Protein Kinase C is Involved in Cyclic Adenosine Monophosphate Formation Due to PGF$_{2\alpha}$ Desensitization in Bovine Iris Sphincter

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**Purpose.** To examine the mechanisms underlying the effects of PGF$_{2\alpha}$ receptor desensitization on agonist-induced second messenger formation and contraction in bovine iris sphincter.

**Methods.** Short-term PGF$_{2\alpha}$ receptor desensitization of the bovine iris sphincter was carried out by incubating the tissue in Krebs-Ringer bicarbonate buffer containing 25 μM PGF$_{2\alpha}$ for 45 min at 37°C. The effects of PGF$_{2\alpha}$ and other pharmacologic agents on inostol 1,4,5-triphosphate (IP$_3$) production and cyclic adenosine monophosphate (cAMP) formation in desensitized and nondesensitized tissues were monitored by anion-exchange chromatography and radioimmunounasssay.

**Results.** In the isolated bovine iris sphincter, protein kinase C (PKC) is involved in the activation of adenylate cyclase and the desensitization of prostaglandin F$_{2\alpha}$ receptor-mediated responses supported by these findings. (A) Exposure of the tissue to phorbol 12,13-dibutyrate, used to activate PKC, enhanced basal cAMP formation in a dose (EC$_{50} = 8.8 \times 10^{-8}$ M) and time (t = 7.5 min) dependent manner. Phorbol 12,13-dibutyrate increased cAMP levels by twofold and it potentiated the isoproterenol-induced cAMP formation. The biologically inactive phorbol ester, 4a-phorbol had no effect. Staurosporine, a potent PKC inhibitor, inhibited phorbol 12,13-dibutyrate-induced cAMP formation in a dose-dependent manner (IC$_{50}$ of 0.25 μM). The increase in cAMP levels by phorbol 12,13-dibutyrate results from stimulation of adenylate cyclase, rather than from inhibition of cAMP phosphodiesterase, and it is not mediated through Ca$^{2+}$ mobilization. Pretreatment of the tissue with phorbol 12,13-dibutyrate inhibited IP$_3$ production in response to PGF$_{2\alpha}$. (B) Desensitization of the sphincter with PGF$_{2\alpha}$ for 45 min increased cAMP formation and attenuated IP$_3$ production and contraction. The effects of PGF$_{2\alpha}$ desensitization were reversed by pretreatment of the tissue with staurosporine. Down-regulation of PKC prevented the PGF$_{2\alpha}$-stimulated increase in cAMP formation. In the desensitized tissue, diacylglycerol, the endogenous activator of PKC, may arise from phosphatidylcholine, via phospholipase D.

**Conclusions.** (A) Activation of PKC in the bovine iris sphincter leads to stimulation of adenylate cyclase and to an increase in cAMP formation. The cAMP formed inhibits IP$_3$ production and muscle contraction. (B) PGF$_{2\alpha}$ desensitization results in adenylate cyclase activation, mediated through PKC. (C) PGF$_{2\alpha}$ desensitization could uncouple the receptor from the Gq and Gi proteins and enhance PG stimulation of adenylate cyclase activity through the Gs protein. (D) Uncoupling of the G proteins from the PG receptor and activation of PKC, both of which...
In the anterior segment of the eye, prostaglandins (PG) are synthesized mainly in the iris/ciliary body. They have been reported to mediate various physiologic processes, including smooth muscle contraction and relaxation, inflammation, intraocular pressure regulation, intracellular calcium mobilization, and cAMP formation.1-2 These actions result from the interactions of PGs with specific receptors. PG receptors are coupled to the stimulation of adenylate cyclase (AC) through the G protein, Gs, to the inhibition of AC through the G protein, Gi, and to the stimulation of phospholipase C (PLC) through the G protein Gq. In the iris sphincter the coupling of PG receptors to AC or to PLC is species-specific.3 Thus, in the rabbit sphincter activation of PG receptors results in the stimulation of AC and generation of cAMP, whereas in the bovine sphincter it stimulates PLC and this leads to the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) and to muscle contraction. There is a reciprocal interaction between the two second messenger systems in a wide variety of tissues, including the iris.4-6 Cyclic adenosine monophosphate (cAMP), through protein kinase A (PKA), inhibits the IP_3 system, probably at the G protein-PLC level, and relaxes the muscle.

