Protein Kinase C Is Involved in Adrenergic Stimulation of Pineal cGMP Accumulation*

Anthony K. Ho†, Constance L. Chik‡‡, and David C. Klein§

From the †Section on Neuroendocrinology, Laboratory of Developmental Neurobiology and the §Developmental Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

The amounts of cAMP and cGMP in the rat pinealectocyte are regulated by norepinephrine acting through synergistic dual receptor mechanisms involving α1- and β-adrenoceptors (Vanecek, J., Sugden, D., Weller, J. L., and Klein, D. C. (1985) Endocrinology 116, 2167-2173; Sugden, L., Sugden, D., and Klein, D. C. (1986) J. Biol. Chem. 261, 11608-11612). Based on the available evidence, it appears that Ca²⁺/phospholipid-dependent protein kinase is involved in the α1-adrenergic potentiation of β-adrenergic stimulation of cAMP, but not in the stimulation of cGMP (Sugden, D., Vanecek, J., Klein, D. C., Thomas, T. P., and Anderson, W. B. (1985) Nature 314, 359-361). In the present study the role of protein kinase C in the adrenergic stimulation of cGMP was reinvestigated, with the purpose of determining whether protein kinase C activators would potentiate the effects of β-adrenergic agonists on cGMP if cells were also treated with agents known to elevate intracellular free Ca²⁺. The protein kinase C activator 4β-phorbol 12-myristate 13-acetate (PMA) markedly elevated the cGMP content of β-adrenergically stimulated pinealectocytes that had also been treated with 1 μM A23187, 15 mM K⁺, or 1 μM ouabain. The effects of A23187 were blocked by EGTA and those of K⁺ were blocked by nifedipine, establishing the involvement of Ca²⁺. The stimulatory effects of PMA on cGMP accumulation were mimicked by other protein kinase C activators. PMA also stimulated cGMP accumulation in cells treated with cholera toxin (1 μg/ml) and A23187 (1 μM), but not in cells treated only with cholera toxin. These results suggest that protein kinase C, which is activated in the pinealectocyte by the α-adrenergic agonist phenylephrine, is probably involved in the adrenergic regulation of cGMP accumulation at a step distal to receptor activation.

Dual receptor regulatory mechanisms generate large and highly selective changes in cytosolic concentrations of cyclic nucleotides (1-5). An example of this occurs in the rat pinealectocyte where norepinephrine stimulates cAMP and cGMP accumulation by concurrent activation of α1- and β-adrenoceptors (6). β-Adrenergic activation produces a 7-10-fold increase in cAMP and a 2-4-fold increase in cGMP. Selective of α1-adrenoceptor stimulation alone has no effect on cAMP or cGMP, but potentiates β-adrenergic stimulation of cAMP by about 10-fold and β-adrenergic stimulation of cGMP about 50-100-fold (6). These interactions generate >100-fold increases in pinealectocyte cyclic nucleotides.

α1-Adrenoceptor potentiation of β-adrenergic stimulation of cAMP appears to be mediated by Ca²⁺-phospholipid-dependent protein kinase C, as indicated by the finding that the α1-adrenergic agonist phenylephrine activates/translocates protein kinase C (7), and that activators of protein kinase C potentiate β-adrenergic stimulation of cAMP accumulation (7). In contrast, protein kinase C activators have not been found to potentiate β-adrenergic stimulation of cGMP accumulation in pinealectocytes (6). Based on this, it has been assumed that protein kinase C is not involved in the regulation of cGMP.

However, there is reason to suspect that this assumption might be wrong. First, protein kinase C has been found to phosphorylate purified brain guanylyl cyclase in vitro, resulting in an increase in catalytic activity (8). Second, a compound which inhibits protein kinase C activity reduces adrenergic stimulation of pinealectocyte cAMP and cGMP accumulation. Third, protein kinase C activators stimulate pineal phospholipase A₂, which is thought to be involved in the adrenergic regulation of pinealectocyte cGMP accumulation (10); involvement of phospholipase A₂ in cGMP regulation is consistent with published reports linking arachidonic acid metabolites and guanylyl cyclase in other tissues (11, 12). However, although these indications suggest that protein kinase C might be involved in regulation of cGMP accumulation, they are unconvincing light of the observation that protein kinase C activators do not potentiate the effects of β-adrenergic stimulation of cGMP accumulation (7).

