Phorbol Ester: Effect on Intraocular Pressure, Adenylate Cyclase, and Protein Kinase in the Rabbit Eye

Thomas W. Mittag,* Nagahisa Yoshimura,*‡ and Steven M. Podos*

Protein kinase C was identified as a major protein kinase enzyme activity in rabbit ciliary processes. Phorbol myristate acetate (4β-PMA) in the presence of Ca2+ activated protein kinase C but did not directly affect the cyclic AMP-dependent protein kinase enzyme isolated from ciliary processes. To elucidate possible roles of protein kinase C, PMA was injected intravitreally into rabbit eyes. Fifty pmoles of PMA produced approximately a 40% decrease of the intraocular pressure relative to the control eye lasting for more than 72 hr. A reduction of intraocular pressure was still elicited by this dose of PMA in animals pretreated with systemic indomethacin given to suppress a possible inflammatory response. The biologically inactive analogue, 4α-phorbol didecanoate (100 pmoles/eye) had no significant effect on intraocular pressure. In vivo and in vitro treatment with PMA had no significant effect on adenylate cyclase in ciliary process membranes assayed in vitro. However, protein kinase C isolated from rat brain, when added together with cofactors to membranes in vitro, augmented adenylate cyclase activation by isoproterenol, vasoactive intestinal peptide and aluminum fluoride. A slight increase in the basal activity and in the forskolin response was not statistically significant. The effect of protein kinase C to increase responsiveness of ciliary process adenylate cyclase was totally dependent on the presence of Ca2+ and was augmented by addition of PMA. These findings indicate modulation of adenylate cyclase activity by protein kinase C acting at the level of the G-proteins and suggest a possible role for this enzyme in water and electrolyte transport in the ciliary processes.


Phorbol myristate acetate (4β-PMA) is a potent tumor promoter present in croton oil. This compound elicits a wide variety of biological and biochemical responses in many cells and tissues (see review).1 PMA has been identified as an activator of Ca2+-activated, phospholipid-dependent protein kinase (protein kinase C), not only in vitro but also in vivo.2–5 This interaction is highly specific and protein kinase C has also been termed the PMA receptor.6 For this reason, PMA is now widely used to elucidate possible physiological role(s) of protein kinase C in many different cells and organs. Protein kinase C is generally thought to play a pivotal role in the transmembrane control of important cellular functions including growth, transformation, and secretion.6–8

In ciliary processes, cyclic AMP and cyclic AMP-dependent protein kinase have been proposed to play key roles in the control of aqueous humor production.9–11 Much less attention has been paid thus far to the possible roles of Ca2+ and protein kinase C. In rabbit ciliary processes, receptor binding studies show the occurrence of both α1- and α2-adrenergic receptors in this tissue.12–14 Drugs that are either α-adrenergic antagonists or α2-agonists are known to lower intraocular pressure in rabbit eyes.15–16 The α1-subclass of adrenergic receptor differs from β-adrenergic receptors because it does not activate the adenylate cyclase enzyme but may in fact be inhibitory on adenylate cyclase in some cells.17 However, in most tissues α1-adrenergic receptors are coupled to phosphoinositide turnover18 and the mobilization of Ca2+. This has also been recently reported for ciliary processes.19 Activation of these receptors can thus lead to stimulation of Ca2+-dependent enzymes such as protein kinase C or calmodulin-dependent protein kinase(s). Therefore, because of the IOP effects of α-adrenergic drugs and the occurrence of α-adrenergic receptors linked to phosphatidyl inositol turnover in ciliary

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processes, we have initiated studies on possible role(s) of protein kinase C in this tissue. In this paper we describe identification and characterization of protein kinase C in rabbit ciliary processes, and experiments using PMA which suggest a possible role of protein kinase C in modulating the adenylate cyclase system and in aqueous humor production.

Materials and Methods

Materials

DEAE-cellulose (DE 52, Whatman) and nitrocellulose membrane filters (MicronSep, pore size 0.45 μm) were obtained from Fisher Scientific Co. (Pittsburgh, PA). H1 histone (histone IIIS), phosphatidylserine, diolein, 4β-phorbol 12-myristate 13-acetate (PMA) and 4α-phorbol 12,13-didecanoate (PDD) were purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin was obtained from CalBiochem (La Jolla, CA). 3H-cyclic AMP was a product of New England Nuclear Corp. (Boston, MA) and the [α- or γ-32P]-labelled ATP was purchased from Amersham (Arlington Heights, IL). Other chemicals were obtained from Sigma or Fisher Scientific.