The basal release of PGF_2a and PGE_2 in rabbit and human irises is several times as high as that of the bovine.3 In addition, PGF_2a receptors in rabbit and human iris sphincters are coupled to adenylate cyclase and muscle relaxation, whereas in the bovine sphincter they are linked to phospholipase C and muscle contraction.5 It was speculated from these findings that we are dealing either with different receptor subtypes of the PGF_2a receptor or with the PG receptors in rabbit and human sphincters are desensitized because of their exposure to high concentrations of the PG.5 Previously, to resolve this we performed experiments on the desensitization of PGF_2a receptors in the bovine sphincter, in which we showed that incubation of the tissue with PGF_2a (25 μM) for short periods of time (45 min) attenuated IP_3 production and contraction and increased cAMP formation.7 The current study was designed to examine the mechanism of PGF_2a desensitization in this tissue. We show that: (A) protein kinase C (PKC) activation by phorbol esters leads to stimulation of AC; (b) short-term PGF_2a desensitization causes an increase in cAMP formation, a reduction in IP_3 production, and an attenuation in muscle contraction; and (C) the observed effects caused by PGF_2a desensitization are mediated through PKC.

**MATERIALS AND METHODS**

In general, the methods used here are essentially the same as described previously.5-7 Myo-[3H]inositol (80-120 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL); [3H]-myristic acid (39.3 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Phorbol esters, carbachol (CCh), L-isoproterenol-HCl and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO). Sources for other reagents were as follows: antibody to cyclic AMP from ICN ImmunoBiologicals (Lisle, IL); reagents for radioimmunoassay (RIA) of cyclic AMP including succinyl (methyl ester-125I) cyclic AMP tyrosine (2200 Ci/mmol) from New England Nuclear; prostaglandins from Cayman Chemical Co. (Ann Arbor, MI); staurosporine and ionophore A23187 from Calbiochem Corp. (San Diego, CA); and phosphatidylethanol and phosphatidic acid from AVANTI Polar Lipids, Inc. (Alabaster, AL).

**Preparation of Iris Sphincter Muscle**

Bovine eyes were obtained from a local slaughterhouse. Eyes were enucleated immediately after death and transported to the laboratory packed in ice. The irises were removed and placed flat in a petri dish. The sphincter muscle was dissected out carefully and placed in modified Krebs-Ringer bicarbonate buffer (millimolar): NaCl, 118; NaHCO_3, 25; KCl, 4.7; KH_2PO_4, 1.2; MgSO_4, 1.2; CaCl_2, 1.25; cuitidine, 1.6; D-glucose, 10; and 1 μM indomethacin. Indomethacin, a cyclooxygenase inhibitor, was added to the incubation medium in all experiments to prevent the formation of endogenous PGs. The pH of the buffer was adjusted to 7.4 with 97% O_2-3% CO_2. In our opinion, methods for securing animal tissue were humane, included proper approval, and complied with the ARVO statement.

**Short-term Prostaglandin Receptor Desensitization of the Iris Sphincter**

Short-term prostaglandin receptor desensitization of the iris sphincter was carried out as described else-
where. Briefly, the iris sphincters were cut into two halves, then incubated in pairs in the absence and presence of 25 μM PGF_{2α} for 45 min at 37°C. Both nondesensitized and desensitized iris sphincter pairs were then washed three times with buffer. The effectiveness of the washing procedure was monitored by including \(^3\)H-PGF_{2α} in the incubation medium.

**Incubation of Sphincter Muscle with myo[\(^3\)H]inositol and Analysis of Inositol Phosphates**

To label the tissue with myo[\(^3\)H]inositol, the paired sphincter strips were incubated for 90 min at 37°C in 1 ml of Krebs-Ringer bicarbonate buffer containing 10 μCi of myo[\(^3\)H]inositol. At this time the sphincters were washed four times with 3 ml nonradioactive Krebs-Ringer bicarbonate buffer and then suspended individually in 1 ml of fresh nonradioactive buffer. LiCl (10 mM, final concentration) was added to each incubation and 10 min later PGF_{2α}, CCh or other agents were added and incubations continued for the time indicated. The incubations were terminated by the addition of 1 ml 10% (w/v) TCA. The tissues were homogenized, centrifugated, and the supernatant was extracted with 4X anhydrous diethyl ether. The water-soluble tissue extract was then neutralized to pH 7.0 with NaOH and myo[\(^3\)H]inositol phosphates were analyzed by anion exchange chromatography using Biorad 1 × 8 resin (formate form, 200-400 mesh) as described previously.\(^5\)

