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EVIDENCE FOR A ROLE OF CALMODULIN IN REGULATION OF PINEALOCYTE CYCLIC NUCLEOTIDES

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Abstract—In rat pinealocytes, norepinephrine (NE) increases cAMP and cGMP accumulation through a synergistic dual receptor mechanism involving α_1- and β-adrenergic receptors. The available evidence indicates that both increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$, and activation of protein kinase C are involved in the α_1-adrenergic potentiation of β-adrenergically stimulated cAMP and cGMP responses. In this study, the role of Ca^{2+} was further investigated using three agents with known anti-calmodulin activities: trifluoperazine, pimozide and W7. It was found that none of these inhibitors had any effects on the isoproterenol (ISO)-stimulated cAMP and cGMP responses. By contrast, all three Ca^{2+}/calmodulin inhibitors reduced the NE-stimulated cAMP and cGMP responses. These results suggested that an α_1-adrenergic mediated mechanism was likely sensitive to the inhibitory action of the Ca^{2+}/calmodulin inhibitors. To determine the possible site of action of the inhibitors, an activator of protein kinase C, 4β-phorbol 12-myristate 13-acetate (PMA), was used to potentiate the ISO-stimulated cAMP response. The PMA potentiation of ISO-stimulated cAMP response was only inhibited by a high concentration of pimozide, and not by trifluoperazine or W7, suggesting that pimozide may have other actions distinct from those of trifluoperazine or W7. Full potentiation of the cGMP response can be achieved by adding both PMA and a depolarizing concentration of K^+. All three calmodulin antagonists were effective in inhibiting the PMA and K^+ potentiation of cGMP responses in ISO-stimulated cells. These three compounds were also effective in inhibiting the potentiating effects of $[Ca^{2+}]_i$ elevating agents on the β-adrenergically stimulated cAMP and cGMP responses. The three inhibitors, at the concentrations used in the present study, were found to have no effect on the protein kinase C activity determined *in vitro*. These results indicated that apart from the protein kinase C pathway, the Ca^{2+}/calmodulin pathway of signal transduction may be of importance in the regulation of pinealocyte cGMP and, to a lesser degree, cAMP responses.

The production of cAMP and cGMP in rat pinealocytes is regulated by norepinephrine (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- to 10-fold increase in the accumulation of cAMP and a 2- to 4-fold increase in the accumulation of cGMP. Selective activation of the α_1-adrenoceptor alone, while having no effect on its own, potentiates the β-adrenergically stimulated cyclic nucleotide responses. Stimulation by both the β- and α_1-adrenoceptors results in 100-fold increases in both cAMP and cGMP accumulation. One of the post-receptor mechanisms that is of importance to this dual receptor regulation of pineal cyclic nucleotides is elevation of $[Ca^{2+}]_i$ [4]. This is based on the following findings: activation of α_1-adrenergic receptor leads to elevation of $[Ca^{2+}]_i$ [4]; the potentiation effects of α_1-adrenoceptor activation on both cAMP and cGMP responses can be replaced by agents that elevate $[Ca^{2+}]_i$, including depolarizing concentrations of K^+, Ca^{2+} ionophores and ouabain [5]; and the potentiating effects of α_1-adrenergic agonists can be blocked by inorganic Ca^{2+} channel blockers and a Ca^{2+} chelating agent [5].

The mechanism through which elevation of $[Ca^{2+}]_i$ potentiates the β-adrenergically stimulated cAMP and cGMP responses remains unclear. One possible explanation is that elevation of $[Ca^{2+}]_i$ leads to activation of protein kinase C. Indeed, we have demonstrated that agents that elevate $[Ca^{2+}]_i$ also activate protein kinase C in rat pinealocytes and activators of protein kinase C potentiate the β-adrenergically stimulated cAMP response [6]. However, there is a major difference between the potentiation mechanism for cAMP and cGMP accumulation. While activation of protein kinase C alone is sufficient for the full potentiation of cAMP, the potentiation of cGMP requires both activation of protein kinase C and a simultaneous elevation of $[Ca^{2+}]_i$ [7, 8]. Furthermore, studies using a protein kinase C inhibitor H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] indicate that inhibition of protein kinase C alone was only partially effective in inhibiting the NE-stimulated cyclic nucleotide responses [7]. Taken together, these findings suggest that an intracellular mechanism other than activation of protein kinase C may participate in the regulation of cAMP and cGMP in the pinealocytes. One such