Ciliary Process Membrane Preparation

Ciliary processes were dissected from albino rabbit eyes within 2 hr of death (sodium pentobarbital overdose). Processes from three rabbits were homogenized in 3 ml of 20 mM Tris-HCl (pH 7.5), 0.32 M sucrose, 5 mM EDTA, 5 mM ethylene glycol bis (aminoethyl ether) N,N,N',N'-tetraacetic acid), 1 mM diethio-teritol, 0.1 mM indomethacin, 50 μg/ml leupeptin and 35.7 μg/ml adenosine deaminase (homogenizing buffer) in a teflon/glass homogenizer using 25 strokes of the pestle. The latter three ingredients were included in the homogenizing buffer to preclude effects on the adenylate cyclase system by endogenously formed prostaglandins, adenosine, and proteolytic activity respectively. The homogenate was centrifuged at 27,000 g for 15 min and the supernatant was discarded. The pellet was suspended and vortexed in 3 ml of the homogenizing buffer and centrifuged again. The recovered pellet was rehomogenized using 3 ml of the same buffer and the suspension passed through a single layer of nylon mesh.

Preparation of Protein Kinase C and Chromatographic Separation of Protein Kinases

Partially purified protein kinase C was prepared from rabbit ciliary processes or from rat brain. Ciliary processes from 20 eyes were dissected and homogenized by a motor-driven teflon/glass homogenizer with 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EGTA, 10 mM EDTA, 5 mM 2-mercaptoethanol, 10 μg/ml leupeptin, and 0.5 mM phenylmethyl sulfonyl fluoride. The homogenate was centrifuged at 100,000 g for 60 min and the supernatant fraction (about 23 mg protein) directly applied to a column of DE 52 cellulose (0.7 x 4.0 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol and 1 μg/ml leupeptin (buffer A). After washing the column with the same buffer, the protein was eluted by a linear gradient (0 to 0.4 M) of KCl. Fractions containing protein kinase C activity devoid of cyclic AMP-dependent protein kinase activity were collected and dialyzed against buffer A. Rat brain protein kinase C was prepared essentially by the same method. All the preparative procedures were done at 4°C.

Enzyme Assay

Protein kinase C and cyclic AMP-dependent protein kinase were measured by the method of Kikkawa et al. The reaction mixture for protein kinase C assay (total volume 250 μl) contained 20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 10 μM [γ-32P]-ATP (8-12 x 10⁶ cpm), 2 μg of phosphatidylserine, 0.2 μg of diolein, 50 μg H1 histone and 1 mM CaCl₂. Both phosphatidylserine and diolein were stored in chloroform at −20°C and before use the chloroform was evaporated under a stream of nitrogen gas. Lipids were dispersed in 20 mM Tris-HCl, pH 7.5 by sonication for 5 min to make the micelles. Cyclic AMP-dependent protein kinase activity was measured in a reaction mixture (total volume 250 μl) containing 20 mM Tris-HCl (pH 7.5) 5 mM magnesium acetate, 10 μM [γ-32P]-ATP (8-12 x 10⁶ cpm), 50 μg H1 histone and 1 μM cyclic AMP. After a 3 min preincubation at 30°C, the reaction was started by adding 50 μl of enzyme solution and terminated after 5 min by adding 2 ml of 25% trichloroacetic acid (TCA). Controls for protein kinase C contained 1 mM EGTA instead of CaCl₂ and controls for cyclic AMP-dependent protein kinase were carried out using reaction mixtures without cyclic AMP. TCA-insoluble materials were recovered by membrane filtration. After washing the filter with 4 x 2 ml of 25% TCA, recovered radioactivity was counted using a scintillation counter. One unit of protein kinase activity is defined as the transfer 1 pmole of 32P to H1 histone in 1 min. Adenylate cyclase activity was determined in a reaction mixture, total volume 250 μl, containing 60 mM sucrose, 80 mM Tris pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 0.2 mM EGTA, 5 mM creatine phosphate, 125 μg creatine phosphokinase, 20 μM GTP, 1
mM $^3$H-cyclic AMP (2–3 × 10$^4$ cpm), 4 mM theophylline, 0.2 mM ATP. Fifty µl aliquots of the membrane suspension (30–70 µg protein) were added to triplicate tubes in ice. Usually cyclase activities were determined without activator (basal), and with $10^{-5}$ M isoproterenol (ISO), $10^{-4}$ M endothelin peptide (VIP), 0.1 mM aluminum sulfate plus 2 mM sodium fluoride (Al/F), and with 60 µM forskolin (FSK), measured as one set. After a 3 min preincubation with drugs and all other buffer ingredients, the assay was started by adding [$\alpha$-$^3$P]-ATP, terminated after 3 min by placing the tubes in boiling water (10 min), and the c-AMP isolated by the double column method (Dowex 50, alumina) of Salomon et al.$^{21}$