**Measurement of Cyclic AMP**

The paired strips were incubated for 90 min at 37°C in 1 ml of Krebs-Ringer bicarbonate buffer. At this time the sphincters were washed four times with 3 ml Krebs-Ringer bicarbonate buffer and then suspended individually in 1 ml of fresh buffer. IBMX (0.1 mM, final concentration) was added to each incubation and 10 min later PGF_{2α}, CCh or other agents were added and incubations continued for the time indicated. The incubations were terminated by the addition of 1 ml 10% (w/v) TCA. The tissues were homogenized, centrifugated and the supernatant was extracted with 4X anhydrous diethyl ether. A 50 μl aliquot from each sample was then succinylated and after appropriate dilution cAMP was assayed by RIA as described by Frandsen and Krishna.\(^8\)

**Assay for Phospholipase D (PLD) Activity**

Phospholipase D is known to catalyze the transfer of phosphatidyl groups to various acceptors; in the presence of an alcohol, this transphosphatidylation produces phosphatidyl-alcohol.\(^9\) The production of phosphatidylethanol is considered a good indication of PLD activity. The assay for PLD activity was as described by Liscovitch and Amsterdam.\(^9\) Briefly, the paired sphincter strips were incubated for 90 min at 37°C in 1 ml of Krebs-Ringer bicarbonate buffer containing 0.05% bovine serum albumin and 5 μCi of \(^3\)H-myristic acid. At this time the sphincters were washed four times with 3 ml buffer that contained 0.05% bovine serum albumin. The sphincters were preincubated singly in 1 ml buffer containing 0.5% (v/v) ethanol at 37°C for 5 min. The agonist was then added as indicated and incubation continued for an additional 5 min. Reactions were terminated by addition of 2 ml of ice-cold chloroform-methanol (1:2, v/v). Phospholipids were extracted according to the procedure of Bligh and Dyer.\(^10\) The phospholipids in the lipid extract were separated by thin layer chromatography using silica gel K6 plates (Whatman) and a solvent system consisting of the organic phase of ethylacetate/isooctane/acetic acid/water (130/20/30/100, by volume). Individual lipids were localized by iodine staining and identified by co-migration with standards. The spot corresponding to phosphatidylethanol was scraped into scintillation vials and the \(^3\)H-radioactivity measured by liquid scintillation counting. Data are expressed as dpm/μmol lipid phosphorus.

**Measurement of Agonist-induced Tension Response in the Iris Sphincter**

For measurement of tension response, the two sphincter strips from the same iris were mounted in two separate 30 ml water-jacketed tissue baths that contained Krebs-Ringer bicarbonate buffer at 37°C. A mixture of O₂ (97%) and CO₂ (3%) was continuously bubbled through the solution. The tissue was allowed to equilibrate for 90 min under a resting tension of 50 mg. To inhibit the endogenous formation of prostaglandins, which can cause inherent tone in the tissue, 1 μM indomethacin was routinely added to the tissue baths. During the equilibration period the physiologic solution was changed every 20 min. At the end of equilibration, the test agents were added and the changes in tension were recorded isometrically using a force-displacement transducer (Grass model FT.03) coupled to a polygraph (Grass model 79D). Dose-response curves for mechanical responses were constructed by cumulative addition of agonist in the tissue bath. The concentration of the agonist was increased only after the effect of the previous concentration had stabilized.

**RESULTS**

**Effects of Phorbol Esters and Other Pharmacologic Agents on cAMP Formation in Bovine Iris Sphincter**

The phorbol esters are tumor promoters that stimulate PKC.\(^11\) They substitute for DAG, which is the phys-
TABLE 1. Effects of Phorbol Esters and Other Pharmacological Agents on cAMP Formation in Bovine Iris Sphincter

