is independent of $[Ca^{2+}]_i$. Similar results were obtained when cyclopiazonic acid was used to inhibit the Ca^{2+}-ATPase. Taken together, our results indicate that thapsigargin enhances the β-adrenergic-stimulated cAMP accumulation through its action in elevating $[Ca^{2+}]_i$ but inhibits the potentiation of the β-adrenergic-stimulated cAMP response by protein kinase C, as a consequence of Ca^{2+}-ATPase inhibition.

The production of cAMP in rat pinealocytes is regulated by NE acting through synergistic dual-receptor mechanisms involving both α_1- and β-adrenoceptors (1–3). Activation of the β-adrenoceptor by itself produces a 7–10-fold increase in the accumulation of cAMP. Selective activation of the α_1-adrenoceptor alone, although having no effect on its own, potentiates the β-adrenergic-stimulated cAMP response. The α_1-adrenergic-mediated potentiation has been shown to involve elevation of $[Ca^{2+}]_i$ (4, 5) and activation of PKC (6, 7). This is based on the findings that α_1-adrenergic activation elevates $[Ca^{2+}]_i$ (5), which in turn causes translocation of PKC (6). Both experimental elevation of $[Ca^{2+}]_i$ (8) and activation of PKC (7) potentiate the β-adrenergic-stimulated cAMP response. However, in the case of elevated $[Ca^{2+}]_i$, the Ca^{2+}/calmodulin-dependent kinase has also been shown to be involved (9). This is based on the finding that although inhibitors of Ca^{2+}/calmodulin-dependent kinase have no effect on the potentiation by an activator of PKC, they significantly inhibit the potentiation by $[Ca^{2+}]_i$-elevating agents (9). Thus, it appears that $[Ca^{2+}]_i$ plays a central role in the regulation of cAMP in rat pinealocytes.

Thapsigargin, a selective inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (10), has been used to differentiate between Ca^{2+}-dependent and -independent consequences of agonist stimulation of phospholipase C (10–12). The acute effect of thapsigargin on intact cells occurs through elevation of $[Ca^{2+}]_i$ without formation of inositol phosphates (11, 12). Although thapsigargin is a tumor promoter, unlike phorbol esters it does not directly bind to or activate PKC (13). Therefore, through its ability to increase $[Ca^{2+}]_i$ by a mechanism that is not dependent on activation of phospholipase C, thapsigargin has been used extensively to investigate the role of $[Ca^{2+}]_i$ in Ca^{2+}-regulated physiological processes.

A.K.H. is a scholar and C.L.C. is a clinical investigator of the Alberta Heritage Foundation of Medical Research (AHFMR). This work was supported by a grant from the Medical Research Council of Canada.

ABBREVIATIONS: NE, norepinephrine; $[Ca^{2+}]_i$, cytosolic calcium concentration; PKC, protein kinase C; ISO, isoproterenol; IBMX, isobutylmethylxanthine; PMA, 4β-phorbol-12-myristate-13-acetate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; AM, acetoxymethyl ester.

In the current study, we used thapsigargin to characterize the specific role of $[Ca^{2+}]_i$ in the adrenergic regulation of cAMP in rat pinealocytes by comparing its effect with those of other $[Ca^{2+}]_i$-elevating agents. We found that although thapsigargin, like other $[Ca^{2+}]_i$-elevating agents, enhanced the β-adrenergic-stimulated cAMP response, it inhibited the PKC-mediated potentiation of the β-adrenergic-stimulated cAMP response. The inhibitory effect of thapsigargin was shared by another inhibitor of Ca^{2+}-ATPase, cyclopiazonic acid, but not by other $[Ca^{2+}]_i$-elevating agents.

Experimental Procedures

Materials. NE, ISO, IBMX, cyclopiazonic acid, PMA, phosphatidylserine, and W7 were obtained from Sigma Chemical Co. (St. Louis, MO). Thapsigargin and ionomycin were purchased from Calbiochem (San Diego, CA). Fura-2 and the acetoxymethyl esters of Fura-2 and of EGTA were purchased from Molecular Probes (Eugene, OR). The PKC substrate peptide Ac-MBP(4–14) was obtained from GIBCO-BRL (Gaithersburg, MD). DMEM was purchased from Biofluids (Rockville, MD). [^{125}I]cAMP and [γ^{32}P]ATP were obtained from ICN (Costa Mesa, CA). All other chemicals were of the purest grades available and were obtained commercially. Antibody for the radioimmunoassay of cAMP was a gift from Dr. A. Baukal (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD).

Preparation and treatment of rat pinealocytes. Pinealocytes were prepared from male Sprague-Dawley rats (150 g; University of Alberta Animal Unit, Edmonton, Alberta, Canada) through trypsinization as described previously (14–16). The cells were suspended in DMEM containing 10% fetal calf serum and maintained at 37° for 24 hr in a gas mixture of 95% air/5% CO_2 before use.

Aliquots of cells (2×10^4 cells/0.4 ml) were treated with drugs that had been prepared in 200× concentrated solutions in water or dimethylsulfoxide. The final concentration of the latter never exceeded 0.5%. The duration of the drug treatment period was 15 min for cAMP determination. At the end of the treatment period, cells were collected through centrifugation (2 min at 10,000 × *g*), and the supernatant was aspirated. The cell pellets were immediately frozen by being placed on solid CO_2 and lysed by the addition of 5 mM acetic acid (100 μl). The lysates were then boiled for 5 min and stored frozen at −20° until analysis.

For the determination of the PKC translocation, aliquots of cells (2×10^5 cells/0.5 ml) were treated with drugs for 6 min, and the cells were collected through centrifugation. To separate the membrane and cytosol fractions, the pinealocytes were permeabilized through resuspension and incubation (7 min, 4°) in 100 μl of 50 μM digitonin in buffer A (20 mM Tris·HCl containing 0.5 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin and aprotinin, pH 7.5) as described previously (6, 17). The samples were then centrifuged (1 min at 12,000 × *g*) to separate the cytosol from the membrane fraction. The membrane fraction was solubilized in 0.1% Triton-X (in buffer A) before assay. The presence of EGTA and EDTA ensured that only the chelator-stable form of membrane-associated PKC was analyzed.

cAMP assay. The lysates from the cell pellets were centrifuged (10 min at 12,000 × *g*), and samples of the supernatant were used to estimate cellular cAMP content according to a radioimmunoassay procedure in which samples were acetylated before analysis (18–20). Because there was a small batch-to-batch variation in the cyclic nucleotide responses between cell preparations, all comparisons were performed within the same batch of cells.