### Intravitreal Injections, Intraocular Pressure Measurements and Aqueous Humor Protein Determination

PMA was dissolved in sterile 1% dimethylsulfoxide (DMSO) in normal saline and 20 µl of the solution (2.5–5.0 µM) was injected at 4 PM using a microsyringe with a 26 gauge disposable needle through the inferotemporal sclera 3 mm from the limbus into the vitreous cavity of one eye of six albino rabbits under anesthesia with systemic ketamine (12.5 mg/kg) and acepromazine (2.5 mg/kg), and with topically administered 0.5% proparacaine. Another group of six rabbits were similarly injected with 20 µl of 5 µM 4-α-phorbol didecanoate (PDD) in 1% DMSO/normal saline in one eye. The contralateral eye received intravitreal injections of 20 µl of the vehicle in all cases. Solutions were made up and coded by one person and the injections and pressure measurements made by another person on a masked basis. Intraocular pressure measurements were made on rabbits between 3 and 4 PM on the day of and just prior to injection and also beginning the next day at 9 AM, noon, and 5 PM for 3 successive days after injection, using a manometrically calibrated pneumotonograph (Digilab Model 30 R) as previously reported.$^{15}$ In other groups of rabbits similarly treated, aqueous humor protein concentration was determined 42 hr after intravitreal injection of drugs, which is the time of maximum IOP effect. In other trials, rabbits were pretreated 4 hr before the intravitreal drug with 20 mg/kg indomethacin (administered intraperitoneally) and 10 mg/kg i.p. indomethacin twice a day thereafter (Table 1). The indomethacin regimen did not itself affect IOP.

### In Vitro PMA Treatment of Whole Ciliary Processes or Membranes With or Without CaCl$_2$

Whole ciliary processes dissected from three rabbit eyes were incubated at 30°C for 45 min in 1 ml of continuously gassed (95% O$_2$, 5% CO$_2$) Krebs-Ringer solution which contains 1.8 mM CaCl$_2$. PMA was added to a final concentration of 0.5 µM, and after a 45 min incubation the membrane fraction was prepared and adenylate cyclase responses determined as described above. Control ciliary processes were incubated in the same solution containing the PMA vehicle (0.1% DMSO).

In the case of ciliary process membranes, which are prepared in a Ca$^{2+}$-free medium, two kinds of PMA pretreatments were given immediately prior to adenylate cyclase assays. Membranes were preincubated for 3 min with or without 1 µM PMA in the absence of Ca$^{2+}$, or they were similarly preincubated with or without PMA in the presence of 1 mM CaCl$_2$ added to the adenylate cyclase buffer (Tables 2 and 3). In each case, the control membrane incubation contained the PMA vehicle (0.1% DMSO).

### Reconstitution With Protein Kinase C

The membrane fraction from rabbit ciliary processes was incubated with partially purified protein kinase C from rat ciliary processes (15 pmol/min/tube) or from rat brain (150 pmol/min/tube) at 30°C for 3 min. The incubation mixture contained all necessary ingredients for the measurement of adenylate cyclase and 1 mM added CaCl$_2$. The free Ca$^{2+}$ concentration was estimated to be 2–5 µM due to the Ca$^{2+}$ buffering capacity of the EDTA + EGTA in the assay medium.$^{22}$ Protein kinase C and 1 µM PMA were added as indicated (Table 4). After a 3 min preincubation, the adenylate cyclase assay was initiated by adding $\alpha$-$^3$P-ATP and the reaction terminated after 3 min as described above. In the controls, buffer A was used in place of protein kinase C.