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentrations (µmol/l)</th>
<th>cAMP Formed (pmol/mg Protein)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>26.2 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td>Phorbol 12,13-dibutyrate (PDBu)</td>
<td>0.1</td>
<td>77.5 ± 4</td>
<td>296</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>0.1</td>
<td>35.7 ± 5</td>
<td>136</td>
</tr>
<tr>
<td>4α-Phorbol</td>
<td>0.1</td>
<td>26.5 ± 1</td>
<td>101</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>1</td>
<td>74.5 ± 5</td>
<td>284</td>
</tr>
<tr>
<td>PDBu (0.1 µmol/l) + isoproterenol (1 µmol/l)</td>
<td>1</td>
<td>102.7 ± 1</td>
<td>392</td>
</tr>
<tr>
<td>Ionophore A23187</td>
<td>1</td>
<td>26.5 ± 0.2</td>
<td>101</td>
</tr>
<tr>
<td>PDBu (0.1 µmol/l) + ionophore A23187 (1 µmol/l)</td>
<td>1</td>
<td>80.0 ± 3</td>
<td>305</td>
</tr>
</tbody>
</table>

The data are mean ± SEM of two experiments, each conducted in triplicate.

The biologic activator of PKC and is produced from agonist-induced turnover of PIP2. In some cells, PKC activation results in potentiation of receptor-mediated cAMP formation, and in others PKC activation results in inhibition of cAMP formation.4,12 As with brain, the iris sphincter is enriched with PKC.13 As shown in Table 1, the phorbol ester, PDBu increased cAMP levels in the sphincter by twofold. The phorbol ester, PMA, which is less permeable, had a modest increase (36%) in cAMP levels. The biologically inactive analogue, 4α-phorbol exerted no effect on the formation of the cyclic nucleotide. Because IBMX was included in the incubation medium, the stimulatory effect of the phorbol ester on cAMP formation appears to result from stimulation of AC rather than from inhibition of cAMP phosphodiesterase. PDBu potentiated (38% increase) the isoproterenol-mediated cAMP formation. Ionophore A23187 had no influence on cAMP levels and it did not augment the PDBu-induced cAMP formation. This indicates that the effect of the phorbol ester on the cyclic nucleotide is not mediated through Ca2+ mobilization.

Dose-Response Effects of PDBu on cAMP Formation

As can be seen from Fig. 1, PDBu increased cAMP formation in the bovine sphincter in a dose-dependent manner, over a concentration range of 0.01 to 10 µM (EC50 = 8.8 × 10−8 M). Under the same experimental conditions PDBu had no effect on cAMP formation in the rabbit iris sphincter (data not shown). In the rabbit sphincter PDBu induces contraction in a dose-dependent manner, over a range of 10–200 nM.15 The phorbol ester did not contract the bovine sphincter (data not shown). In the following studies we have employed 100 nM PDBu.

Time Course of PDBu-Induced cAMP Formation

Time course studies revealed that PDBu increased cAMP formation in a time dependent manner (Fig. 2). Cyclic AMP formation by the phorbol ester leveled off between 15 to 30 min, the t½ value was 7.5 min. In the following studies we have employed 15 min preincubations with PDBu.

Dose-Response Effects of Staurosporine on PDBu-Induced cAMP Formation

Staurosporine, a microbial alkaloid isolated from Streptomyces sp, has been reported to be a very potent and selective inhibitor of PKC in various tissues, including smooth muscle.14,15 As shown in Fig. 3, in the bovine sphincter, staurosporine inhibited the PDBu-
induced cAMP formation in a dose-dependent manner (IC50 = 0.25 μM). At 1 μM, staurosporine inhibited cAMP formation by 60%. These data support a role for PKC in PDBu-induced cAMP formation in this tissue.

**Dose-Response Effects of PGF2α on the Contractile Response in Bovine Iris Sphincter Desensitized in the Absence and Presence of Staurosporine**

The ability of PGF2α to induce contraction in non-desensitized and desensitized bovine sphincter is given in Fig. 4. In the desensitized muscle there was both a shift to the right in the dose-response curve (the EC50 for the non-desensitized and desensitized tissues were: 9 × 10⁻⁸ and 1.5 × 10⁻⁷ M, respectively), and a significant decrease in the maximal response (67%). Attenuation of the contractile response caused by desensitization was reversed by staurosporine. There was both a shift to the left of the dose-response curve (EC50 = 1.2 × 10⁻⁷ M) and a smaller decrease in the maximal response (11%). These data suggest: (a) that pretreatment of the tissue with the PKC inhibitor blocks PGF2α desensitization, and (b) that PKC plays a role in the mechanism of PGF2α desensitization in this tissue.