Determination of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined with the use of a fluorescent Ca^{2+} indicator, Fura-2 (21, 22). Briefly, 5×10^5 cells were pelleted and resuspended in culture medium (DMEM with 25 mM HEPES, pH 7.4). The cells were loaded with Fura-2 through incubating with 5 μM Fura-2/AM for 45 min at 37°. After being washed twice with DMEM, the pinealocytes were suspended in a fresh buffered salt solution (buffer B: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, 6 mM glucose, pH 7.4). In some experiments, a low Ca^{2+} medium was used, which was identical in composition to buffer B with the exception that it contained only 0.1 mM $CaCl_2$. Fura-2-loaded cells (3×10^5 cells/1.5 ml) were transferred to a cuvette for fluorescence signal determination with the use of a SLM Aminco DMX-1000 fluorescence spectrophotometer with a thermostatically controlled cell holder fitted with a magnetic stirrer. The excitation wavelengths that were used were 340 and 380 nm, and the emission was monitored at 510 nm. Free Ca^{2+} concentration was calculated according to the equation established by Poenie *et al.* (23): $[Ca^{2+}]_i = K^d \times F_o/F_s \times (R - R_o)/(R_s - R)$, where K_d is the dissociation constant of Fura-2/Ca^{2+} complex (225 nM); F_o and F_s are the fluorescence intensities at 380 nm for free (*o*) and Ca^{2+}-saturated (*s*) dye; and R, R_o, and R_s are the ratios of the dye fluorescence intensities at 340 nm and 380 nm for unknown, free, and Ca^{2+}-saturated dye, respectively. Both F_s and R_s were determined by lysing the cells with Triton X-100 (0.1%), whereas F_o and R_o were determined through the addition of 5 mM EGTA to the lysed cell suspension.

PKC assay. PKC activity was measured in duplicate (6, 17). The reaction mixture contained 20 mM Tris·HCl, 1.0 mM $CaCl_2$, 20 mM $MgCl_2$, 50 μM Ac-MBP(4–14) as substrate, 0.5 mg/ml leupeptin, and 0.1 mM ATP ($1–5 \times 10^6$ cpm of [γ-^{32}P]ATP). Phosphatidylserine (280 μg/ml) and PMA (10 μM) were added to some tubes to demonstrate phospholipid-dependent protein kinase activity. The reaction was initiated by the addition of 1–2 μg of protein, and the incubation (6 min at 37°) was stopped by immediate spotting of the reaction mixture onto phosphocellulose discs. The disks were then washed twice with 1% phosphoric acid and three times with distilled water. The radioactivity retained by the filter disc was determined through scintillation counting. PKC activity was calculated from the difference in ^{32}P incorporated into the PKC substrate peptide in the presence and the absence of added phospholipids.

Statistical analysis. Data were presented as mean ± standard error based on four aliquots of cells for the cAMP measurements and on three aliquots of cells for the PKC measurements. Each experiment was repeated at least twice. Statistical comparisons were analyzed through analysis of variance with the Newman-Keuls test for the cAMP and PKC measurements. The paired *t* test was used for the analysis of $[Ca^{2+}]_i$ measurements. Statistical significance was set at $p = 0.05$.

Results

Effect of thapsigargin on basal and adrenergic-stimulated $[Ca^{2+}]_i$. The effects of thapsigargin on the basal and NE-stimulated $[Ca^{2+}]_i$ were examined with the use of fura-2 as a fluorescent $[Ca^{2+}]_i$ indicator. The basal $[Ca^{2+}]_i$ in the pinealocytes was 153 ± 18 nM, which increased rapidly after treatment with NE (10 μM) as in previous reports (5, 24). It reached the maximal level within 1 min (318 ± 26 nM) and stabilized at 283 ± 22 nM, which was sustained for ≥10 min (Fig. 1a). Treatment with thapsigargin (100 nM) caused a more gradual increase in $[Ca^{2+}]_i$, which reached a sustained plateau level (545 ± 35 nM) after 5 min (Fig. 1b). The effect of thapsigargin on $[Ca^{2+}]_i$ was dose dependent. The addition of NE (10 μM) during the plateau phase of the thapsigargin-stimulated elevation of $[Ca^{2+}]_i$ did not further increase the $[Ca^{2+}]_i$ level (Fig. 1c).

We next determined the effect of thapsigargin on the intracellular Ca^{2+} store (Fig. 2). In this experiment, a low Ca^{2+} (0.1 mM) buffer B was used. As shown in Fig. 2a, chelation of extracellular Ca^{2+} by EGTA (1 mM) caused a gradual decrease in $[Ca^{2+}]_i$, and the addition of ionomycin (1 μM) 4 min

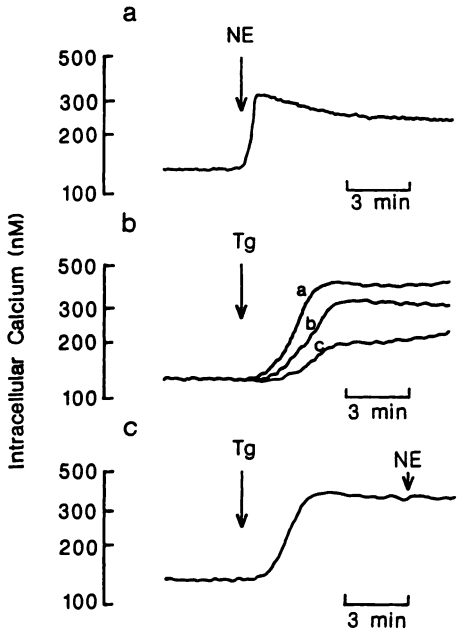

Fig. 1. Effects of NE and thapsigargin on $[Ca^{2+}]_i$ in rat pinealocytes. Rat pinealocytes were prepared and loaded with the fluorescent Ca^{2+} indicator Fura-2. Ratio of the fluorescence emission signal at 510 nm, excited at 340 and 380 nm, was continuously recorded and calibrated as described. *Traces*, representative of at least three experiments. a, The addition of NE (10 μM) alone. b, The addition of thapsigargin [(*Tg*) *a* = 100 nM, *b* = 30 nM, *c* = 10 nM]. c, the addition of thapsigargin (0.1 μM) followed by NE (10 μM). For further details, see Experimental Procedures.

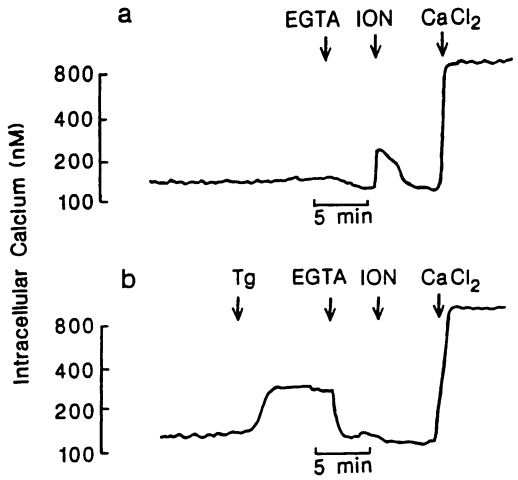

Fig. 2. Effects of thapsigargin on intracellular Ca^{2+} stores in rat pinealocytes. Rat pinealocytes were prepared and loaded with the fluorescent Ca^{2+} indicator Fura-2. Experiments were performed in low Ca^{2+} (0.1 mM) medium. Ratio of the fluorescence emission signal at 510 nm, excited at 340 and 380 nm, was continuously recorded and calibrated as described. *Traces*, representative of at least three experiments. a, The addition of EGTA (1 mM) followed by ionomycin [(*ION*) 1 μM] and $CaCl_2$ (2 mM). b, The same as in a, except that thapsigargin [(*Tg*) 0.1 μM] was added 8 min before EGTA. For further details, see Experimental Procedures.

later caused a small and transient elevation in $[Ca^{2+}]_i$. This transient elevation probably occurred through release of intracellular Ca^{2+} stores because the full ionomycin response (see Fig. 7) was restored when 2 mM $CaCl_2$ was added to the medium. In comparison, in parallel experiments, when the pinealocytes were first treated with 0.1 μM thapsigargin for 8 min before the addition of EGTA and ionomycin (Fig. 2b), ionomycin had no effect on $[Ca^{2+}]_i$. These data indicate that thapsigargin causes depletion of intracellular Ca^{2+} store in rat pinealocytes within 8 min of treatment.

