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EFFECTS OF PROTEIN KINASE INHIBITOR (1-(5-ISOQUINOLINESULFONYL)-2-METHYLPIPERAZINE (H7) ON PROTEIN KINASE C ACTIVITY AND ADRENERGIC STIMULATION OF cAMP AND cGMP IN RAT PINEALOCYTES

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Abstract—Protein kinase C is thought to be involved in the adrenergic regulation of pineal function. In this tissue, norepinephrine increases cAMP and cGMP accumulation through a synergistic dual receptor mechanism involving α_1- and β-adrenergic receptors; the available evidence indicates that the α_1-adrenergic stimulation activates protein kinase C, and that this potentiates β-adrenergic stimulation of pineal cAMP. The role of protein kinase C in the regulation of cGMP is unclear. In the present report, we determined whether an inhibitor of protein kinase C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), inhibits pineal protein kinase C and the adrenergic stimulation of pineal cAMP and cGMP. H7 (10^{-4} M) reduced soluble protein kinase C activity by 40%. Treatment of intact pinealocytes with H7 for 0–240 min reduced the effects of subsequent norepinephrine (NE) stimulation of cAMP and cGMP accumulation by at least 25%. H7 also inhibited 25–30% the maximum stimulation of both cAMP and cGMP produced by concurrent treatment with isoproterenol and two agents which elevate intracellular Ca^{2+}, ouabain and A23187. However, H7 did not reduce the effects of selective β-adrenergic activation, indicating that H7 was probably inhibiting the effects of NE by blocking α_1-adrenergic potentiation of β-adrenergic stimulation, not β-adrenergically activated mechanisms. H7 also reduced the stimulation of cAMP accumulation produced by the combined treatment of isoproterenol and an activator of protein kinase C, 4-β-phorbol 12-myristate, 13-acetate, which is consistent with the view that H7 is acting by inhibiting protein kinase C activity. These observations are in agreement with the conclusion that potentiation of β-adrenergic stimulation of cAMP by α_1-adrenergic agonists, protein kinase C activators, or $[Ca^{2+}]_i$ elevating agents involves protein kinase C. In addition, these results are of special interest because they point to the possibility that protein kinase C is involved in the regulation of cGMP accumulation.

The amounts of cAMP and cGMP in the rat pinealocyte are elevated by norepinephrine (NE)§, acting through a synergistic dual receptor mechanism [1, 2]. Activation of β-adrenoceptors is an absolute requirement; selective β-adrenergic agents produce a 7- to 10-fold increase in cAMP accumulation, and a 2- to 4-fold increase in cGMP accumulation. α_1-Adrenoceptor activation by itself has no effect on cAMP or cGMP accumulation, but it potentiates the effects of β-adrenergic stimulation, resulting in 100- to 300-fold increases in cAMP and cGMP accumulation.

Activation of pineal α_1-adrenoceptors appears to activate protein kinase C through elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$ [3]) and phospholipase C activity [4–6]. Protein kinase C seems to be involved in the regulation of cAMP accumulation because established activators of protein kinase C, including 4-β-phorbol 12-myristate,13-acetate (PMA), mimic the effects of α_1-adrenergic activation. Similarly, $[Ca^{2+}]_i$ elevating agents, which are thought to activate pineal protein kinase C, also mimic the effects of selective α_1-activation on the accumulation of cAMP in β-adrenergically stimulated pinealocytes [7].

In contrast to the existing evidence indicating that protein kinase C is involved in the regulation of cAMP accumulation, the evidence of a role of protein kinase C in the regulation of cGMP content is in conflict: PMA does not potentiate β-adrenergic stimulation of cGMP accumulation [8], but $[Ca^{2+}]_i$ elevating treatments do have this α_1-adrenergic-like potentiating effect [7]. The conflicting evidence regarding the role of protein kinase C in the regulation of cGMP accumulation and our general interest in the regulation of cAMP accumulation have stimulated us to evaluate the effects of a reported inhibitor of protein kinase C. This compound, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7; [9]),

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§ Abbreviations: NE, norepinephrine; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; $[Ca^{2+}]_i$, intracellular free Ca^{2+}; PMA, 4-β-phorbol 12-myristate,13-acetate; ED_{50}, dose of drug producing a 50% maximal response; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; and ISO, isoproterenol.

has been used to study the role of protein kinase C in various physiological responses [10, 11]. In the present studies, we evaluated the effects of H7 on pineal protein kinase C activity and on stimulation of cAMP and cGMP accumulation.