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† Abbreviations: NE, norepinephrine; $[Ca^{2+}]_i$, intracellular Ca^{2+}; ISO, isoproterenol; PE, phenylephrine; PMA, 4β-phorbol 12-myristate 13-acetate; IC_{50}, concentration of drug inhibiting response by 50%; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; and EGTA, ethyleneglycolbis(aminoethylether)tetra acetate.

mechanism could be the Ca^{2+}-mediated activation of the Ca^{2+}/calmodulin pathway.

In the present study, the possible role of Ca^{2+}/calmodulin-dependent kinase in the regulation of pinealocyte cAMP and cGMP was investigated using Ca^{2+}/calmodulin inhibitors belonging to three different classes of drugs: trifluoperazine, pimozide and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7). To exclude the possibility that their site of action may include inhibition of the protein kinase C pathway, the *in vitro* action of these agents on protein kinase C activity was also determined.

MATERIALS AND METHODS

Materials. The naphthalene sulphonamide derivative, W7, was obtained from Seikagaku America Inc. (St. Petersburg, FL). Pimozide, trifluoperazine, NE, ISO, ionomycin, phosphatidylserine and diolien were obtained from Sigma (St. Louis, MO). PMA was purchased from Calbiochem (San Diego, CA), [γ-^{32}P]ATP was obtained from Amersham (Oakville, Ontario). ^{125}I-Labeled cAMP and cGMP were obtained from Bionetics Laboratories (Bethesda, MD). All other chemicals were of the purest grade available and were obtained commercially. Antibodies for the radioimmunoassays of cAMP and cGMP were gifts from Dr. A. Baukal (NICHD, NIH, Bethesda, MD).

Preparation and treatment of rat pinealocytes. Pinealocytes were prepared from male Sprague–Dawley rats (150 g, University of Alberta Animal Unit) by trypsinization as previously described [9]. The cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and maintained (37°) for 24 hr in a gas mixture of 95% air and 5% CO_2 before experimental treatment.

Aliquots of cells (2×10^4 cells/0.35 mL) were treated with chemicals which had been prepared in 100× concentrated solutions in water or dimethyl sulfoxide. The final concentration of the latter never exceeded 0.5%. The duration of the drug treatment period was 15 min. At the end of the treatment period, cells were collected by centrifugation (2 min, 10,000 *g*), the supernatant was aspirated, and the tube was placed on solid CO_2. The cell pellet was then stored at −70° until analysis.

In the experiment where protein kinase C activity was determined, pinealocytes were collected by centrifugation and the pellet was sonicated in a buffer containing 20 mM Tris–HCl, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, pH 7.5. The homogenate was then centrifuged at 12,000 *g* for 10 min, and the supernatant fraction was used as an enzyme source for protein kinase C determination.

Protein kinase C assay. Protein kinase C activity was assayed in triplicate as described previously [10]. The reaction mixture contained 20 mM Tris–HCl, 0.75 mM $CaCl_2$, 10 mM $MgCl_2$, 0.2 mg/mL histone, 0.5 mg/mL leupeptin, 0.1 mM ATP (100,000–500,000 cpm of [γ-^{32}P]ATP). Phosphatidylserine (96 μg/mL) and diolien (6.4 μg/mL) were added to some tubes to demonstrate phospholipid-dependent protein kinase activity; the three Ca^{2+}/calmodulin inhibitors (1–100 μM) were added directly to the assay solution as required. The reaction was initiated by adding 2 μg of pineal protein; the incubation (6 min, 30°) was stopped by the addition of 1 mL of trichloroacetic acid (25%, v/v; 4°); the precipitate was collected by filtration through a membrane filter (Whatman HA, 0.45 μm) which was washed five times with 2 mL of 5% trichloroacetic acid. ^{32}P bound to the filter was determined; protein kinase C activity was calculated from the difference in ^{32}P incorporated into histone, in the presence and absence of added phospholipids, and was expressed as nanomoles ^{32}P incorporated per 10 minutes per milligram protein.