Effect of thapsigargin on ISO-stimulated cAMP accumulation. In the first series of experiments, we investigated the effect of thapsigargin on the β-adrenergic-stimulated cAMP response. Stimulation with ISO (1 μM) alone increased the cAMP level by 10-fold (Fig. 3). The addition of thapsigargin alone, although having no effect on basal cAMP level, dose-dependently (1 nM–1 μM) enhanced the ISO (1 μM)-stimulated cAMP response. At 1 μM, thapsigargin increased the ISO-stimulated cAMP response by ~2-fold ($p < 0.05$) (Fig. 3). To determine whether the potentiating effect of thapsigargin was due to increased synthesis or decreased breakdown of cAMP, we used IBMX (1 mM), a phosphodiesterase inhibitor. As shown in Table 1, the potentiating effect of thapsigargin persisted in the presence of IBMX, indicating that this effect of thapsigargin was due to increased synthesis of cAMP.

Our previous studies have established that $[Ca^{2+}]_i$-elevating agents potentiate the stimulated cAMP responses by activating both PKC (6) and Ca^{2+}/calmodulin-dependent kinase (9). To determine whether these two kinases are also involved in the potentiating effect of thapsigargin, we used a specific PKC inhibitor, calphostin C, and a Ca^{2+}/calmodulin-dependent kinase inhibitor, W7. As shown in Table 2, calphostin C (0.1 μM) was most effective in reducing the PMA potentiation of the ISO-stimulated cAMP response (a 70% inhibition; $p < 0.05$). Calphostin C also reduced the potentiating effects of thapsigargin and ionomycin by 30% and 40%, respectively ($p < 0.05$ for both). In comparison, W7 had no effect on the potentiation by PMA but reduced the potentiating effect of both thapsigargin and ionomycin by 45% ($p < 0.05$). These data indicate that both PKC and Ca^{2+}/calmodulin-dependent kinase are involved in the potentiating effect

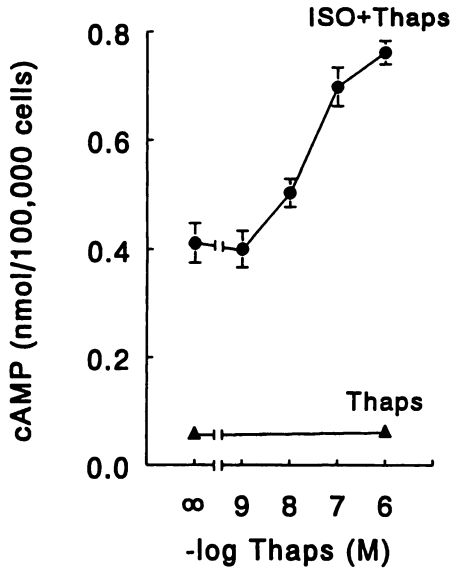

Fig. 3. Dose responses of thapsigargin on ISO-stimulated cAMP accumulation. Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) in the presence or absence of graded concentrations of thapsigargin (*Thaps*). *Points*, mean ± standard error of cAMP determinations done in duplicate on four samples of cells. For further details, see Experimental Procedures.

TABLE 1

Effects of thapsigargin on the cAMP responses stimulated by ISO and ISO plus PMA in the presence of a phosphodiesterase inhibitor

Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) or ISO (1 μM) plus PMA (0.1 μM) and thapsigargin (0.1 μM) in the presence or absence of BMX (1 mM). Neither PMA nor thapsigargin alone had a significant effect on the basal cAMP accumulation. Each value represents the mean ± standard error of four samples.

Treatment	cAMP	
	−IBMX	+IBMX
	pmol/10⁵ cells	
ISO	438 ± 28	1,520 ± 182
ISO + thapsigargin	936 ± 38[a]	2,411 ± 96[a]
ISO + PMA	3,313 ± 261	11,473 ± 423
ISO + PMA + thapsigargin	1,661 ± 96[a]	8,380 ± 731[a]
Control	63 ± 13	183 ± 21

[a] Significantly different from the corresponding treatments without thapsigargin ($p < 0.05$).

TABLE 2

Effects of protein kinase inhibitors on the potentiating effect of thapsigargin on the cAMP responses stimulated by ISO

Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) or ISO (1 μM) plus thapsigargin (0.1 μM) in the presence or absence of calphostin C (0.1 μM) or W7 (10 μM). Treatments with ISO plus PMA (0.1 μM) and ISO plus ionomycin (10 μM) were also included for comparison. Thapsigargin, PMA, and ionomycin alone had no significant effect on the basal cAMP accumulation. Each value represents the mean ± standard error of four samples.

Treatment	cAMP		
	Alone	+ Calphostin C	+ W7
	pmol/10⁵ cells		
ISO	443 ± 33	467 ± 29	421 ± 33
ISO + thapsigargin	886 ± 42[a]	646 ± 76[b]	546 ± 36[b]
ISO + PMA	3,183 ± 31	1,033 ± 110[b]	3,011 ± 321
ISO + ionomycin	2,413 ± 142[a]	1,546 ± 96[b]	867 ± 29[b]
Control	58 ± 9	63 ± 11	44 ± 5

[a] Significantly different from the corresponding treatments with ISO ($p < 0.05$).
[b] Significantly different from the corresponding treatments without protein kinase inhibitor ($p < 0.05$).

of thapsigargin, whereas the potentiating effect of PMA involves only PKC.

Effect of thapsigargin on NE-stimulated cAMP accumulation. To investigate the effect of thapsigargin on the combined β- and α_1-adrenergic-stimulated cAMP accumulation, we used NE. NE (10 μM) alone stimulated the cAMP accumulation up to 50-fold as reported previously (3, 19) (Fig. 4). In contrast to the ISO-stimulated cAMP response, the addition of thapsigargin (1 nM–1 μM) dose-dependently inhibited the NE-stimulated cAMP response (Fig. 4). At 1 μM, thapsigargin inhibited the NE-stimulated cAMP response by ~45% ($p < 0.05$) (Fig. 4). Thapsigargin reduced the maximal NE-stimulated cAMP response without shifting the EC_{50} of this response (Fig. 5).