In searching for an explanation of why protein kinase C activators have not been found to potentiate β-adrenergic stimulation of cGMP content of pinealectocytes, it became apparent that the potential importance of [Ca²⁺]i (13, 14) was ignored. Although both α₁-adrenergic activators and PMA activate protein kinase C, only the former elevates [Ca²⁺]i (13, 14) with this in mind and with the knowledge that stimulation of cGMP accumulation is generally recognized to be more Ca²⁺-dependent.

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed; National Institutes of Health, Bldg. 10, Rm. 8D42C, Bethesda, MD 20892.

‡§Canadian Medical Research Council Fellow.

1The protein kinase inhibitor 1-(5-isquinolinylsulfonyl)-2-methylpiperazine (H7) (14) significantly reduces the stimulation of pineal cAMP and cGMP accumulation by norepinephrine (A. K. Ho, C. L. Chik, and D. C. Klein, unpublished results).


3The abbreviations used are: [Ca²⁺]i, intracellular free Ca²⁺; EGTA, ethylene bis(oxyethylene)nitrotriacetic acid; PMA, 4β-phorbol 12-myristate 13-acetate; PDD, 4β-phorbol 12,13-didecanoate; PDBu, 4β-phorbol 12,13-dibutyrate; 8AG, 1-thio, 2-acetylglycerol.


5In unpublished studies we have found that the α1-adrenoceptor mediates the adrenergic stimulation of protein kinase C (A. K. Ho, T. P. Thorsaas, W. Anderson, and D. C. Klein, unpublished studies).
dependent than stimulation of cAMP accumulation (15), we
decided to determine whether protein kinase C activators
might elevate cGMP content of β-adrenergically stimulated
pinealocytes if [Ca2+]i was elevated. Several agents that are
known to increase pinealocyte [Ca2+]i, (13) were used;
the results of this study are presented here.

EXPERIMENTAL PROCEDURES

Materials—Isoproterenol, nifedipine, diolein, 1-octeyl-2-acetyl-
glycerol (OAG), ouabain, A23187, and EGTA were obtained from
Sigma. Phorbol esters were purchased from Behring Diagnostics.
Cholera toxin was purchased from List Biological Laboratories
(Campbell, CA). All other drugs and chemicals were from other
commercial sources and were of the purest grade available. Antibodies
for the radioimmunoassays of cAMP and cGMP were gifts from Dr.
K. Catt (National Institute of Child Health and Human Development,
National Institutes of Health, Bethesda, MD). Sprague-Dawley rats
(female, 200 g) were obtained from Charles River Breeding Labora-
tories.

Preparation and Treatment of Rat Pinealocytes—Pinealocytes were
prepared from glands by trypsinization as previously described (6,
16). Cells were then suspended in Dulbecco’s modified Eagle’s medium
(containing 10% fetal calf serum) and incubated for 24 h in a gas
mixture of 95% air, 5% carbon dioxide. Aliquots of cells (106 cells/0.5
ml) were prepared and treated with drugs for 15 min unless indicated
otherwise. Drugs were dissolved in water, ethanol or dimethyl sul-
foxide and added at less than 1% of the total volume. At this concen-
tration, ethanol or dimethyl sulfoxide has no effect on the cAMP and
cGMP responses of pinealocytes to norepinephrine. After treatment,
the cells were collected by centrifugation (2 min, 1000 × g), the
supernatant was aspirated, and the pellet was immediately frozen in
solid CO2.

Assays—The frozen cell pellet was lysed by boiling for 3 min in 5
ml acetic acid (100 µl). The preparation was then centrifuged (12,000
× g, 10 min), and the supernatant was used for determinations of
cAMP and cGMP by radioimmunoassay (6, 17). When necessary,
samples were further diluted with the acetic acid. Protein in the cell
pellets was determined by a dye-binding method using bovine serum
albumin as a standard (18).