Cyclic nucleotide assays. The frozen cell pellets were lysed by the addition of 5 mM acetic acid (100 μL) and boiling (5 min). The lysates were centrifuged (12,000 *g*, 10 min) and samples of the supernatant were used to estimate cellular cAMP and cGMP, using a radioimmunoassay procedure in which samples were acetylated prior to analysis [11]. Protein in the cell pellets was determined by a dye-binding method using bovine serum albumin as a standard [12].

Statistical analysis. Data are presented as the means ± SEM of the amount of cAMP or cGMP in three aliquots of cells. The amount of cyclic nucleotide in each cell pellet was based on duplicate determinations. Data were analyzed by Duncan's multiple range test [13].

RESULTS

Effects of Ca^{2+}/calmodulin inhibitors on ISO- and NE-stimulated cAMP and cGMP accumulation in dispersed pinealocytes. The three Ca^{2+}/calmodulin inhibitors used in the present study were pimozide, trifluoperazine and W7. To determine whether these inhibitors may act as adrenergic antagonists, their effects on ISO (a β-adrenergic agonist) and NE (a mixed α- and β-adrenergic agonist) stimulated cAMP and cGMP responses were investigated. None of these inhibitors had any effects on basal or β-adrenergically stimulated cAMP or cGMP responses (Table 1), suggesting that neither adenylate nor guanylate cyclase was sensitive to the inhibitors. NE stimulated cAMP and cGMP responses 100-fold as reported previously [3, 8] (Figs. 1 and 2). The inhibitors, trifluoperazine, pimozide and W7*, markedly reduced the NE-stimulated cAMP response in a concentration-dependent manner (Fig. 1) with estimated IC_{50} values of 0.1, 0.3 and 10 μM respectively. In the case of cGMP, all three compounds were also effective in inhibiting the NE-stimulated responses with respective IC_{50} values of 0.01, 0.1 and 3 μM (Fig. 2). Thus, it appears that the Ca^{2+}/calmodulin pathway is involved in both the NE-stimulated cAMP and cGMP responses.

Effects of Ca^{2+}/calmodulin inhibitors on cAMP and cGMP accumulation stimulated by ISO and PMA. PMA, an activator of protein kinase C,

* W5 [*N*-6-aminohexyl)-1-naphthalenesulfonamide], a related compound, was less potent than W7 in inhibiting the NE-stimulated cAMP and cGMP responses. W5 (30 μM) reduced the NE-stimulated cAMP and cGMP responses only by 10%.

Table 1. Effects of Ca^{2+}/calmodulin inhibitors on ISO-stimulated cAMP and cGMP accumulation

Treatment	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
Control	24.1 ± 1.8	2.7 ± 0.3
Isoproterenol (1 μM)	315.9 ± 5.1*	34.7 ± 3.4*
+Trifluoperazine (1 μM)	279.6 ± 26.7*†	27.6 ± 3.4*†
+Pimozide (10 μM)	287.7 ± 18.7*†	32.9 ± 1.2*†
+W7 (30 μM)	312.4 ± 17.9*†	26.1 ± 5.1*†
Trifluoperazine (1 μM)	20.6 ± 1.8	2.8 ± 0.6
Pimozide (10 μM)	24.8 ± 4.2	2.4 ± 0.6
W7 (30 μM)	22.6 ± 2.5	2.3 ± 0.8

Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with ISO (1 μM) in the presence or absence of the three calmodulin inhibitors. The inhibitors were added 30 sec prior to the addition of ISO. Each value is the mean ± SEM of cAMP or cGMP determinations done in duplicate on three samples of cells.

* Significantly different from control ($P < 0.05$).

† Not significantly different from isoproterenol-treated group ($P > 0.05$).

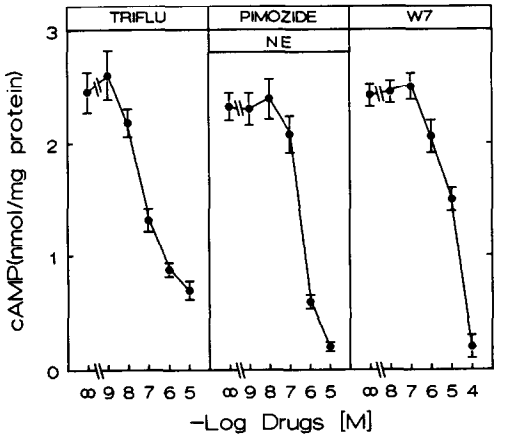

Fig. 1. Effects of trifluoperazine, pimozide, and W7 on NE-stimulatcd cAMP accumulation. Pincalocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with NE (10 μM) in the presence or absence of graded concentrations of calmodulin inhibitors. Inhibitors were added 30 sec before NE. Each point is the mean ± SEM of cAMP determinations done in duplicate on three samples of cells. cAMP was measured by radioimmunoassay. Similar results were obtained from at least two independent experiments.