**Effects of PGF2α-Desensitization on IP3 Production and cAMP Formation in Bovine Iris Sphincter**

To clarify the biochemical mechanisms underlying the PGF2α-desensitization of the physiological response in
TABLE 2. Effects of PGF<sub>2α</sub> Desensitization on IP<sub>3</sub> Production and cAMP Formation in Bovine Iris Sphincter

<table>
<thead>
<tr>
<th>PGF&lt;sub&gt;2α&lt;/sub&gt; (μmol/l)</th>
<th>IP&lt;sub&gt;3&lt;/sub&gt; Production (% of Control)</th>
<th>cAMP Formation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nondensitized</td>
<td>Desensitized</td>
</tr>
<tr>
<td></td>
<td>J10 ± 3</td>
<td>104 ± 5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>193 ± 7</td>
</tr>
</tbody>
</table>

In the IP<sub>3</sub> and cAMP assays, the nondesensitized and desensitized tissues were exposed to the agonist for 5 min. The basal values for IP<sub>3</sub> production for nondesensitized and desensitized bovine iris sphincters were (dpm/mg of total tissue proteins) 3848 ± 235 and 3904 ± 252, respectively. The basal values for cAMP formation for nondesensitized and desensitized bovine iris sphincters were (pmol/mg of total tissue proteins) 21.5 ± 0.6 and 21.6 ± 0.5, respectively. The data are mean ± SEM and obtained from eight to 12 determinations.

In the bovine sphincter we have investigated the effects of PGF<sub>2α</sub> on IP<sub>3</sub> production and cAMP formation. As can be seen from Table 2, PGF<sub>2α</sub>-desensitization attenuated IP<sub>3</sub> production and stimulated cAMP formation at the two concentrations of PG investigated. Thus, PGF<sub>2α</sub> (5 μM) enhanced IP<sub>3</sub> production and inhibited cAMP formation in the nondesensitized tissue by 93 and 20%, respectively, and in the desensitized tissue it stimulated the production of IP<sub>3</sub> and cAMP by 29 and 73%, respectively. These data show that in the nondesensitized tissue PGF<sub>2α</sub> receptors are coupled to the inhibition of AC and stimulation of PLC and contraction, and in the desensitized tissue they are coupled to the stimulation of AC and inhibition of PLC and contraction.

Effects of Staurosporine on PGF<sub>2α</sub>-Induced cAMP Formation in Desensitized Bovine Iris Sphincter

Staurosporine (5 μM) inhibited significantly (48%) PGF<sub>2α</sub>-induced cAMP formation in the desensitized tissue (Table 3). These effects of the PKC inhibitor occurred at all concentrations of the PG investigated.

This finding demonstrates that PKC is involved in the PGF<sub>2α</sub>-stimulated cAMP formation in the desensitized tissue.

Effects of PKC Down Regulation on PGF<sub>2α</sub>-Induced cAMP Formation in PGF<sub>2α</sub>-Desensitized Bovine Iris Sphincter

Further support for the concept that PKC is involved in the mechanism of PGF<sub>2α</sub> desensitization comes from the studies on the effects of down regulation of the enzyme. In this experiment PKC was down regulated with PDBu and the tissue was then desensitized with PGF<sub>2α</sub>. As can be seen from Fig. 5, down regulation of the enzyme blocked PGF<sub>2α</sub>-induced cAMP formation in the desensitized tissue at all concentrations of the PG investigated (0.01–10 μM). In the control desensitized tissue PGF<sub>2α</sub> caused a dose-dependent accumulation of cAMP (EC<sub>50</sub> = 6 × 10<sup>-8</sup> M).

TABLE 3. Effects of Staurosporine on PGF<sub>2α</sub>-Induced cAMP Formation in PGF<sub>2α</sub>-Desensitized Bovine Iris Sphincter

<table>
<thead>
<tr>
<th>PGF&lt;sub&gt;2α&lt;/sub&gt; (μmol/l)</th>
<th>cAMP Formed (% Increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>26 ± 3</td>
</tr>
<tr>
<td></td>
<td>64 ± 5</td>
</tr>
</tbody>
</table>

The desensitized tissue was preincubated with staurosporine (1 μmol/l) as indicated for 10 min. The tissue was exposed to the agonist for 5 min. The data are mean ± SEM of two experiments each conducted in triplicate.