### Protein Determination

Protein was determined by the method of Bradford$^{23}$ using bovine serum albumin as a standard. All experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

### Results

#### Characterization of Protein Kinase C of the Rabbit Ciliary Processes

Using H1 histone as an exogenous substrate, Ca$^{2+}$-dependent protein kinase C and cyclic AMP-dependent protein kinase represented the two major protein kinase activities of the three activities measured by this substrate in the rabbit ciliary processes.$^{24}$ Figure 1 shows a typical DEAE-cellulose chromatographic separation of the two protein kinases in the 100,000 g supernatant fraction from the ciliary pro-
rabbit ciliary processes was thus obtained. Chromatographically separated protein kinase C activity of the rabbit ciliary processes, while totally dependent on the presence of Ca2+, showed increased activity with phosphatidyl serine and required phosphatidyl serine together with a diacylglycerol (such as diolein) to show maximal activity (Fig. 2). Low concentrations of PMA could activate protein kinase C in the presence of Ca2+ and phosphatidylerine, but PDD, which has no tumor-promoting activity, was ineffective on protein kinase C of the rabbit ciliary processes under the same conditions (Fig. 2). Phosphatidylerine, PMA or PDD were neither activators or inhibitors of cyclic AMP-dependent protein kinase activity isolated from rabbit ciliary processes at the same concentrations that affected protein kinase C (Fig. 2).

In Vivo Effects of PMA and PDD on the Intraocular Pressure and Aqueous Humor Protein of Rabbit Eyes

After 50 pmoles of PMA was injected into the vitreous of rabbit eyes, the intraocular pressure (IOP) had fallen by 20 to 30% by the next day relative to the IOP of the contralateral vehicle-injected eyes measured at the same time (Fig. 3). This reduction of IOP reached its maximum (almost 40% change) at about 40 hr after the injection and continued for the period of measurement (72 hr). At the time of maximum PMA effect (40 hr) there was no contralateral effect in the vehicle-injected eyes relative to the baseline.

Fig. 1. DEAE-cellulose chromatography of the supernatant fraction of the rabbit ciliary processes. \( \begin{array}{c} \text{protein kinase C activity} \\ \text{cyclic AMP-dependent protein kinase activity} \end{array} \)

Fig. 2. Activator, phospholipid and diacylglycerol dependence of protein kinase C and cyclic AMP-dependent protein kinase isolated from rabbit ciliary processes. PKC, protein kinase C activity; PKA, cyclic AMP-dependent protein kinase activity; PS, phosphatidyl serine; DO diolein; PMA, 48-phorbol 12-myristate 13-acetate at 40 and 0.4 nM; 4aPDD, 4a-phorbol 12,13-didecanoate. Baseline activity for PKA activity was 150 cpm. See Methods for concentrations of cofactors and activators not shown in figure.

Fig. 3. Effects of intravitreal injection of phorbol esters on the intraocular pressure of rabbit eyes. Fifty pmoles of PMA (closed symbols) or 100 pmoles of PDD (open symbols) were injected intravitreally into one eye in groups of six rabbit eyes and vehicle injected into the contralateral eyes. IOP change represents the % difference in pressure of PMA-treated eyes relative to the pressure of the contralateral eyes injected with vehicle measured at the same time. Pressures in vehicle- and drug-treated eyes respectively in the PMA experiment: baseline (preinjection) 22.00 ± 1.18, 21.87 ± 1.16; at 42 hr 21.98 ± 0.87, 14.63 ± 0.63. Corresponding pressures in the PDD experiment: baseline 20.00 ± 1.09, 20.50 ± 1.26; at 42 hr 19.33 ± 1.43, 18.34 ± 1.65.
(preinjection) intraocular pressures. By comparison, a higher dose (100 pmoles) of PDD did not produce any significant change of IOP (Fig. 3). Aqueous humor protein concentration in the 50 pmoles PMA-injected eyes tended to be higher than the vehicle-injected contralateral eyes ($P < 0.10$, Table 1). All eyes, whether injected with vehicle or PMA, showed significantly increased protein ($P < 0.05$) relative to un.injected normal rabbit eyes, which had a protein content of 0.95 ± 0.1 mg/ml, n = 5. However, after systemic indomethacin pretreatment the aqueous humor protein content both in PMA-treated and vehicle-treated eyes was less than in animals not pretreated with indomethacin. The decrease in protein was significant ($P < 0.02$) in the case of eyes injected with vehicle, but not in eyes injected with PMA. Differences in protein content between PMA-treated and vehicle-treated eyes were also not statistically significant in the indomethacin-pretreated group ($P < 0.10$). In contrast to the protein data, the IOP change at 24 hr in response to 75 pmoles PMA, although significantly less, was not abolished by indomethacin pretreatment when compared to the IOP change in animals not given indomethacin (Table 1).