Statistical Analysis—Data are presented as the mean ± S.E. of
cAMP or cGMP in three aliquots of cells. Each analysis was performed
in duplicate. Statistical comparisons were by Bartlett’s test for het-
 ergogeneity of variance and Duncan’s multiple range test (19).

RESULTS

Effects of A23187 on the cAMP Response to Protein Kinase
C Activators in β-Adrenergically Stimulated Pinealocytes—
The purpose of this first series of studies was to determine
whether elevation of [Ca2+]i, with Ca2+ ionophore A23187
would reveal an effect of protein kinase C activators on cGMP
accumulation in β-adrenergically stimulated pinealocytes. High concentrations of A23187 alone (EC50 3 pM) mimic
the effects of α1-adrenergic agonists on the cGMP content of
β-adrenergically stimulated pinealocytes (20). Accordingly, in
these experiments, a concentration of A23187 (1 µM) was
chosen which produces a small effect on the cGMP content of iso-
proterenol-treated cells (20). The concentration of isopro-
terol (1 µM) chosen selectively activates β-adrenoceptors (6).
Cellular cAMP was also measured in this and the follow-
ing studies to provide a basis of comparison and to determine
the relative effects of the experimental treatments on cAMP.

As previously reported, 4β-phorbol 12-myristate 13-acetate
(PMA, 0.1-100 nM) alone had no effect on cellular cGMP in
isoproterenol-treated pinealocytes, but stimulated cellular
cAMP about 100-fold (6). In the presence of 1 µM A23187,
PMA produced a dose-dependent increase in cGMP (Fig. 1).
This is the first indication that PMA treatment will increase
cGMP in β-adrenergically stimulated cells, providing that
[Ca2+]i is also elevated. The influence of A23187 (1 µM) on the
PMA stimulation of the cAMP content of isoproterenol-
treated cells was less dramatic; it appeared to reduce the ED50
for PMA slightly. This finding indicates that [Ca2+]i, in these
cells is nearly sufficient to support a maximal or near maximal
increase in cAMP content in response to PMA. Precise de-
termination of the ED50 for PMA for the cGMP response in
these experiments is not possible because an unequivocal
maximum response was not observed. However, assuming that
the maximum response was achieved at 100 nM, then it would
appear that the dose required to produce half-maximal cAMP
and cGMP responses is about 3 nM.

The stimulatory effect of PMA on the cGMP content of
A23187- and isoproterenol-treated pinealocytes was mimicked by
two other protein kinase C activators, OAG and 4β-phorbol
12,13-dibutyrate (PDBu), but not by diolein or 4α-phorbol
13,15-didecanoate (PDD), which are known not to activate
protein kinase C (21, Fig. 2). This suggests to us that the
Effect of PMA on cGMP accumulation is probably due to protein kinase C activation.

Effects of K+ (15 mM) on the cGMP Response to Protein Kinase C Activators in β-Adrenergically Stimulated Pinealocytes—K+ (15 mM) treatment produces a small elevation of rat pinealocyte [Ca2+]. (10) and a partial potentiation of isoproterenol-stimulated cGMP accumulation (ED50 = 19 mM) (Fig. 3). PMA produced a dose-dependent increase in cellular cGMP in K+- and isoproterenol-treated pinealocytes (ED50 = 3 nm). The K+ (15 mM) treatment also slightly reduced the PMA ED50 for stimulation of cellular cAMP (Fig. 3). The effects of PMA treatment in these experiments with K+ (15 mM) were mimicked by OAG and PDBu, but not by PDD or by diolein (Table I), indicating the involvement of protein kinase C.