FIGURE 5. Dose-response effects of PGF<sub>2α</sub> on cAMP formation in bovine iris sphincters which were either desensitized with PGF<sub>2α</sub> (□) or first pretreated with PDBu (0.1 μM) for 1 hr then desensitized with the PG (●). The data are mean ± SEM of three experiments each conducted in triplicate.
TABLE 4. Effects of PDBu on PGF$_{2a}$-Induced IP$_3$ Production and cAMP Formation in Nondesensitized Bovine Iris Sphincter

<table>
<thead>
<tr>
<th>Additions</th>
<th>IP$_3$ Production (% of Control)</th>
<th>cAMP Formation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGF$_{2a}$ (1 μmol/l)</td>
<td>161 ± 7</td>
<td>84 ± 5</td>
</tr>
<tr>
<td>PDBu 90.1 μmol/l</td>
<td>100 ± 3</td>
<td>281 ± 8</td>
</tr>
<tr>
<td>PGF$_{2a}$ (1 μmol/l) + PDBu (0.1 μmol/l)</td>
<td>108 ± 5</td>
<td>265 ± 9</td>
</tr>
</tbody>
</table>

The tissue was preincubated with or without PDBu for 15 min as indicated and then exposed to the agonist for 5 min. The data are mean ± SEM of three experiments, each conducted in triplicate.

Effects of PDBu on PGF$_{2a}$-Induced IP$_3$ Production and cAMP Formation in Nondesensitized Bovine Iris Sphincter

Activation of PKC by PDBu blocked IP$_3$ production by PGF$_{2a}$ and reversed the inhibitory effect of the PG on cAMP formation (Table 4). In the presence of PGF$_{2a}$, PDBu increased cAMP formation by 165%. These data show that PKC is also involved in feedback regulation of PLC activity.

Effects of PGF$_{2a}$ Desensitization on Carbachol-Induced IP$_3$ Production and on Carbachol Inhibition of Isoproterenol-Stimulated cAMP Formation

In both nondesensitized and desensitized tissues carbachol (10 μM) increased IP$_3$ production by about 80% and inhibited isoproterenol-stimulated cAMP formation by 36 and 33%, respectively (Table 5). Therefore, these muscarinic effects are not influenced by PGF$_{2a}$ desensitization. In addition, as with its effects on IP$_3$, carbachol-evoked muscle contraction was not affected by PGF$_{2a}$ desensitization (data not shown).

Effects of PGF$_{2a}$ Desensitization on Phospholipase D Activity in the Bovine Iris Sphincter

Diacylglycerol, the endogenous activator of PKC, could arise either from PIP$_2$, via PLC, and/or from phosphatidylcholine, via phospholipase D. While PGF$_{2a}$ desensitization attenuated PIP$_2$ hydrolysis (Table 2 above) it increased, although modestly (32%), phospholipase D activity (Table 6). These data demonstrate: (a) the presence of phospholipase D in the bovine iris sphincter, and (b) that DAG could arise, through the phospholipase D pathway, in the desensitized tissue. Activators of PKC have been reported to stimulate phospholipase D activity in various tissues, including the iris sphincter (Y. Zhang and A.A. Abdel-Latif, unpublished observations).

DISCUSSION

In the present work we have used the bovine iris sphincter, which produces very little PG as compared to other species, to answer two questions: (a) Does activation of PKC leads to stimulation of AC and consequently to an increase in cAMP production?, and (b) Is PKC involved in the mechanisms of PGF$_{2a}$ desensitization in this tissue?. We found that: (a) Activation of PKC in the bovine iris sphincter by the phorbol ester, PDBu, results in the stimulation of AC and increase in cAMP levels, and (b) that desensitization of the smooth muscle with PGF$_{2a}$ increases cAMP accumulation by the PG and attenuates the PGF$_{2a}$-induced IP$_3$ production and contraction. Thus, the stimulatory effect of PDBu on basal cAMP formation was dose- and time-dependent, was not potentiated by Ca$^{2+}$, and it was

TABLE 5. Effects of PGF$_{2a}$ Desensitization on Carbachol-Induced IP$_3$ Production and on Carbachol Inhibition of Isoproterenol-Induced cAMP Formation in Bovine Iris Sphincter