MATERIALS AND METHODS

Materials. H7 was obtained from Seikagaku America Inc. (St. Petersburg, FL). NE, isoproterenol (ISO), ouabain, A23187, phosphatidylserine and diolien were obtained from Sigma (St. Louis, MO). 4-β-Phorbol 12-myristate, 13-acetate (PMA) was purchased from Calbiochem (San Diego, CA). [γ-^{32}P]ATP was obtained from ICN (Irvine, CA). Iodinated cAMP and cGMP were obtained from Meloy Laboratories (Springfield, VA). All other chemicals were of the purest grade available and were obtained commercially. Antibodies for the radioimmunoassays of cAMP and cGMP were a gift from Dr. K. Catt (NICHD, NIH, Bethesda, MD).

Preparation and treatment of rat pinealocytes. Pinealocytes were prepared from female Sprague–Dawley rats (200 g) by trypsinization as previously described [1, 12]. The cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and maintained (37°) for 24 hr in a gas mixture of 95% air and 5% CO_2 before experimental treatment.

Aliquots of cells (10^5 cells/0.5 ml) were treated with chemicals that had been prepared in 100× concentrated solutions in water or dimethyl sulfoxide. The final concentration of the latter never exceeded 1%. When H7 was used, the treatment period was 10 min, unless otherwise indicated (see Table 2), and preceded the initiation of treatments with other chemicals. The duration of the subsequent treatment period was 15 min, except in the case of the time course experiment.

At the end of the treatment period, cells were collected by centrifugation (2 min, 1000 *g*), the supernatant fraction 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 2×10^{-2} M Tris–HCl, 2×10^{-3} M EDTA, 5×10^{-4} M EGTA, 2×10^{-3} M phenylmethylsulphonyl 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 [8, 13]. The reaction mixture contained 2×10^{-2} M Tris–HCl, 7.5×10^{-4} M $CaCl_2$, 10^{-2} M $MgCl_2$, 0.2 mg/ml histone, 0.5 mg/ml leupeptin, and 10^{-4} M 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 (+ phospholipids, legend to Table 1); H7 (10^{-4} to 10^{-6} M) was 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 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 nmol ^{32}P incorporated per 6 min per mg protein.

Cyclic nucleotide assay. 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 fraction were used to estimate cellular cAMP and cGMP, using a radioimmunoassay procedure in which samples are acetylated prior to analysis [1, 14]. Protein in the cell pellets was determined by a dye binding method using bovine serum albumin as a standard [15]. There was a small batch-to-batch variation of the cyclic nucleotide responses between cell preparations. However, all comparisons were performed within the same batch of cells.

Statistical analysis. Data are presented as the mean ± 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 [16].

RESULTS

Effect of H7 on protein kinase C activity in broken cell preparations. Addition of H7 (10^{-4}–10^{-6} M) to protein kinase C assays caused a dose-dependent

Table 1. Effect of H7 on protein kinase activity in broken cell preparations

Treatment	Protein kinase activity (nmol/mg protein/6 min) −Phospholipids	+Phospholipids
Control	4.4 ± 0.14	23.1 ± 0.57
+H7 (10^{-4} M)	4.1 ± 0.28	13.4 ± 0.54*
+H7 (10^{-5} M)	4.8 ± 0.37	20.0 ± 0.44*
+H7 (10^{-6} M)	4.3 ± 0.28	22.0 ± 0.40

Phospholipid-dependent (protein kinase C) and -independent protein kinase activities in the supernatant fraction (10,000 *g*, 6 min) were determined in the presence or absence of the indicated concentration of H7. See Materials and Methods for further details. Each value represents the mean ± SEM of three determinations.