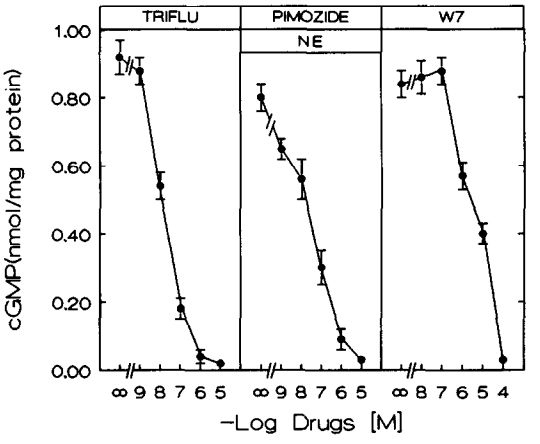

Fig. 2. Effects of trifluoperazine, pimozide, and W7 on NE-stimulated cGMP accumulation. Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with NE (10 μM) in the presence or absence of graded concentrations of calmodulin inhibitors. Inhibitors were added 30 sec before NE. Each point is the mean ± SEM of cGMP determinations done in duplicate on three samples of cells. Similar results were obtained from at least two independent experiments.

potentiates the ISO-stimulated cAMP accumulation more than 100-fold but has little potentiation effect on the cGMP response in rat pinealocytes [7]. Among the three Ca^{2+}/calmodulin inhibitors tested, trifluoperazine (10 nM to 10 μM) and W7 (1 to 30 μM) were ineffective (Fig. 3). Pimozide (>1 μM) inhibited the cAMP response to the combined treatment with ISO and PMA, indicating that at these concentrations, pimozide may have actions distinct from trifluoperazine or W7. These findings suggested that the Ca^{2+}/calmodulin pathway may not be an absolute requirement for the potentiation of cAMP response when protein kinase C has already been activated.

Effects of Ca^{2+}/calmodulin inhibitors on the K^+ and PMA potentiation of cAMP and cGMP accumulation in β-adrenergically stimulated pinealocytes. For the full potentiation of the ISO-stimulated cGMP response, both activation of protein kinase C and elevation of $[Ca^{2+}]_i$ are required [8]. This was achieved in the present study by treatment with PMA and a depolarizing concentration of K^+. In the presence of 12.5 mM K^+, PMA potentiated the ISO-stimulated cAMP and cGMP responses 100–fold (Figs. 4 and 5). The effects of the three calmodulin inhibitors on the cAMP responses were similar to those observed previously when the cells were stimulated by ISO and PMA. Pimozide reduced the cAMP response at

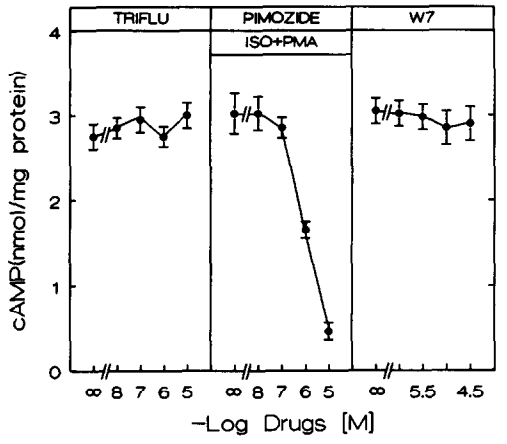

Fig. 3. Effects of trifluoperazine, pimozide, and W7 on PMA potentiation of cAMP response in β-adrenergically stimulated cells. Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with PMA (0.1 μM) and ISO (1 μM) in the presence or absence of graded concentrations of calmodulin inhibitors. Inhibitors were added 30 sec before ISO and PMA. Each point is the mean ± SEM of cAMP determinations done in duplicate on three samples of cells. Similar results were obtained from at least two independent experiments.