Effect of In Vivo and In Vitro PMA Treatment of Ciliary Processes on Adenylate Cyclase

Rabbits were injected intravitreally with 50 pmoles of PMA into one eye and with an equal volume of vehicle in the other eye. Seventy-two hours after the injection, animals were sacrificed and adenylate cyclase activities of the ciliary process membranes from PMA-treated eyes and vehicle-treated eyes were measured and compared. As shown in Figure 4, basal adenylate cyclase activities were almost the same. No significant differences in net adenylate cyclase activations by ISO, VIP, Al/F and FSK were found comparing the PMA-treated and untreated (vehicle-injected) groups (Fig. 4). This result was reproducible in three separate experiments, and in each case, the reduction in IOP was present only in the PMA-treated eyes measured immediately before sacrifice.

For in vitro exposure to PMA, ciliary processes from normal rabbit eyes were dissected and half of the processes were incubated with 0.5 μM PMA in physiological buffer and the remaining half were incubated in buffer containing the PMA-vehicle as described in Materials and Methods. Membrane fractions were prepared after the in vitro incubation and adenylate cyclase activities were then measured on

![Graph](https://example.com/graph.png)

Fig. 4. Effects of in vivo PMA treatment on adenylate cyclase responsiveness of rabbit ciliary process membranes determined in vitro. Adenylate cyclase activities were measured in vitro on the membrane fractions prepared from rabbit ciliary processes dissected from eyes 72 hr after intravitreal injection of vehicle, 20 μl 1% DMSO in one eye (untreated) and 50 pmoles of PMA (PMA treated) contained in 20 μl 1% DMSO into the other eye. Activities are shown without any activator (B) or as net responses above basal in the presence of 10−6M isoproterenol (ISO), 10−6M vasoactive intestinal peptide (VIP), 0.1 mM aluminum sulfate plus 2 mM sodium fluoride (F), or with 60 μM forskolin (FSK).
Table 2. Effects of phorbol ester (PMA) in 1 mM CaCl$_2$ on basal adenylate cyclase activity in ciliary process membranes (pmoles/min/mg protein ± SEM, n = 3)

<table>
<thead>
<tr>
<th>Addition</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle†</td>
<td>9.9 ± 2.1</td>
<td>30.4 ± 3.2</td>
</tr>
<tr>
<td>1 μM PMA</td>
<td>10.2 ± 0.8</td>
<td>36.2 ± 1.9</td>
</tr>
<tr>
<td>Vehicle + Ca</td>
<td>20.9 ± 0.8</td>
<td>69.1 ± 5.6</td>
</tr>
<tr>
<td>1 μM PMA + Ca</td>
<td>24.3 ± 0.7</td>
<td>77.4 ± 5.7</td>
</tr>
</tbody>
</table>

* 1 mM CaCl$_2$ added in presence of 1.2 mM EDTA + EGTA and 3 mM MgCl$_2$. Free Ca$^{2+}$ is approximately 2-5 μM.
† Vehicle is 0.1% dimethyl sulfoxide.

these membranes. The ratio of adenylate cyclase activations by ISO, VIP, Al/F and FSK in PMA-treated and vehicle-treated processes were determined in two separate experiments (data shown in Table 3, column 1). The mean activities of the treated tissues were 80-90% of the control activities except for VIP where the treated tissue response was 132%. However, the experimental variability in the level of adenylate cyclase enzyme activity was largely due to instability of the adenylate cyclase system when tissues were incubated for 45 min. Therefore, this type of experiment was discontinued in favor of short incubation experiments on membranes reconstituted with the protein kinase enzyme and/or its required cofactors (Tables 2, 3, 4).