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

Table 2. Relationship between the duration of the H7 pretreatment period prior to NE stimulation and the effects of H7 on cAMP and cGMP accumulation

Treatment	Length of H7 treatment (min)	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
Control		10.1 ± 1.7	0.8 ± 0.2
NE (3×10^{-7} M)		521 ± 55.6	181 ± 23.5
+H7 (10^{-4} M)	0	271 ± 17.1*	41.5 ± 7.5*
+H7 (10^{-4} M)	10	287 ± 2.5*	39.5 ± 4.8*
+H7 (10^{-4} M)	30	274 ± 4.1*	42.0 ± 6.1*
+H7 (10^{-4} M)	60	268 ± 12.9*	30.9 ± 6.6*
+H7 (10^{-4} M)	240	295 ± 3.4*	31.7 ± 5.4*

Pinealocytes were treated with H7 (10^{-4} M) for up to 240 min prior to addition of NE (3×10^{-7} M). Each value represents the mean ± SEM of cAMP or cGMP in three aliquots of cells (10^5 cells/0.5 ml); each determination was performed in duplicate.
* Significantly different from the NE-treated groups ($P < 0.05$).

reduction in phospholipid-dependent kinase activity, but it had no effect on phospholipid-independent kinase activity (Table 1). A 10^{-4} M concentration of H7 reduced protein kinase C activity by 40%; at a concentration of 10^{-6} M, H7 had no significant effect. This indicates that H7 inhibits pineal protein kinase C activity with similar potency as reported using platelet homogenates [17].

Effect of H7 treatment on NE stimulation of cAMP and cGMP accumulation in intact pinealocytes. H7 (10^{-4} M) alone did not alter cAMP or cGMP accumulation. However, it inhibited NE (3×10^{-7} M) stimulation of cAMP accumulation by about 50% and that of cGMP accumulation by about 75%; the effect was similar when treatment was initiated 0–240 min before NE was added (Table 2). In the subsequent experiments, H7 was added 10 min before the initiation of other treatments.

The effects of H7 (10^{-4} M) on the NE-cAMP and NE-cGMP dose–response curves were determined. H7 reduced the maximum cAMP and cGMP responses produced by NE (10^{-5} M) treatment by about 25%, but it had little effect on the apparent ED_{50} (Fig. 1). This inhibitory effect of H7 was dose dependent (Table 3). The effect of H7 on the time course of the NE stimulation of cAMP and cGMP was determined (Fig. 2). H7 (10^{-4} M, 10 min) reduced the cAMP and cGMP responses to NE (3×10^{-7} M) at all times examined.

To determine if H7 was inhibiting β-adrenergically stimulated events, we examined the effects of H7 on cAMP and cGMP accumulation in cells treated with ISO (10^{-6} M), which should selectively activate β-adrenergic systems [2]. H7 did not inhibit the effects of ISO (Table 3). This indicates that H7 has no direct effect on cyclic nucleotide phosphodiesterase.

Effect of H7 on cAMP and cGMP accumulation in pinealocytes treated with ISO and agents which elevate $[Ca^{2+}]_i$. The cAMP and cGMP contents of ISO-treated pinealocytes increased markedly in

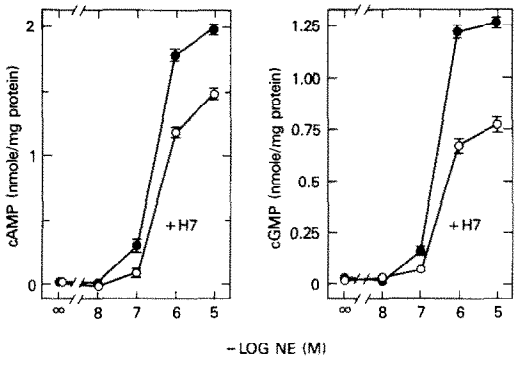

Fig. 1. Effect of H7 (10^{-4} M) on the NE-cAMP and NE-cGMP dose–response curves in rat pinealocytes. Pinealocytes were treated with H7 for 10 min before the initiation of a 15-min treatment period with NE. For further technical details, see Materials and Methods. Each point represents the mean ± SEM of cAMP or cGMP in three aliquots of cells; each determination was performed in duplicate. The absence of an error bar indicates that the SEM fell within the area of the symbol.