K+ is thought to elevate [Ca2+], through depolarization of the cell, which opens a voltage-dependent Ca2+ channel (13). If the elevation of [Ca2+], rather than depolarization or another effect of K+ (15 mM) treatment, is essential for PMA to stimulate the cGMP content of β-adrenergically treated pinealocytes, then the effect of K+ should be inhibited if Ca2+ entry is blocked. To test this possibility, nifedipine, a selective blocker of this channel, was used; nifedipine is known to prevent the increase in pinealocyte [Ca2+], produced by K+ (13) and the K+ potentiation of isoproterenol stimulation of cAMP and cGMP accumulation (20). In the present studies nifedipine was found to abolish the K+-induced stimulation of cGMP content of PMA- and isoproterenol-treated pinealocytes (Table II). This result indicates that a net increase in [Ca2+], is required for the effect of PMA on cGMP to be observed.

Nifedipine did not block the stimulation of pinealocyte cAMP in this series of studies (Table II). This is consistent with other evidence (Fig. 1, Table I) that the stimulatory effect of PMA on cAMP in β-adrenergically treated pinealocytes appears to be relatively independent of [Ca2+]. In addition, it indicates that the effect of nifedipine on cGMP is relatively specific and does not reflect toxicity or nonselective interruption of transmembrane signaling.

Effects of PMA on cAMP Accumulation in β-Adrenergically Stimulated Pinealocytes Treated with Ouabain—Ouabain-treatment is known to produce a very gradual and small increase in pinealocyte [Ca2+], (13) and to potentiate β-adrenergic stimulation of cAMP and cGMP accumulation (ED50 = 1.8 × 10−8 M) (20). For these studies a dose of ouabain (1 μM) was chosen which produces only a small potentiation of isoproterenol stimulation of cAMP and cGMP accumulation (17, Fig. 4). PMA produced a dose-dependent increase in the cGMP content of isoproterenol- and ouabain-treated pinealocytes. A significant effect of 0.1 nM PMA was detected; a half-maximal response was generated with a concentration of 0.4
cytes-The were measured cGMP Content of β-Adrenergically Stimulated Pinealocytes-A23187 was the same as that produced by a combined treatment with high concentrations of PMA (0.1 nM), and A23187 (0.1-10 μM), as indicated. Each point represents the mean ± S.E. of cAMP or cGMP determinations done in duplicate on three samples of cells. cAMP and cGMP were measured by radioimmunoassay. The absence of an error bar indicates that S.E. fell within the symbol. For further details see "Experimental Procedures."

FIG. 4. Effect of PMA on cAMP and cGMP accumulation in β-adrenergically stimulated and ouabain-treated pinealocytes. Pinealocytes were isolated from rat pineal glands and incubated under control conditions for 24 h. They were then aliquoted (10⁶ cells per 0.5 ml) and incubated for 15 min with isoproterenol (ISO, 1 μM), PMA (0.1-100 nM), and ouabain (1 μM), as indicated. Each point represents the mean ± S.E. of three samples of cells. cAMP and cGMP were measured by radioimmunoassay. The absence of an error bar indicates that S.E. fell within the symbol. For further details see "Experimental Procedures."

Effect of High Concentrations of PMA and A23187 on the cGMP Content of β-Adrenergically Stimulated Pinealocytes—High concentrations of A23187 (10 μM) produce a large potentiation of the isoproterenol stimulation of cellular cAMP and cGMP. It was of interest to determine whether the maximum response produced by high concentrations of A23187 was the same as that produced by a combined treatment with high concentrations of PMA and A23187. To examine this question, a dose-response study was performed using isoproterenol-treated cells (Fig. 5). It was found that the maximum cGMP response produced by 10 μM A23187 was about one-third of that produced by a combined treatment with PMA (0.1 μM) and A23187 (1 μM). In addition, PMA appeared to reduce the ED₅₀ for A23187, indicating that a lower [Ca²⁺], concentration is required for this effect. This is consistent with the finding that PMA reduces the Ca²⁺ requirement of protein kinase C (23, 24).