Comparison of the effects of thapsigargin with other $[Ca^{2+}]_i$-elevating agents. The effects of thapsigargin on the ISO- and NE-stimulated cAMP responses were compared with those of a depolarizing concentration of K^+ and ionomycin, two established $[Ca^{2+}]_i$-elevating agents. Both ionomycin (10 μM) and K^+ (30 mM) enhanced the ISO-stimulated cAMP response by >5-fold, which was substantially higher than that for thapsigargin (Table 3). Regarding the NE-stimulated cAMP response, in contrast to thapsigargin, neither ionomycin nor a depolarizing concentration of K^+ had a significant effect on the NE-stimulated cAMP response (Table 3).

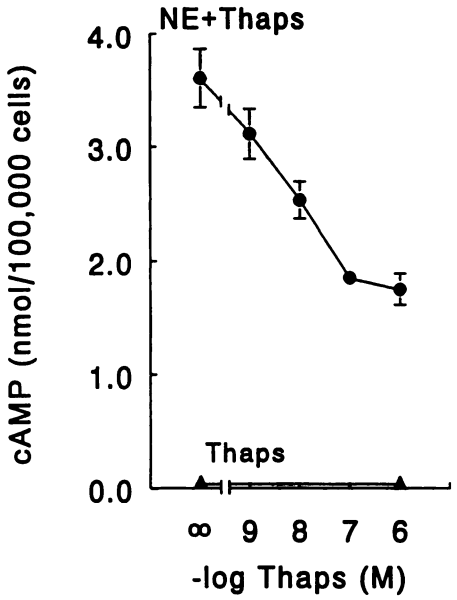

Fig. 4. Dose responses of thapsigargin on NE-stimulated cAMP accumulation. Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with NE (10 μM) in the presence or absence of graded concentrations of thapsigargin (*Thaps*). *Points*, mean ± standard error of cAMP determinations done in duplicate on four samples of cells. For further details, see Experimental Procedures.

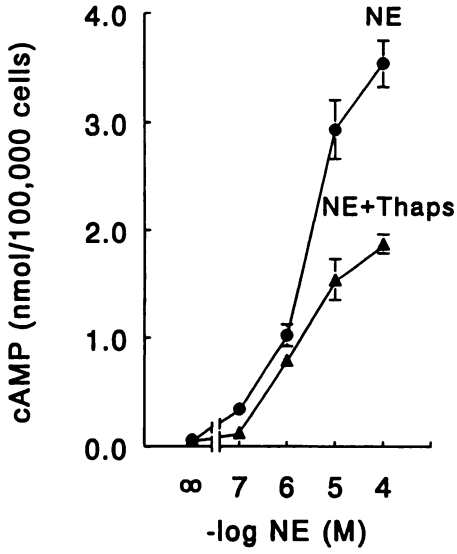

Fig. 5. Effects of thapsigargin on NE-stimulated cAMP accumulation. Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with varying doses of NE in the presence or absence of thapsigargin [(*Thaps*) 0.1 μM]. *Points*, mean ± standard error of cAMP determinations done in duplicate on four samples of cells. For further details, see Experimental Procedures.

Comparison of the effects of thapsigargin with cyclopiazonic acid. To determine whether the effects of thapsigargin are due to Ca^{2+}-ATPase inhibition, we used another Ca^{2+}-ATPase inhibitor, cyclopiazonic acid. As shown in Table 4, similar to thapsigargin, cyclopiazonic acid (50 μM) was effective in enhancing the ISO-stimulated cAMP response 2-fold ($p < 0.05$) while inhibiting the NE-stimulated cAMP response by ~40% ($p < 0.05$).

TABLE 3

Effect of different $[Ca^{2+}]_i$-elevating agents on the ISO- and NE-stimulated cAMP response

Pinealocytes (2×10^4 cells/0.4 ml) were treated with ISO (1 μM) or NE (10 μM) in the presence or absence of different $[Ca^{2+}]_i$-elevating agents for 15 min with ISO (1 mM). Each value represents the mean ± standard error of four samples of cells.

Treatment	cAMP
	pmol/10^5 cells
Control	39 ± 13
ISO	414 ± 31
+Thapsigargin (1 μM)	716 ± 44[a]
+K^+ (30 mM)	2,037 ± 183[a]
+Ionomycin (10 μM)	2,238 ± 114[a]
NE	2,897 ± 261
+Thapsigargin (1 μM)	1,735 ± 116[b]
+K^+ (30 mM)	3,177 ± 305
+Ionomycin (10 μM)	3,316 ± 189

[a] Significantly different from ISO-stimulated response ($p < 0.05$).
[b] Significantly different from NE-stimulated response ($p < 0.05$).

TABLE 4

Effect of cyclopiazonic acid on the ISO-, NE-, and ISO plus PMA-stimulated cAMP responses

Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) or ISO plus PMA (0.1 μM) in the presence or absence of cyclopiazonic acid [(CPA) 50 μM]. Each value represents the mean ± standard error of four samples.

Treatment	cAMP	
	−CPA	+CPA
	pmol/10^5 cells	
ISO	427 ± 35	1,005 ± 16[a]
NE	2,312 ± 103	1,324 ± 96[a]
ISO + PMA	3,525 ± 157	2,192 ± 103[a]
Control	34 ± 12	41 ± 5

[a] Significantly different from the corresponding treatment without CPA ($p < 0.05$).

Effect of thapsigargin on the potentiation of the ISO-stimulated cAMP accumulation. One possible explanation for the differential effects of thapsigargin on the ISO- and NE-stimulated cAMP responses is that thapsigargin may specifically inhibit a component of the α_1-adrenergic-mediated potentiating mechanism of the NE stimulation. This possibility was tested in the following studies with the use of PMA, which activates PKC directly (4, 7), and ionomycin, which elevates $[Ca^{2+}]_i$ directly (8) as substitutes for the α_1-adrenergic component of the NE response.

As shown in previous studies (6, 7), the addition of PMA (100 nM) enhanced the ISO (1 μM)-stimulated cAMP response 10-fold (Fig. 6). However, cotreatment with thapsigargin (1 nM–1 μM) dose-dependently inhibited the cAMP response stimulated by ISO plus PMA. At 100 nM, the ISO-plus-PMA-stimulated cAMP response was reduced by 50% ($p < 0.05$) (Fig. 6). This inhibition was not due to enhanced degradation of cAMP because a similar degree of inhibition was observed in the presence of IBMX (Table 1). Similar to thapsigargin, cyclopiazonic acid (50 μM), another Ca^{2+}-ATPase inhibitor, inhibited the ISO-plus-PMA-stimulated cAMP response (Table 4).