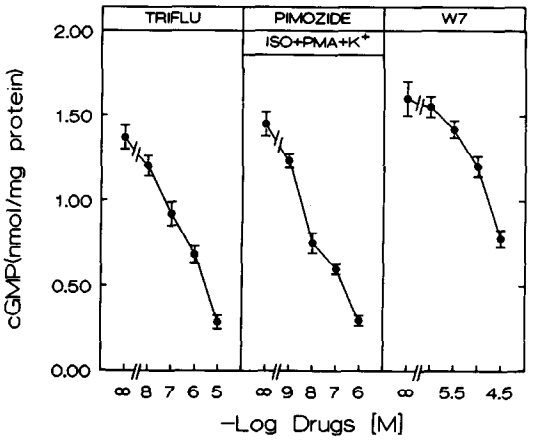

Fig. 5. Effects of trifluoperazine, pimozide, and W7 on PMA and K^+ potentiation of cGMP response in β-adrenergically stimulated cells. Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with PMA (0.1 μM), K^+ (12.5 mM) and ISO (1 μM) in the presence or absence of graded concuntrations of calmodulin inhibitors. Inhibitors were added 30 sec before ISO and PMA. Each point is the mean ± SEM of cGMP determinations done in duplicate on three samples of cells. Similar results were obtained from at least two independent experiments.

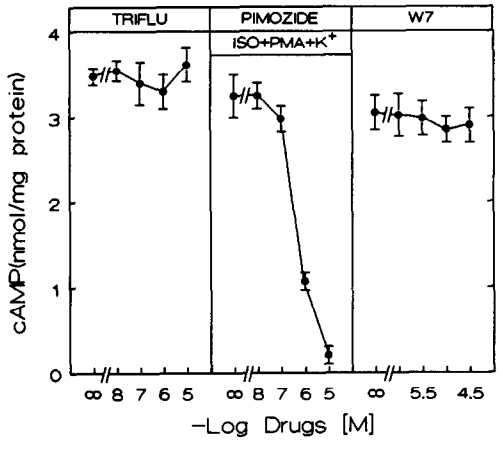

Fig. 4. Effects of trifluoperazine, pimozide, and W7 on PMA and K^+ potentiation of cAMP response in β-adrenergically stimulated cells. Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with PMA (0.1 μM), K^+ (12.5 mM) and ISO (1 μM) in the presence or absence of graded concentrations of calmodulin inhibitors. Inhibitors were added 30 sec before ISO, PMA and K^+. Each point is the mean ± SEM of cAMP determinations done in duplicate on three samples of cells. Similar results were obtained from at least two independent experiments.

high concentration and neither trifluoperazine nor W7 was effective (Fig. 4). This lack of effect of W7 and trifluoperazine on the potentiation effect of PMA, a protein kinase C activator, on the cAMP response suggested that at the concentration used, these two inhibitors had little effect on protein kinase C activities. In contrast, cGMP determination from these pinealocytes indicated that all three compounds were effective in inhibiting the cGMP responses in a concentration-dependent manner. Significant inhibition was observed with 0.1 μM trifluoperazine or pimozide and 10 μM W7 (Fig. 5). These data suggested that activation of the Ca^{2+}/calmodulin pathway may be required for the full potentiation of the cGMP response.

Effects of Ca^{2+}/calmodulin inhibitors on the potentiation by $[Ca^{2+}]_i$ elevating agents of cAMP and cGMP accumulation in ISO-stimulated pinealocytes. $[Ca^{2+}]_i$ elevating agents such as depolarizing concentrations of K^+, which activate a voltage-dependent Ca^{2+} channel, or Ca^{2+} ionophores can potentiate the ISO-stimulated cAMP response [5]. They differ from protein kinase C activators in that they are equally effective in potentiating the ISO-stimulated cGMP response [6]. Treatments with the three calmodulin antagonists inhibited the K^+ (45 mM) potentiation of ISO-stimulated cAMP and cGMP responses (Figs. 6 and 7) with similar IC_{50} values for both responses. The respective IC_{50} values for trifluoperazine, pimozide and W7 were 0.3, 0.1 and 3 μM (Figs. 6 and 7). To exclude the possibility that these antagonists may act via the voltage-dependent Ca^{2+} channel, ionomycin, which directly elevates $[Ca^{2+}]_i$, was used. All three Ca^{2+}/calmodulin inhibitors were effective in inhibiting the ionomycin potentiation of ISO-stimulated cAMP and cGMP responses (Table 2). In contrast, H7, an established inhibitor of protein kinase C, only had a small inhibiting action on the ionomycin potentiation of ISO-stimulated cyclic nucleotide responses (Table 2). Taken together, these results indicated that the site of action of these inhibitors was likely to be distal to elevation of $[Ca^{2+}]_i$ since the