<table>
<thead>
<tr>
<th>Additions</th>
<th>IP$_3$ Production (% of Control)</th>
<th>cAMP Formation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nondesensitized</td>
<td>Desensitized</td>
</tr>
<tr>
<td>Carbachol (1 μmol/l)</td>
<td>179 ± 3</td>
<td>180 ± 4</td>
</tr>
<tr>
<td>Isoproterenol (1 μmol/l)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isoproterenol (1 μmol/l) + carbachol (10 μmol/l)</td>
<td>229 ± 4</td>
<td>238 ± 5</td>
</tr>
</tbody>
</table>

The nondesensitized and desensitized sphincters were exposed to the agonists for 5 min. The data are mean ± SEM and obtained from four determinations.
inhibited by staurosporine, a potent PKC inhibitor, in a dose-dependent manner (IC_{50} of 0.25 \mu M). The biologically inactive analogue 4a-phorbol had no effect on cAMP formation. PDBu potentiated cAMP accumulation by isoproterenol by 38% (Table 1). This potentiation was small because we have used maximal doses of both agonists, and since both agonists use the same effector system (AC) there was no additivity. PKC activation by phorbol esters potentiated the isoproterenol-mediated cAMP production in J774 cells.17 These effects suggest that phorbol esters, by activating PKC, potentiate cAMP formation by influencing a component of the AC system beyond the receptor site. Because our studies were carried out in the presence of IBMX, the observed increase in cAMP levels by PDBu results from stimulation of AC, rather than from inhibition of CAMP phosphodiesterase. The mechanism underlying the stimulation of cAMP accumulation by PKC activators is unknown. Activation of PKC appears to lead to multiple changes in the receptor-stimulated AC signal transduction pathway.18 In frog erythrocytes, the phorbol ester, TPA, produces phosphorylation of the catalytic unit of AC.19 It has been suggested that phosphorylation of the catalytic unit of AC by PKC may be involved in the phorbol ester-induced enhancement of AC activity. Intravitreal injection of 50 pmol of the phorbol ester, PMA, into rabbit eyes produced 40% of the intraocular pressure relative to the control eye lasting for more than 72 hr.20 Studies on in vivo and in vitro treatment with PMA had no significant effect on AC in ciliary process membranes assayed in vitro. In the current study PDBu had no effect on cAMP levels in rabbit iris sphincter. There is accumulating evidence that indicates modulation of AC by PKC at the level of G proteins.12,20,21

Having demonstrated that activation of PKC leads to accumulation of cAMP, we proceeded to answer the second question as to whether PKC is involved in the mechanism of PGF_{2\alpha} desensitization in this tissue. Our data show that activation of PKC may mediate the observed effects of PGF_{2\alpha} desensitization in the bovine iris sphincter. This conclusion is based on the following: (A) In non-desensitized bovine iris sphincter PGF_{2\alpha} dose-dependently induced IP_3 production and contraction and inhibited cAMP formation. (B) Desensitization of the tissue with PGF_{2\alpha} slightly attenuated muscle contraction by PGF_{2\alpha} (increased the EC_{50} from 9 \times 10^{-8} to 1.5 \times 10^{-7} M) and depressed the maximal response by 67%. The finding that PGF_{2\alpha} reduced the maximal response by PGF_{2\alpha} significantly but not the EC_{50} (Fig. 4) indicates that PKC is acting, probably by phosphorylation, at the G-protein-PLC sites. The effects of desensitization were reversed by staurosporine, thus suggesting the involvement of PKC. (C) Desensitization of the tissue inhibited IP_3 production and stimulated cAMP formation induced by PGF_{2\alpha}. The PGF_{2\alpha}-induced cAMP formation was reversed by staurosporine. Furthermore, PKC down-regulation blocked the PGF_{2\alpha}-stimulated increase in cAMP formation in the desensitized tissue. (D) Activation of PKC by PDBu blocked PGF_{2\alpha}-induced IP_3 production.