Table 3. Dose–response study on the effects of H7 on the NE (10^{-6} M) stimulation of cAMP and cGMP accumulation in rat pinealocytes

Treatment	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
Control	18.1 ± 2.0	1.2 ± 0.4
H7 (10^{-4} M)	15.6 ± 1.7	1.5 ± 0.1
NE (10^{-6} M)	1810 ± 75.5	1210 ± 34.9
+H7 (10^{-4} M)	1390 ± 85.3*	760 ± 7.5*
+H7 (10^{-5} M)	1530 ± 129.4*	930 ± 44.0*
+H7 (10^{-6} M)	1870 ± 112.9	1130 ± 42.2
ISO (10^{-6} M)	120 ± 1.8	12.5 ± 1.7
+H7 (10^{-4} M)	109 ± 10.6	11.7 ± 2.2

Pinealocytes were treated with H7 for 10 min before the addition of NE. For further details see Materials and Methods. Each value represents the mean ± SEM of cAMP or cGMP in three aliquots of cells; each determination was performed in duplicate.
* Significantly different from the NE-treated group ($P < 0.05$).

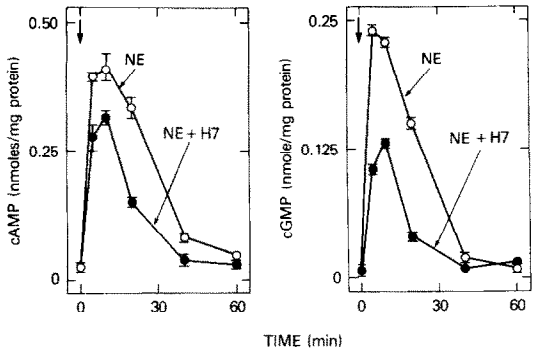

Fig. 2. Effect of H7 (10^{-4} M) on the time-course of NE (3×10^{-7} M) stimulation of cAMP and cGMP accumulation in rat pinealocytes. Pinealocytes were treated with H7 for 10 min before the addition of NE. The arrows indicate when NE was added. For further technical details, see Materials and Methods. Each point represents the mean ± SEM of cAMP or cGMP in three aliquots of cells; each determination was performed in duplicate. The absence of an error bar indicates that the SEM fell within the area of the symbol.

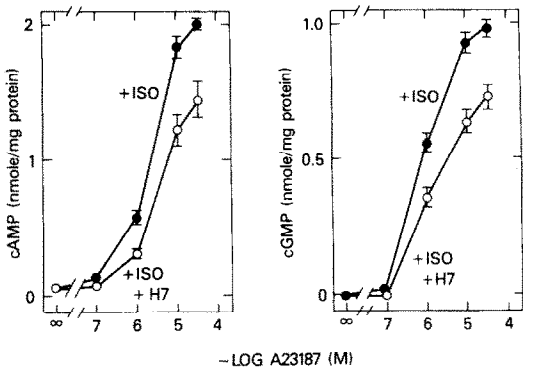

Fig. 4. Effect of H7 (10^{-4} M) on the A23187-cAMP and A23187-cGMP dose-response curves in ISO (3×10^{-7} M) -treated rat pinealocytes. Pinealocytes were treated with H7 for 10 min before the initiation of a 15-min treatment period with ISO and the indicated concentration of A23187. For further technical details, see Materials and Methods. Each point represents the mean ± SEM of cAMP or cGMP in three aliquots of cells; each determination was performed in duplicate. The absence of an error bar indicates that the SEM fell within the area of the symbol.

response to treatment with agents that elevate $[Ca^{2+}]_i$ [7]. Two of these are the Ca^{2+} ionophore A23187 and the Na^+, K^+-ATPase inhibitor ouabain. Ouabain had a very gradual and weak effect on pinealocyte $[Ca^{2+}]_i$*, but also activates phospholipase C activity [18]. In this series of studies we found that H7 (10^{-4} M) treatment reduced the maximal stimulation of cAMP and cGMP produced by combined treatment with ISO and A23187 or ISO and ouabain by 25–35%, without any apparent changes in the ED_{50} of A23187 or ouabain (Figs 3 and 4).