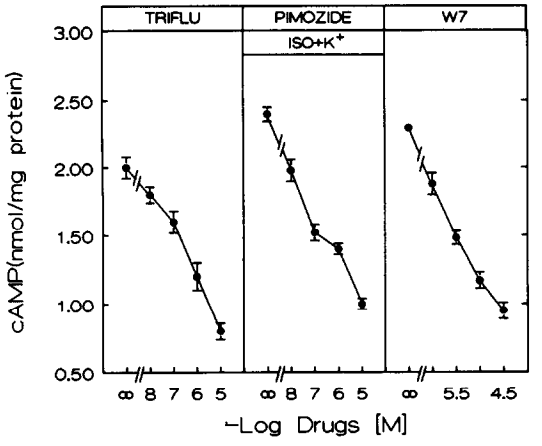

Fig. 6. Effects of trifluoperazine, pimozide, and W7 on K^+ potentiation of cAMP response in β-adrenergically stimulated cells. Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with K^+ (45 mM) and ISO (1 μM) in the presence or absence of graded concentrations of calmodulin inhibitors. Inhibitors were added 30 sec before ISO and K^+. Each point is the mean ± SEM of cAMP determinations done in duplicate on three samples of cells. Similar results were obtained from at least two independent experiments.

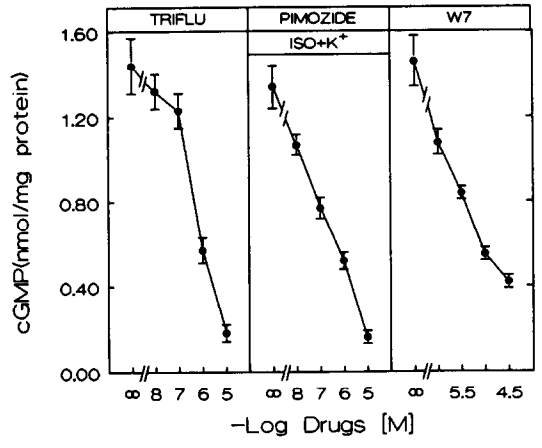

Fig. 7. Effects of trifluoperazine, pimozide, and W7 on K^+ potentiation of cGMP response in β-adrenergically stimulated cells. Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with K^+ (45 mM) and ISO (1 μM) in the presence or absence of graded concentrations of calmodulin inhibitors. Inhibitors were added 30 sec before ISO and K^+. Each point is the mean ± SEM of cGMP determinations done in duplicate on three samples of cells. Similar results were obtained from at least two independent experiments.

inhibitors were equally effective in inhibiting the potentiating effects of the Ca^{2+} ionophore.

Effects of Ca^{2+}/calmodulin inhibitors on protein kinase C activity. To exclude the possibility that these calcium/calmodulin inhibitors are acting via protein kinase C, the *in vitro* effects of these inhibitors on pinealocyte protein kinase C activity were determined. Addition of graded concentrations (1–100 μM) of trifluoperazine or W7 had little effects on the protein kinase C activity prepared from rat pineal glands (Table 3). Pimozide inhibited the protein kinase C activity at 100 μM, well above the concentrations used in the present study. W7 ($\leq$100 μM) had no effect on the *in vitro* protein kinase C assay though previously this compound has been shown to inhibit protein kinase C activities in heart and spleen tissues with IC_{50} values around 230 μM [14].