Studies on the Effects of Ca²⁺ Chelation on PMA Stimulation of the cGMP Content of β-Adrenergically Stimulated Pinealocytes—The above studies with β-adrenergically treated cells indicate that cGMP content of pinealocytes is regulated by protein kinase C and that PMA stimulation of cAMP accumulation is far less dependent upon Ca²⁺ than is stimulation of cGMP accumulation. In those studies [Ca²⁺], was increased with three agents. It was of interest to approach the issue of Ca²⁺ requirement by lowering [Ca²⁺]. To do this cells were treated with EGTA (3 mM), which is known to lower pinealocyte [Ca²⁺], (13) and to block β₁-adrenergic potentiation of β-adrenergic stimulation of cAMP and cGMP (Table III) (15, 20). EGTA (3 mM) treatment blocked the PMA stimulation
of the cGMP content of A23187 (1 \(\mu\)M)- and isoproterenol (1 
\(\mu\)M)-treated pinealocytes. This is consistent with the evidence 
(Figs. 1-5), indicating a marked dependence for Ca\(^{2+}\) requirement 
for PMA stimulation of the cGMP content of -adrenergically treated 
-pinealocytes.

EGTA did not inhibit the PMA stimulation of cAMP in 
-pinealocytes (Table III). This confirms our suspicion from the 
above studies that the effects of PMA on cAMP are generally 
Ca\(^{2+}\)-insensitive. However, in those experiments, [Ca\(^{2+}\)], was 
at normal levels (-100 nM, 13). In contrast, [Ca\(^{2+}\)], in EGTA 
(3 mM)-treated pinealocytes is <10 nM (13), indicating that the 
[Ca\(^{2+}\)], required for PMA stimulation of cAMP is below 
physiological levels.

Effects of PMA on the cGMP Content of K\(^{+}\) and Cholera 
Toxin-treated Pinealocytes—The regulation of pinealocyte 
cGMP accumulation appears to involve a GTP-binding protein, 
similar to the adenylyl cyclase regulatory protein. This was indicated by the finding that the combined treatment of 
cholera toxin and either the \(\alpha\)-adrenergic agonist phenylephrine (10 \(\mu\)M), 10 \(\mu\)M A23187, 45 mM K\(^{+}\), or 10 \(\mu\)M ouabain causes a large increase in pineal cGMP content (25). We 
tested the effects of PMA on pinealocytes treated with cholera 
toxin or with cholera toxin and a concentration of K\(^{+}\) (15 
mm) which has a small effect on cGMP accumulation (Fig. 6). 
PMA produced a dose-dependent increase in the cGMP 
content of cells treated with cholera toxin alone, and a larger 
effect in cells treated with cholera toxin and K\(^{+}\). This suggests 
that protein kinase C might act by increasing the efficiency of the GTP-binding protein involved in stimulation of cGMP 
accumulation.

**DISCUSSION**

The results of this study provide a clear indication that 
selective activators of protein kinase C increase the cGMP 
content of \(\beta\)-adrenergic or cholera toxin-stimulated pinealocytes if [Ca\(^{2+}\)], is sufficiently elevated. Accordingly, it appears 
that our previous attempts to elevate pineal cGMP content 
(7) in \(\beta\)-adrenergically stimulated pinealocytes by treatment 
with protein kinase C activators was unsuccessful because 
[Ca\(^{2+}\)], was not elevated.

The evidence in this report together with the available 
knowledge (7, 21) provides compelling reason to believe that protein kinase C is involved in the regulation of cGMP 
accumulation. As detailed in the Introduction, it is known 
that \(\alpha\)-adrenergic activation increases [Ca\(^{2+}\)], (13) and 
activates protein kinase C in the pinealocyte (7). The new 
evidence in this report indicates that protein kinase C activators 
can elevate the cGMP content of \(\beta\)-adrenergic or cholera 
toxin-treated cells, providing [Ca\(^{2+}\)], is also elevated. Thus, it 
seems likely that protein kinase C mediates, at least in part, 
the \(\alpha\)-adrenergic potentiation of \(\beta\)-adrenergic stimulation of 
pineal cGMP accumulation. The finding that protein kinase 
C activators also potentiate the effects of cholera toxin stim-
ulation of cGMP accumulation points to the possibility that 
protein kinase C interacts with the cGMP regulatory system 
at a site distal to the adrenoceptor (28), and that it may 
influence the efficiency of the GTP-binding protein involved in 
elevating pinealocyte cGMP levels.