Effect of H7 on cAMP and cGMP in pinealocytes treated with ISO and PMA. As discussed at the beginning of the paper, pinealocyte cAMP is stimulated by the combined treatment of ISO and PMA; the former activates adenylyl cyclase via β-adrenoceptors and the latter activates pineal protein kinase C [8, 19]. H7 (10^{-4} M) reduced by 25% the increase in cAMP produced by the combined treatment of PMA (10^{-9} to 10^{-6} M) and ISO (10^{-6} M) (Fig. 5).

* A. L. Sugden, D. Sugden, A. K. Ho and D. C. Klein, unpublished observations.

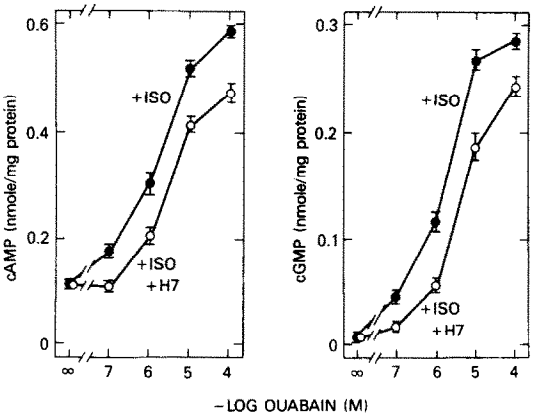

Fig. 3. Effect of H7 (10^{-4} M) on the ouabain-cAMP and ouabain-cGMP dose-response curves in ISO (3×10^{-7} M) -treated rat pinealocytes. Pinealocytes were treated with H7 for 10 min before the initiation of a 15-min treatment period with ISO and the indicated concentration of ouabain. For further technical details, see Materials and Methods. Each point represents the mean ± SEM of cAMP or cGMP in three aliquots of cells; each determination was performed in duplicate. The absence of an error bar indicates that the SEM fell within the area of the symbol.

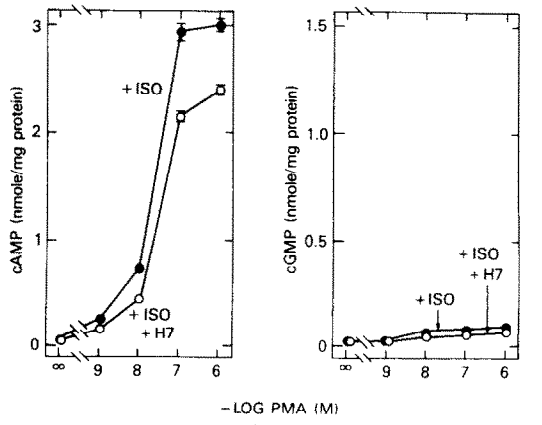

Fig. 5. Effect of H7 (10^{-4} M) on the PMA-cAMP and PMA-cGMP dose-response curves in ISO (3×10^{-7} M) -treated rat pinealocytes. Pinealocytes were treated with H7 for 10 min before the initiation of a 15-min treatment period with ISO and the indicated concentration of PMA. For further technical details, see Materials and Methods. Each point represents the mean ± SEM of cAMP or cGMP in three aliquots of cells; each determination was performed in duplicate. The absence of an error bar indicates that the SEM fell within the area of the symbol.

This reduction is similar to that seen above (Figs. 1–4). There was no apparent change in the ED_{50} of the potentiating effect of PMA on cAMP accumulation in ISO-treated pinealocytes.

As previously reported [8], PMA had no effect on cGMP accumulation in ISO-treated pinealocytes (Fig. 5).

DISCUSSION

This investigation revealed that H7 (10^{-4} M) partially inhibited pineal protein kinase C activity and the NE stimulation of cAMP and cGMP accumulation in intact cells. This set of observations is consistent with the hypothesis that the effects of NE involve protein kinase C; other evidence is consistent with the interpretation that protein kinase C mediates the α_1-adrenergic potentiation of the effects of β-adrenergic stimulation.