Treatment with 1 μM ionomycin alone elevated $[Ca^{2+}]_i$ from 143 ± 12 to 785 ± 89 nM (Fig. 7b), and at 10 μM, the elevated $[Ca^{2+}]_i$ reached a level that probably saturated the Fura-2 present inside the cell and therefore cannot be determined accurately. Cotreatment with thapsigargin did not significantly affect the ionomycin-stimulated elevation of

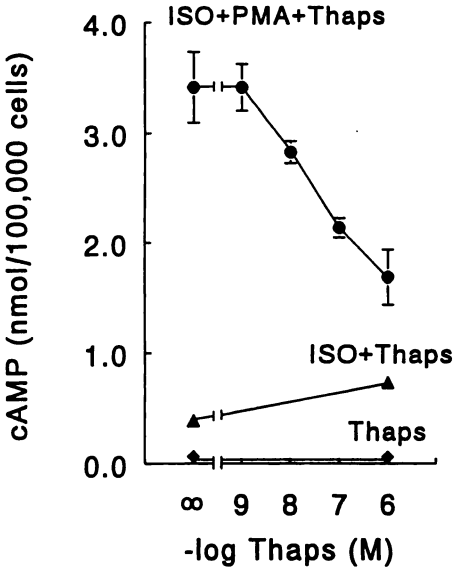

Fig. 6. Dose responses of thapsigargin on ISO and PMA-stimulated cAMP accumulation. Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) and PMA (0.1 μM) in the presence or absence of graded concentrations of thapsigargin (*Thaps*). *Points*, mean ± standard error of cAMP determinations done in duplicate on four samples of cells. For further details, see Experimental Procedures.

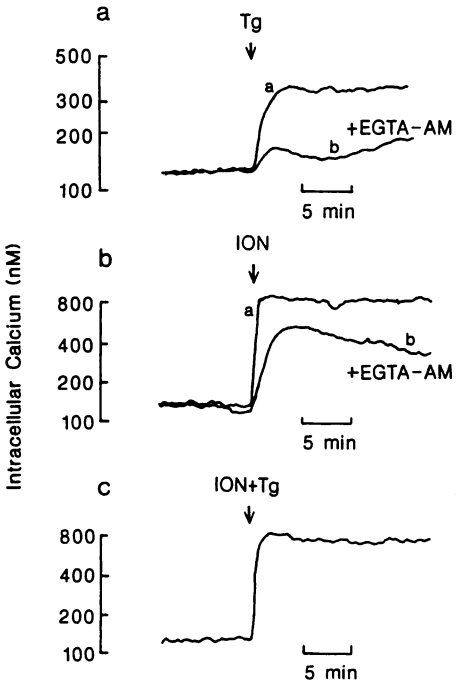

Fig. 7. Effects of ionomycin and EGTA-AM on thapsigargin stimulated $[Ca^{2+}]_i$ elevation in rat pinealocytes. Rat pinealocytes were prepared and loaded with the fluorescent Ca^{2+} indicator Fura-2. Ratio of the fluorescence emission signal at 510 nm, excited at 340 and 380 nm, was continuously recorded and calibrated as described. *Traces*, representative of at least three experiments. a, Thapsigargin [(*Tg*) 0.1 μM] in the presence and absence of EGTA-AM (10 μM). b, Ionomycin [(*ION*) 1 μM] in the presence or absence of EGTA-AM (added 2 min before the addition of ionomycin). c, Ionomycin (1 μM) plus thapsigargin (0.1 μM). For further details, see Experimental Procedures.

$[Ca^{2+}]_i$ (Fig. 7c). Similar to previous reports (4, 8), the addition of ionomycin (10 μM) enhanced the ISO (1 μM)-stimulated cAMP response 5-fold (Fig. 8). Under this condition,

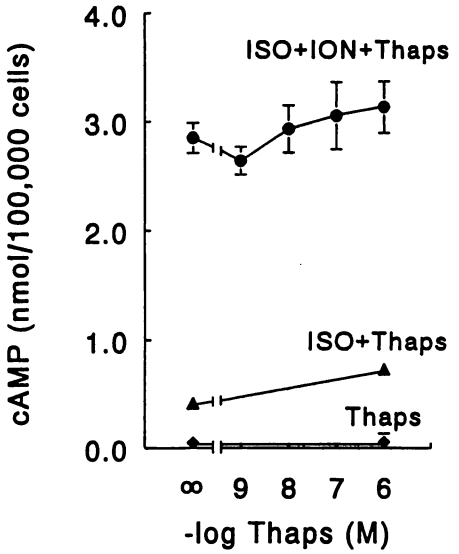

Fig. 8. Dose responses of thapsigargin on ISO- and ionomycin-stimulated cAMP accumulation. Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) and ionomycin [(*ION*) 10 μM] in the presence or absence of graded concentrations of thapsigargin (*Thaps*). *Points*, mean ± standard error of cAMP determinations done in duplicate on four samples of cells. For further details, see Experimental Procedures.

thapsigargin (1 μM) had no effect on the ISO-plus-ionomycin-stimulated cAMP response ($p > 0.05$) (Fig. 8). A similar result was obtained when a depolarizing concentration of K^+ (30 mM) was used to elevate $[Ca^{2+}]_i$ (data not shown).

The role of $[Ca^{2+}]_i$ in the effects of thapsigargin on the ISO- and ISO-plus-PMA-stimulated cAMP in pinealocytes. To investigate the role of changes in $[Ca^{2+}]_i$ on the effects of thapsigargin on the ISO- and ISO-plus-PMA-stimulated cAMP responses, the membrane-permeable Ca^{2+} chelator EGTA-AM was used to suppress the changes in $[Ca^{2+}]_i$. Direct $[Ca^{2+}]_i$ measurement showed that the simultaneous addition of EGTA-AM (10 μM) significantly blunted the thapsigargin-stimulated elevation of $[Ca^{2+}]_i$ (Fig. 7a). Pretreatmnet with EGTA-AM (10 μM) for 2 min was also effective in reducing the ionomycin (1 μM)-stimulated $[Ca^{2+}]_i$ elevation, indicating rapid hydrolysis of EGTA-AM in the pinealocytes (Fig. 7b). Treatment with EGTA-AM (10 μM) had little effect on the ISO-stimulated cAMP response (Table 5). However, EGTA-AM treatment abolished the potentiating effect of thapsigargin on the ISO-stimulated cAMP response, suggesting that this effect of thapsigargin was due to its action on elevating $[Ca^{2+}]_i$. In comparison, the inhibitory effect of thapsigargin on the ISO-plus-PMA-stimulated cAMP response was not affected by treatment with EGTA-AM, suggesting that the inhibitory effect may not be related to the thapsigargin-mediated elevation of $[Ca^{2+}]_i$ (Table 5).

To further investigate the inhibitory effect of thapsigargin, the ISO-plus-PMA-stimulated pinealocytes were cotreated with ionomycin (10 μM). The addition of ionomycin had no significant effect on the ISO-plus-PMA-stimulated cAMP response. Under these conditions, thapsigargin remained effective in reducing the cAMP response (Table 6). These results further indicate that the inhibitory effect of thapsigargin on the cAMP response may be independent of changes in $[Ca^{2+}]_i$.

TABLE 5

Effect of EGTA-AM on the effect of thapsigargin on the ISO- and ISO plus PMA-stimulated cAMP responses

Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) or ISO plus PMA (0.1 μM) in the presence or absence of thapsigargin (0.1 μM) and/or EGTA-AM (10 μM). Each value represents the mean ± standard error of four samples.