Our studies have indicated that there is a marked difference 
in the Ca\(^{2+}\) dependence of the effects of protein kinase C 
activators on cAMP and cGMP levels in the pinealocyte, 
consistent with the general knowledge in the field that stim-
ulation of cGMP is highly Ca\(^{2+}\)-dependent (27-29). One hy-
pothetical explanation of this difference is that the protein 
kinase C-dependent phosphorylation required for stimulation 
of cAMP content is relatively Ca\(^{2+}\)-independent and that the 
phosphorylation required for stimulation of cGMP content is 
highly Ca\(^{2+}\)-dependent.

Another hypothetical explanation assumes that elevation 
of both cAMP and cGMP content by PMA is similar in that 
they require the same protein kinase C-dependent, Ca\(^{2+}\)- 
independent phosphorylation; in addition, this explanation 
proposes that the difference in Ca\(^{2+}\) dependence resides at 
another point in the activation cascade. Ca\(^{2+}\) may be an 
essential requirement for full catalytic activity of guanylyl 
cyclase, but not adenylyl cyclase. Consistent with this is the 
idea that an increase in [Ca\(^{2+}\)], is the stimulus for guanylyl 
cyclase activation (28). Alternatively, guanylyl cyclase might 
have an essential requirement for a compound whose synthe-
sis is strongly Ca\(^{2+}\)-dependent.

A candidate for such a compound is an arachidonic acid 
metabolite, as discussed in the Introduction. The evidence 
supporting this hypothesis is that arachidonic acid production 
appears to be required for stimulation of pineal cGMP 
content, because the phospholipase A\(_2\) inhibitor mepacrine blocks 
\(\alpha\)-adrenergic stimulation of cGMP (15). This effect is somewhat 
specific, because mepacrine does not inhibit \(\alpha\)-adrenergic stim-
ulation of pinealocyte cAMP content (15). In addition, we 
have found that stimulation of arachidonic acid production 
by norepinephrine E is also highly Ca\(^{2+}\)-dependent (9).

Arachidonic acid metabolism in the pineal gland has been 
found to be unusual because it generates lipoxygenase products 
at 20-fold higher rates than in other tissues (30). This 
points to the possibility that lipoxygenase products might be 
included in the regulation of the cGMP content of the pine-
aleocyte, as has been suggested in other cell systems (11, 12).

The above discussions have focused on guanylyl cyclase as the 
putative molecule whose full activity requires Ca\(^{2+}\), either 
directly or indirectly. However, there are other possible fac-
tors. One is the GTP-binding protein which participates in 
the regulation of pineal cGMP content, possibly in a manner 
alogous to the mechanism involved in stimulation of ad-
enylyl cyclase by the GTP-binding protein identified as G\(_{\alpha}\) 
(8). Thus, it is possible that a GTP-binding protein exists 
which regulates guanylyl cyclase and that it requires Ca\(^{2+}\) 
directly or indirectly for full activity.
A significant amount of work will have to be done to understand fully the mechanism involved in regulation of pineal cGMP content. However, the finding that protein kinase C is involved in the regulation of pineal cGMP content is important for ongoing research on cGMP. Relatively little is known about the regulation of cGMP and it may be possible to reveal previously ignored or overlooked regulatory systems by treating tissues of interest with protein kinase C activators and then testing the effects of putative regulatory compounds. In the case of the pinealocyte, it appears that multiple factors, including protein kinase C, [Ca\textsuperscript{2+}], and a Go-like protein, must be activated to fully elevate cGMP content. It will be of interest to determine whether protein kinase C is one of the elements involved in dual receptor regulation of the accumulation of cGMP in other cell types, or whether the pinealocyte is an interesting exception.

REFERENCES
5. Redgate, E. S., Deupree, J. D., and Axelrod, J. (1986) Brain Res. 365, 61-68