First, we obtained indirect evidence that α_1-adrenergic mechanisms involve protein kinase C, because H7 did not inhibit the effects of selective β-adrenergic activation. This is consistent with the evidence that α_1-adrenergic stimulation activates protein kinase C, and that α_1-adrenergic effects on cAMP involve activation of protein kinase C [8]. Second, we found that H7 inhibited the effects of the protein kinase C activator PMA, which indicates that H7 is probably inhibiting protein kinase C activity in these studies. Accordingly, the simplest explanation consistent with these and other observations [8] is that H7 inhibits NE stimulation of cAMP and cGMP by inhibiting protein kinase C activity. However, in view of the high concentration of H7 required, the possibility that calmodulin-, cAMP- or cGMP-dependent kinases are inhibited cannot be excluded [9].

The conclusion that H7 is probably inhibiting protein kinase C activity is also supported by the similar potency of H7 in inhibiting protein kinase C activity in broken cell preparations and in inhibiting the stimulation of cAMP accumulation in intact cells produced by treatment with either NE or the combination of PMA and ISO. It is of interest to point out that the partial nature of H7 inhibition is in agreement with the partial inhibition of protein kinase C protein phosphorylation seen in rabbit platelets [17]. Partial inhibition of protein kinase C activity might reflect the presence of multiple forms of protein kinase C, with differential sensitivity to H7.

The observation that H7 inhibited the effects of the combined treatments with ISO and A23187 or ISO and ouabain supports the hypothesis that $[Ca^{2+}]_i$ elevating agents potentiate the effects of β-adrenergic stimulation through activation of protein kinase C [7]. It appears that an increase in $[Ca^{2+}]_i$ alone may be sufficient to translocate protein kinase C if sufficient diacylglycerol is present [20, 21]. This can be tested directly by measuring protein kinase C translocation.

Although these observations support the view that NE stimulation of cGMP involves protein kinase C, this is inconsistent with the observation that activators of protein kinase C do not potentiate the ISO stimulation of cGMP [8]. This inconsistency clouds our understanding of the mechanism through which α_1-adrenergic receptors potentiate the β-adrenergic stimulated cGMP response.

One explanation of this inconsistency is that α_1-adrenergic potentiation of the ISO stimulation of cGMP not only requires activation of protein kinase C but also another variable. Comparing the effect of the activators of protein kinase C with α_1-adrenergic activators, it is clear that the former do not mimic all the effects of α_1-adrenergic stimulation, which include activation of phospholipase C [4–6], phospholipase A_2 [22], and protein kinase C [8] and elevation of $[Ca^{2+}]_i$ [3]. However, we found that PMA did not stimulate phospholipase C activity or elevate $[Ca^{2+}]_i$.* This points to an increase in phospholipase C activity or in $[Ca^{2+}]_i$, or both, as possible candidates for the additional events required for stimulation of cGMP accumulation. Thus, it may be possible to demonstrate experimentally an effect of activators of protein kinase C on cGMP accumulation in ISO-treated cells by either elevating phospholipase C activity or $[Ca^{2+}]_i$, or both. This is currently being investigated.

Another possibility which should be considered is that the α_1-adrenergic regulation of cGMP does not involve protein kinase C. Perhaps another mechanism is activated by the increase in $[Ca^{2+}]_i$, and that this mechanism is inhibited by H7 acting independently of protein kinase C. Thus, although the present data point to an involvement of protein kinase C in the adrenergic regulation of cGMP, more experimental evidence is required to clearly establish this functional relationship.

Note added in proof: Additional evidence that protein kinase C is involved in the adrenergic regulation of cGMP has been published recently. In these studies we found that activators of protein kinase C can stimulate cGMP accumulation in β-adrenergically stimulated cells if $[Ca^{2+}]_i$ is elevated to a minor degree (Ho A. K., Chik C. L. and Klein D. C., Protein kinase C is involved in regulation of pineal cGMP accumulation. *Journal of Biological Chemistry* **262**, 10059–10064, 1987).

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