Treatment	cAMP	
	−EGTA-AM	+EGTA-AM
	pmol/10⁵ cells	
ISO	383 ± 27	428 ± 25
ISO + thapsigargin	738 ± 60[b]	413 ± 19[a]
ISO + PMA	3,321 ± 250	3,632 ± 257
ISO + PMA + thapsigargin	1,941 ± 116[b]	1,832 ± 156[b]
Control	63 ± 3	83 ± 5

[a] Significantly different from ISO-plus-thapsigargin-treated cells ($p < 0.05$).
[b] Significantly different from the corresponding treatment without thapsigargin ($p < 0.05$).

TABLE 6

Effect of ionomycin on the effect of thapsigargin on the ISO and ISO-plus-PMA-stimulated cAMP response

Pinealocytes (2×10^4 cells/0.4 ml) were incubated for 15 min with ISO (1 μM) or ISO plus PMA (0.1 μM) in the presence or absence of thapsigargin (0.1 μM) and/or ionomycin [(ION) 10 μM]. Each value represents the mean ± standard error of four samples.

Treatment	cAMP	
	−ION	+ION
	pmol/10⁵ cells	
ISO	365 ± 18	2,061 ± 169
ISO + thapsigargin	696 ± 43[a]	2,243 ± 67
ISO + PMA	3,631 ± 245	3,346 ± 216
ISO + PMA + thapsigargin	1,998 ± 96[a]	1,873 ± 169[a]
Control	58 ± 3	78 ± 4

[a] Significantly different from the corresponding treatments without thapsigargin ($p < 0.05$).

Effect of thapsigargin on PKC activity. The above results suggested that thapsigargin selectively inhibits the potentiating effect of a PKC activator, PMA. The possibility that thapsigargin may have a direct inhibitory effect on PKC activity was investigated. As shown in Table 7, H7, an established inhibitor of PKC (25), reduced PKC activity by 85%. Under the same condition, thapsigargin (≤1 μM) had no inhibitory effect on the *in vitro* PKC activity in the pinealocyte. Even at 10 μM, thapsigargin reduced the *in vitro* PKC activity by only 30%.

The effects of thapsigargin on the PMA- and NE-mediated translocation of PKC in intact pinealocytes were also deter-

TABLE 7

Effect of thapsigargin on the *in vitro* PKC activity

PKC activity of the cytosolic fractions of pinealocytes were determined in the absence or presence of graded concentrations of thapsigargin. Ac-MBP(4–14) was used as substrate. H7 was included in this experiment as a positive control. Each value represents the mean ± standard error of determinations done in duplicate on three samples of cell preparations. For further details, see Experimental Procedures.

Treatment	PKC activity
	nmol/mg protein/hr
No treatment	40.6 ± 1.0
+H7 (100 μM)	6.9 ± 0.4[a]
+Thapsigargin (10 μM)	27.5 ± 1.2[a]
+Thapsigargin (1 μM)	40.2 ± 1.6
+Thapsigargin (0.1 μM)	41.3 ± 2.2

[a] Significant inhibition of the *in vitro* PKC activity ($p < 0.05$).

mined. As shown in Table 8, PMA (0.1 μM) caused a 4-fold increase in PKC activity associated with the particulate fraction while reducing the cytosolic fraction by 60% (7, 17). Although thapsigargin alone caused a small but significant ($p < 0.05$) increase in the membrane-associated PKC activity, it had no effect on the redistribution of the PKC activity stimulated by PMA. Thapsigargin also had no significant effect on the NE-stimulated increase in PKC activity associated with the particulate fraction (Table 8). Therefore, it seems unlikely that thapsigargin inhibits the NE- or ISO-plus-PMA-stimulated potentiation of cAMP responses by reducing the translocation of PKC.

Discussion

As with other cell types (12, 27–32), thapsigargin is effective in elevating $[Ca^{2+}]_i$ in rat pinealocytes. The source of the initial increase in $[Ca^{2+}]_i$ is presumably derived from intracellularly sequestered Ca^{2+}, whereas the sustained increase in $[Ca^{2+}]_i$ is related to influx of extracellular Ca^{2+} (29–32). Compared with other Ca^{2+}-mobilizing agonists, such as NE, the initial increase by thapsigargin is slower, but the magnitude of increase by thapsigargin (0.1 μM) is similar to that of NE (10 μM). This slow rate of increase in $[Ca^{2+}]_i$, coupled with the rapid hydrolysis of EGTA-AM in the pinealocytes, could explain why EGTA-AM is effective in blunting the response without pretreatment.

Similar to studies that used a depolarization concentration of K^+, ouabain, or ionomycin to increase $[Ca^{2+}]_i$ (4, 8, 22), thapsigargin was found to potentiate the ISO-stimulated cAMP response in rat pinealocytes. This potentiation by thapsigargin is directly related to its effect on $[Ca^{2+}]_i$ elevation as treatment with EGTA-AM, which significantly blunted the effect of thapsigargin on the $[Ca^{2+}]_i$, abolished this effect. In addition, both PKC and Ca^{2+}/calmodulin-dependent kinase seemed to be involved downstream from the $[Ca^{2+}]_i$ elevation in the potentiating effect of thapsigargin. This is based on the observations that thapsigargin treatment alone caused a small but significant translocation of PKC to the membrane and that calphostin C, a PKC inhibitor, and W7, a Ca^{2+}/calmodulin-dependent kinase inhibitor, were both effective in abolishing the potentiating effect of thapsigargin. Furthermore, the potentiating effect of thapsigargin persisted in the presence of a phosphodiesterase inhibitor, IBMX, indicating that thapsigargin potentiates the cAMP responses by increasing the synthesis rather than by reducing the degradation of cAMP. Because similar effects were observed with other $[Ca^{2+}]_i$-elevating agents, including ionomycin and a depolarizing concentration of K^+ (1, 4, 8, 9), it seems that the potentiation mechanism is similar for thapsigargin and other $[Ca^{2+}]_i$-elevating agents in the adrenergic-stimulated cAMP accumulation. The observation that cyclopiazonic acid, another chemically unrelated Ca^{2+}-ATPase inhibitor, was equally effective as thapsigargin is consistent with the interpretation that the enhancing effects of thapsigargin are related to elevation of $[Ca^{2+}]_i$ through inhibition of Ca^{2+}-ATPase.

TABLE 8

Effect of thapsigargin on the PMA- and NE-mediated translocation of PKC activity in rat pinealocytes

PKC activity of the membrane and cytosolic fractions of pinealocytes was determined in the absence or presence of indicated drugs. Each value represents the mean ± standard error of determinations done in duplicate on three samples of cell preparations. For further details, see Experimental Procedures.