We already reported on biochemical and functional interactions between the cAMP and the IP_3-Ca^{2+}-DAG systems in the iris sphincter.4,6,29 Recently, several articles have been published on “cross talk” between the two signalling pathways in a variety of systems.4,12,19 In the iris sphincter cAMP, through PKA, probably act by inhibiting PLC and/or G proteins and subsequently IP_3 production and contraction (Fig. 6). PKC functions to regulate Ca^{2+}-dependent functions, such as contraction, in two ways: (A) It stimulates AC, and (B) it inhibits PLC activation, both of which were demonstrated in this study. In the bovine sphincter, we suggest that the PGF_{2\alpha} receptor is coupled to the activation of PLC through Gq, and to the stimulation and inhibition of AC through Gs and Gi, respectively. Recently, Fargin et al22 showed the cloned 5-HT1A receptor, which was stably expressed in HeLa cells, to mediate the effects of serotonin by inhibiting cAMP formation and by stimulating the hydrolysis of PIP_2. They suggested that the Gi protein could mediate the effects of serotonin, both to inhibit AC and to stimulate PLC. In the non-desensitized iris sphincter the PGF_{2\alpha} receptor is coupled to stimulation of PLC and to inhibition of AC through G proteins, the nature of which remains to be established (Fig. 6). It is possible that PGF_{2\alpha} desensitization uncouples the receptor from the Gq and Gi proteins. This will result in the stimulation of AC by PGF_{2\alpha} through the Gs protein. In addition, in the desensitized tissue, DAG could arise from phosphatidylethanolamine via phospholipase D (Table 6) to activate PKC; this will inhibit PLC.

### TABLE 6. Effects of PGF\textsubscript{2\alpha} Desensitization on Phospholipase D Activity in Bovine Iris Sphincter

<table>
<thead>
<tr>
<th>PGF\textsubscript{2\alpha} (\mu mol/l)</th>
<th>No. of Experiments</th>
<th>Nondesensitized</th>
<th>Desensitized</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>4</td>
<td>103 ± 0.5</td>
<td>118 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>91 ± 8</td>
<td>125 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>99 ± 11</td>
<td>132 ± 10</td>
</tr>
</tbody>
</table>

The desensitized and non-desensitized tissues were exposed to the agonist for 5 min. Phospholipase D activity was measured as phosphatidylethanol formation. The basal values for phosphatidylethanol formation for non-desensitized and desensitized bovine iris sphincters were (dpm/\mu mol total lipid phosphorus) 3450 ± 200 and 2680 ± 200, respectively.
PGF$_{2\alpha}$ Desensitization and Second Messengers in Bovine Iris

PGF$_{2\alpha}$ Receptor

$\uparrow$

Gs $\rightarrow$ G$_i$, G$_q$

PkC

$\downarrow$

AC PLC

ATP PkA $\downarrow$
P1P$_2$ IP$_3$ $\uparrow$
PkC $\uparrow$
cAMP $\uparrow$$[\text{Ca}^{2+}]_i$

Relaxation Contraction

FIGURE 6. Scheme showing the two second messenger systems IP$_3$-$\text{Ca}^{2+}$, DAG and cAMP, mediating the actions of PGF$_{2\alpha}$ on the contractile and relaxation responses in the bovine iris sphincter smooth muscle. Abbreviations: Gs, stimulatory G protein; Gi, inhibitory G protein; Gq, phospholipase C (PLC) stimulatory G protein; + and −, indicate stimulation and inhibition, respectively.

and stimulate AC and increase cAMP levels. The cAMP formed could then negatively regulate PLC activity and subsequently reduce IP$_3$ production and depress contraction (Fig. 6). In GH4C1 cells, PKC stimulation inactivated the inhibitory GTP-binding protein Gi and stimulated AC activity. It must also be emphasized that we could be dealing with two types of PGF$_{2\alpha}$ receptors, one coupled to AC and the second to PLC. Because of the lack of specific PGF$_{2\alpha}$ receptor antagonists we have no experimental evidence to support the presence of PG receptor subtypes that are linked to either PLC or AC in this tissue.

There is accumulating experimental evidence that indicates that prostaglandins play a normal physiologic role in the iris, and presumably, in the whole anterior segment of the eye, and that exogenous PG may be useful for the pharmacologic manipulation of such physiological functions. Thus, further studies on the biochemical mechanisms underlying the actions of PGs in the iris and other ocular tissues, and the “cross talk” between their respective second messenger systems and the functional responses they control remain an important area for further investigation.

Key Words

bovine iris sphincter, prostaglandin F$_{2\alpha}$ receptor desensitization, protein kinase C, cyclic AMP, inositol trisphosphate

References