DISCUSSION

The two intracellular events that have been shown to be important in the α_1-adrenergic potentiation of β-adrenergically stimulated cAMP and cGMP responses are elevation of $[Ca^{2+}]_i$ and activation of protein kinase C. In the present study, the possible participation of another Ca^{2+}-dependent event, the Ca^{2+}/calmodulin pathway, in the regulation of pineal cyclic nucleotide production was evaluated using three compounds with known anti-calmodulin activity. Three inhibitors were used since most Ca^{2+}/calmodulin inhibitors have been found to be non-selective and had actions other than inhibition of calmodulin. The three compounds used were trifluoperazine, pimozide and W7. Each belonged to a different class of drug and they were chosen for their inhibitory potency of calmodulin [15, 16].

Two of the inhibitors have antipsychotic activity: trifluoperazine, a phenothiazine, and pimozide, a diphenylbutylpiperidine [15, 17]. These compounds have a number of properties distinct from their potent ability to bind and inhibit calmodulin. They include antagonistic actions towards α_1-adrenergic, dopaminergic and cholinergic receptors, alteration of membrane ionic permeability (at concentrations >10 μM), and inhibition of calmodulin-independent enzymes [17–19]. W7, a naphthalene sulfonamide derivative, however, appears to be more selective since it has no effect on either Ca^{2+} flux or adrenergic, dopaminergic, cholinergic or histaminergic receptors [20]. This compound, however, has been shown recently to inhibit the voltage-dependent calcium current in *Paramecia* [21], and in high concentration it also inhibits protein kinase C activity [14]. In the present study, all three compounds had potent inhibitory actions on the NE-stimulated cAMP and cGMP responses while having no effects on the basal or the β-adrenergically stimulated cAMP and cGMP responses. These findings indicate that none of these inhibitors has a direct effect on adenylate or guanylate cyclase activities. The effective inhibitory concentration as well as peak concentrations used (10 μM for trifluoperazine, 10 μM pimozide and 30 μM for W7) are in the selective range for inhibition of the Ca^{2+}/calmodulin pathway [22]. Furthermore, these three compounds are also effective in inhibiting the K^+ and ionomycin potentiation of β-adrenergically stimulated cyclic nucleotide responses. Both K^+ and ionomycin are treatments known to elevate $[Ca^{2+}]_i$ and mimic the α_1-adrenergic potentiation of β-adrenergically stimulated cAMP and cGMP responses. Taken together, these findings are consistent with the Ca^{2+}/calmodulin pathway

Table 2. Effects of calmodulin inhibitors on the ionomycin potentiation of ISO-stimulated cAMP and cGMP accumulation

Treatment	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
Experiment I		
Control	24.1 ± 1.8	2.7 ± 0.3
Ionomycin (10 μM)	27.3 ± 2.4	4.2 ± 1.0
Isoproterenol (1 μM)	329.5 ± 10.3	36.6 ± 4.8
+Ionomycin (10 μM)	2035.5 ± 107.3	1173.1 ± 41.4
+Trifluoperazine (1 μM)	1239.6 ± 144.8*	453.6 ± 53.4*
+Pimozide (1 μM)	1298.7 ± 238.5*	489.6 ± 22.8*
+W7 (10 μM)	513.4 ± 15.3*	151.8 ± 11.4*
Experiment II		
Control	31.6 ± 4.2	3.5 ± 0.8
Isoproterenol (1 μM)	303.6 ± 18.2	40.1 ± 5.3
+Ionomycin (10 μM)	1937.6 ± 83.4	1089.1 ± 67.3
+H7 (100 μM)	1703.1 ± 108.2*	839.2 ± 63.4*

Pinealocytes (2×10^4 cells/0.35 mL) were incubated for 15 min with ISO (1 μM) and ionomycin (10 μM) in the presence or absence of the three calmodulin inhibitors. The inhibitors were added 30 sec prior to the addition of ISO. Each value is the mean ± SEM of cAMP or cGMP determination done in duplicate on three samples of cells.

* Significantly different from isoproterenol and ionomycin-treated cells ($P < 0.05$).

Table 3. Effects of calmodulin inhibitors on protein kinase C activity in broken cell preparations

Treatment	Protein kinase C activity (nmol/mg protein/10 min)
Control	38.6 ± 1.31
Trifluoperazine	
1 μM	38.4 ± 1.78
10 μM	38.6 ± 0.83
100 μM	37.3 ± 0.18
Pimozide	
1 μM	38.5 ± 2.07
10 μM	35.0 ± 1.64
100 μM	31.4 ± 1.66*
W7	
1 μM	36.3 ± 1.03
10 μM	35.7 ± 2.37
100 μM	35.0 ± 2.34

Protein kinase C activity in the supernatant fraction (10,000 *g*, 10 min) was determined in the presence or absence of the indicated concentrations of the calmodulin inhibitors. See Materials and Methods for further details. Each value is the mean ± SEM of three determinations.