Treatment	PKC activity	
	Cytosol	Membrane
	pmol/10⁶ cells/6 min	
Experiment 1		
Control	1,520 ± 38	226 ± 8
PMA (0.1 μM)	643 ± 54[a]	1,073 ± 45[a]
Thapsigargin (0.1 μM)	1,491 ± 26	266 ± 13[a]
PMA (0.1 μM) + thapsigargin (0.1 μM)	589 ± 47[a]	1,149 ± 65[a]
Experiment 2		
Control	1,749 ± 52	253 ± 11
NE (10 μM)	1,673 ± 26	352 ± 18[a]
Thapsigargin (0.1 μM)	1,712 ± 28	313 ± 16[a]
NE (10 μM) + thapsigargin (0.1 μM)	1,653 ± 39	378 ± 13[a]

[a] Significantly different from control ($p < 0.05$).

Although there are major similarities, there also are distinct differences between thapsigargin and other $[Ca^{2+}]_i$-elevating agents. First, thapsigargin significantly inhibits the cAMP response stimulated by the mixed β- and α_1-adrenergic agonist NE, an effect that is not shared by other $[Ca^{2+}]_i$-elevating agents. Second, even though thapsigargin elevates $[Ca^{2+}]_i$ to the same magnitude as NE or a depolarizing concentration of K^+, the magnitude of the enhancing effect of thapsigargin on the ISO-stimulated cAMP response is substantially smaller than that of a depolarizing concentration of K^+. These results suggest that thapsigargin may have a separate inhibitory action on the cAMP accumulation and that this action may be independent of its elevation of $[Ca^{2+}]_i$.

It has been established that α_1-adrenergic activation potentiates the β-adrenergic-stimulated cAMP response via elevation of $[Ca^{2+}]_i$ (8) and activation of PKC (4). Considering that thapsigargin inhibits the mixed α_1- and β-adrenergic-stimulated cAMP response, it is possible that thapsigargin may mediate this inhibition by modulating one of these signalling pathways involved in the potentiation. Our results indicate that thapsigargin has no effect on the NE-stimulated PKC translocation. However, thapsigargin selectively inhibits the potentiating effect by a PKC activator while having little effect on the potentiation by other $[Ca^{2+}]_i$-elevating agents. The lack of effect of thapsigargin on the potentiation by $[Ca^{2+}]_i$-elevating agents may be accounted for by current and earlier findings that, in addition to PKC, $[Ca^{2+}]_i$ elevating agents use Ca^{2+}/calmodulin-dependent kinase in securing its potentiating effect (9). However, unlike its potentiating effect on the ISO-stimulated response, the inhibitory effect of thapsigargin on the PKC-mediated potentiation is not affected by the cotreatment with EGTA-AM (which blunts the $[Ca^{2+}]_i$-elevating effect of thapsigargin) or ionomycin (which raises and maintains $[Ca^{2+}]_i$ at a substantially elevated level). Therefore, thapsigargin, in addition to its $[Ca^{2+}]_i$-elevating effect, seems to inhibit the PKC-mediated potentiation through a mechanism independent of its elevation of $[Ca^{2+}]_i$. This additional effect may account for the inhibitory effect of thapsigargin on the NE-stimulated cAMP response and the smaller enhancing effect of thapsigargin in comparison to other $[Ca^{2+}]_i$-elevating agents.

The observation that thapsigargin does not have a direct inhibitory effect on the PKC activities or the PMA-mediated translocation of PKC indicates that thapsigargin likely inhibits the PMA-mediated potentiation at a site subsequent to PKC activation. Interestingly, another structurally unre-

lated Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, has the same inhibitory effects as thapsigargin. Therefore, rather than a nonspecific effect of the drug, the inhibitory effect of thapsigargin could still be due to its action on Ca^{2+}-ATPase, although the subsequent elevation of $[Ca^{2+}]_i$ may not be directly involved. The precise mechanism through which inhibition of endoplasmic reticulum Ca^{2+}-ATPase leads to a reduction in the PMA-mediated potentiation of the cAMP response remains unresolved.

Apart from elevating $[Ca^{2+}]_i$, another consequence of Ca^{2+}-ATPase inhibition is the depletion of endoplasmic reticulum Ca^{2+} stores. This depletion of endoplasmic reticulum Ca^{2+} stores has been linked to interruption of events occurring both inside and outside the endoplasmic reticulum (33, 34). It is possible that this depletion also affects the PMA-mediated potentiation of the cAMP response. However, if depletion of intracellular Ca^{2+} stores is the mechanism involved, ionomycin, which could also deplete intracellular Ca^{2+} stores, should have a similar effect. Indeed, in one of our earlier studies, we found that the addition of another Ca^{2+} ionophore, A23187, also had no effect on the ISO-plus-PMA-stimulated cAMP response, even when extracellular Ca^{2+} was removed with the use of EGTA (4). In the absence of extracellular Ca^{2+}, the A23187 treatment would be expected to deplete intracellular Ca^{2+} stores. Thus, it seems unlikely that the inhibitory effect of thapsigargin on the potentiation of the cAMP response by PMA is due solely to depletion of intracellular store. Additional experiments are required to clarify the mechanism through which inhibitors of Ca^{2+}-ATPase reduce the potentiating effects of PKC on the adrenergic-stimulated cAMP response.

In summary, our data suggest a complicated interaction between thapsigargin-mediated intracellular Ca^{2+} redistribution and the potentiation of the adrenergic-stimulated cAMP by PKC. Thapsigargin, by elevating $[Ca^{2+}]_i$, potentiates the β-adrenergic-stimulated cAMP response through activation of PKC and Ca^{2+}/calmodulin-dependent kinase. However, due to either its direct inhibition of the endoplasmic reticulum Ca^{2+}-ATPase or other $[Ca^{2+}]_i$-independent mechanism, thapsigargin also acts at a step distal to activation of PKC in reducing the effectiveness of the PKC-mediated potentiation of the cAMP response.

Acknowledgments

The authors thank Dr. Albert Baukal for the supply of antisera for the radioimmunoassay.