* Significantly different from control ($P < 0.05$).

being involved in the α_1-adrenergic potentiation of β-adrenergically stimulated cyclic nucleotide accumulation. Since these compounds are also effective in inhibiting the potentiating effects of ionomycin, the site of action is likely distal to elevation of $[Ca^{2+}]_i$.

The second intracellular mechanism that is of importance to the adrenergic regulation of cyclic nucleotide is activation of protein kinase C [7, 8]. It is possible that the three antagonists may be inhibitory to this pathway as in heart and spleen tissues [14]. This, however, is an unlikely explanation for the following reasons. First, even at a 100 μM concentration, only pimozide, the one compound that inhibited the PMA potentiation of ISO-stimulated cAMP response, had a small inhibitory effect on the *in vitro* kinase C activity. Second, the IC_{50} values for W7 in inhibiting protein kinase C activity in spleen and heart tissues are around 230 μM (approximately 10- to 100-fold greater than our observed IC_{50} values) in inhibiting the cyclic nucleotide responses. Third, the two compounds, W7 and trifluoperazine, which had no effects on protein kinase C activities, also had no effects on the PMA (a protein kinase C activator) potentiation of β-adrenergically stimulated cAMP responses. In contrast, H7, an established inhibitor of protein kinase C, reduces the PMA potentiation of β-adrenergically stimulated cAMP response [10]. It also should be noted that the maximum inhibition of the NE-stimulated cAMP and cGMP responses by H7 (100 μM) is only 50% [7] as compared to the nearly total inhibition by a 10 μM concentration of various Ca^{2+}/calmodulin inhibitors in the present study. Therefore, it appears that during NE stimulation, in addition to the protein kinase C pathway, the Ca^{2+}/calmodulin pathway is also likely to be activated in securing the cAMP and cGMP responses.

Previously, we have shown that while activation of protein kinase C alone is sufficient for the cAMP response, the cGMP response requires both activation of protein kinase C and a second Ca^{2+} mediated process [8]. Our present findings suggest that the Ca^{2+}/calmodulin pathway is a probable candidate for the second Ca^{2+}-mediated process. While the Ca^{2+}/calmodulin pathway appears to be involved in the regulation of cAMP when the response is potentiated by α_1-adrenoceptor activation

or $[Ca^{2+}]_i$ elevating agents, this pathway appears to be of little importance when PMA is used to activate protein kinase C. However, in the case of cGMP, the role of the Ca^{2+}/calmodulin pathway appears to be an absolute requirement even in the presence of protein kinase C activation by PMA. This is illustrated by the study using PMA and K^+ as potentiators of the cAMP and cGMP responses in ISO-treated cells. Apart from pimozide which inhibited protein kinase C activity at high concentration, neither trifluoperazine nor W7 had any effects on the cAMP responses. In contrast, all three compounds potently inhibited the cGMP responses.

The mechanism whereby Ca^{2+}/calmodulin modulates the production of cyclic nucleotides in the pinealocytes remains unclear. In the case of cAMP, it remains possible that the Ca^{2+}/calmodulin kinase may either phosphorylate the same substrate as protein kinase C or enhance the phosphorylation by protein kinase C. Such a substrate may be the adenylate cyclase itself or the G-protein involved in the cyclase activation. This explanation has also taken into account that when protein C is activated pharmacologically by potent agents such as PMA, Ca^{2+}/calmodulin may not be required for the full potentiation. As for the cGMP response, apart from the G-protein and the guanylate cyclase, other possible mechanisms may include an effect of Ca^{2+}/calmodulin on the phospholipase A_2 and/or the Na^+/H^+ antiporter [22, 23]. This is based on the previous demonstration that in contrast to the cAMP response, the cGMP response is extremely sensitive to inhibition of phospholipase A_2 or the Na^+/H^+ antiporter [23, 24]. The precise mechanism, however, remains to be determined.

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