References

1. Chik, C. L., and A. K. Ho. Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. *Prog. Biophys. Mol. Biol.* **53:**197–203 (1989).
2. Klein, D. C. Photoneural regulation of the mammalian pineal gland, in *Photoperiodism, Melatonin and the Pineal* (D. Evered and Clark S., eds.). Pitman, London, 38–56 (1985).
3. Vanecek, J., D. Sugden, J. L. Weller, and D.C. Klein. Atypical synergistic α_1- and β-adrenergic regulation of adenosine 3′5′-monophosphate and guanosine 3′5′-monophosphate in rat pinealocytes. *Endocrinology* **116:** 2167–2173 (1985).
4. Ho, A. K., C. L. Chik, and D. C. Klein. Protein kinase C is involved in adrenergic stimulation of pineal cGMP accumulation. *J. Biol. Chem.* **262:** 10059–10064 (1987).
5. Sugden, A. L., D. Sugden, and D. C. Klein. α_1-Adrenoceptor activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. *J. Biol. Chem.* **262:**741–745 (1987).
6. Ho, A. K., T. P. Thomas, C. L. Chik, W. B. Anderson, and D. C. Klein. Protein kinase C: subcellular redistribution by agents which increase Ca^{2+} influx. *J. Biol. Chem.* **263:**9292–9297 (1988).
7. Sugden, D., J. Vanecek, D. C. Klein, T. P. Thomas, and W. B. Anderson. Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. *Nature (Lond.)* **314:**359–360 (1985).
8. Sugden, A. L., D. Sugden, and D. C. Klein. Essential role of calcium influx in the adrenergic regulation of cAMP and cGMP in rat pinealocytes. *J. Biol. Chem.* **261:**11608–11612 (1988).
9. Ho, A. K., I. Young, and C. L. Chik. Evidence for a role of calmodulin in regulation of pinealocyte cyclic nucleotides. *Biochem. Pharmacol.* **41:**897–903 (1991).
10. Thastrup, O., P. J. Cullen, B. K. Drobak, M. R. Hanley, and A. P. Dawson. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} ATPase. *Proc. Natl. Acad. Sci. USA* **87:**2466–2470 (1990).
11. Law, G. J., J. A. Pachter, O. Thastrup, M. Hanley, and P. S. Dannies. Thapsigargin, but not caffeine, blocks the ability of TRH to release Ca^{2+} from an intracellular store in GH_4C_1 pituitary cells. *Biochem. J.* **267:**359–364 (1990).
12. Jackson, T. R., S. I. Patterson, O. Thastrup, and M. R. Hanley. A novel tumour promoter, thapsigargin, transiently increase cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115–401L neuronal cells. *Biochem. J.* **253:**81–86 (1988).
13. Hakii, H., H. Fugiki, M. Saganuma, M. Nakayasu, T. Tahira, T. Sugimura, P. J. Scheur, and S. B. Christensen. Thapsigargin a histamine secretagogue is a non-12-O-tetradecanoylphorbol-13-acetate type tumour promoter in two stage mouse skin carcinogenesis. *Carcinogenesis* **7:**707–713 (1986).
14. Buda, M., and D. C. Klein. A suspension culture of pinealocytes: regulation of N-acetyltransferase activity. *Endocrinology* **103:**1483–1493 (1978).
15. Ho, A. K., M. Girard, I. Young, and C. L. Chik. Intracellular pH on adrenergic stimulated cAMP and cGMP production in rat pinealocytes. *Am. J. Physiol.* **261:**C642–C649 (1991).
16. Ho, A. K., C. L. Chik, J. L. Weller, E. J. Cragoe, Jr., and D. C. Klein. Evidence of α_1-adrenergic → protein kinase C → Na^+/H^+ antiporter-dependent increase in pinealocyte intracellular pH. *J. Biol. Chem.* **264:** 12983–12988 (1989).
17. Ho, A. K., and C. L. Chik. Acute elevation of intracellular pH translocated protein kinase C activities in rat pinealocytes. *Biochem. Biophys. Res. Commun.* **200:**569–576 (1994).
18. Harper, J. F., and G. J. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′-0 acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1:**207–218 (1975).
19. Ho, A. K., and C. L. Chik. Phosphatase inhibitors potentiate adrenergic-stimulated cAMP and cGMP production in rat pinealocytes. *Am. J. Physiol.* **268:**E458–E466 (1995).
20. Chik, C. L., and A. K. Ho. Pituitary adenylate cyclase-activating polypeptide (PACAP): control of rat pineal cyclic AMP and melatonin but not cyclic GMP. *J. Neurochem.* **64:**2111–2117 (1995).
21. Rink, T. J., R. Y. Tsien, and T. Pozzan. Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J. Cell. Biol.* **95:**189–196 (1982).
22. Ho, A. K., S. S. Lalh, I. Young, E. J. Cragoe, Jr., and C. L. Chik. Inhibitory effects of amilorides on pinealocyte cAMP and cGMP accumulation: involvement of post-receptor mechanisms. *Endocrinology* **127:**460–466 (1990).
23. Poenie, M., J. Alderton, R. Y. Tsien, and R. A. Steinhardt. Changes of free calcium levels with stages of the cell division cycle. *Nature (Lond.)* **315:** 147–149 (1985).
24. Chik, C. L., Q. Y. Liu, M. Girard, E. Karpinski, and A. K. Ho. Inhibitory action of ethanol on the L-type of Ca^{2+} channels and Ca^{2+}-dependent guanosine 3′,5′-monophosphate accumulation in rat pinealocytes. *Endocrinology* **131:**1895–1902 (1992).
25. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. Direct activation of calcium activated, phospholipid-dependent protein kinase by tumour-promoting phorbol esters. *J. Biol. Chem.* **257:**7847–7851 (1982).
26. Ho, A. K., C. L. Chik, and D. C. Klein. Effects of protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine(H7)on pineal protein kinase C activity and adrenergic stimulation of pineal cAMP and cGMP. *Biochem. Pharmacol.* **37:**1015–1020 (1988).
27. Islam, S., and P. O. Berggren. Mobilization of Ca^{2+} by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone in permeabilized insulin-secreting RINm5F cells: evidence for separate uptake and release compartments in inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool. *Biochem. J.* **293:**423–429 (1993)
28. Czarny, M., and J. Baranska. Effect of ionophore A23187, thapsigargin, caffeine and heparin on phosphatidylserine synthesis in rat liver microsomal fraction. *Biochem. Biophys. Res. Commun.* **194:**577–583 (1993).
29. Bird, G. St. J., and J. W. Putney. Inhibition of thapsigargin-induced calcium entry by microinjected guanine nucleotide analogues. *J. Biol. Chem.* **268:**21486–21488 (1993).
30. Thastrup, O., F. Birthe, and O. Scharff. The calcium mobilizing and tumour promoting agent, thapsigargin elevated the platelet cytoplasmic free calcium concentration to a higher steady state level: a possible mechanism of action for the tumor promotion. *Biochem. Biophys. Res. Commun.* **142:**654–650 (1987).
31. Tepel, M., S. Kuhnapfel, G. Theilmeier, C. Teupe, R. Schlotmann, and W.

Zideck. Filling state of intracellular Ca^{2+} pools triggers transplasma membrane Na^+ and Ca^{2+} influx by a tyrosine kinase-dependent pathway. *J. Biol. Chem.* **269**:26239–26242 (1994).
32. Putney, J. W., Jr. Capacitative calcium entry revisited. *Cell Calcium* **11**:611–624 (1990).
33. Banhegyi, G., G. Bellomo, R. Fulceri, J. Mandl, and A. Benedetti. Intraluminal calcium of liver endoplasmic reticulum stimulates the glucuronidation of p-nitropenol. *Biochem J.* **292**:99–104 (1993).
34. Kimball, S. R., and L. S. Jefferson. Regulation of protein synthesis by modulation of intracellular calcium in rat liver. *Am. J. Physiol.* **263**:E958–E964 (1992).

Send reprint requests to: Dr. A. K. Ho, Department of Physiology, Faculty of Medicine, University of Alberta, 7–26 Medical Sciences Building, Edmonton, Alberta, Canada T6G 2H7. E-mail: aho@physio.med.ualberta.ca