Reconstitution of Membranes With Protein Kinase C and Cofactors

To determine effects of exogenous activated protein kinase C on the adenylate cyclase system it was necessary to do separate control experiments using the PKC cofactors (Ca$^{2+}$, PMA) alone. Ciliary process membranes contain some endogenous PKC activity, and therefore the effect of PMA and Ca$^{2+}$ separately or together was determined on basal adenylate cyclase and on responsiveness to a variety of activators. The results showed that Ca$^{2+}$ alone increased basal cyclase activity but that PMA had no significant effect either alone or together with Ca$^{2+}$ (Table 2). Because Ca$^{2+}$ altered the basal, effects of PMA with or without Ca$^{2+}$ on responsiveness of adenylate cyclase to activators were compared using activity ratios (PMA treated/untreated). The cofactors required for PKC activation had no significant effect on activator responsiveness of adenylate cyclase (activity ratios approx. 1.0, data columns 2 and 3 from left in Table 3). As a final control experiment it was established that the PKC preparations from both rat brain and ciliary processes retained about 70% of their normal activity towards histone H1 when assayed in the buffer used for adenylate cyclase determinations to which 1 mM CaCl$_2$ and 1 μM PMA had been added. The decrease in activity was likely due to a lower free Ca$^{2+}$ concentration (2-5 μM) compared to the usual PKC assay buffer (1 mM free Ca$^{2+}$).

Ciliary process membranes from normal rabbits were prepared and either a partially purified protein kinase C preparation (with no measurable cyclic-AMP-dependent protein kinase activity) was added or buffer A was added as the control. After 3 min preincubation in the presence of Ca$^{2+}$ and PMA, adenylate cyclase responsiveness was measured. As shown in Table 4, the addition of 15 pmoles/min/tube of rabbit ciliary process protein kinase C plus 1 μM PMA (in the presence of Ca$^{2+}$) appeared to enhance adenylate cyclase activation compared to the control without protein kinase C. Statistically, however, only the ISO data gave a P value smaller than 0.05. Preincubation with higher activities of protein kinase C prepared from rat brain (150 pmoles/min/tube) augmented ISO, VIP and Al/F adenylate cyclase activations and the differences were all statistically significant. The basal activity and the forskolin response were not significantly affected by reconstitution with

Table 3. Effects of in vitro PMA treatments of ciliary processes or ciliary process membranes on responsiveness of membrane adenylate cyclase to various activators

<table>
<thead>
<tr>
<th>Activators</th>
<th>Ratio of adenylate cyclase activations (PMA treated/untreated control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ciliary processes + PMA* &lt;br&gt;(mean of 2)</td>
</tr>
<tr>
<td>Isoproterenol (10 μM)</td>
<td>0.80</td>
</tr>
<tr>
<td>VIP (1 μM)</td>
<td>1.32</td>
</tr>
<tr>
<td>Al/F (0.1/2 mM)</td>
<td>0.88</td>
</tr>
<tr>
<td>Forskolin (60 μM)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* Whole ciliary processes were incubated in gassed (95% O$_2$, 5% CO$_2$) Krebs-Ringer solution (Ca$^{2+}$ 1.8 mM). Treatment was with or without 0.5 μM PMA, at 30°C for 45 min immediately before membrane preparation.
† Ciliary process membranes were treated with or without 1 μM PMA for 3 min in Ca$^{2+}$-free buffer immediately before determination of adenylate cyclase responses. The untreated control contained 0.1% DMSO instead of PMA.
‡ Ciliary process membrane was treated with or without 1 μM PMA in the presence of 1 mM Ca$^{2+}$ immediately before determination of adenylate cyclase responses. The untreated control contained 0.1% DMSO plus 1 mM CaCl$_2$. 
Table 4. Effects of reconstitution with protein kinase C (PKC) on responsiveness of ciliary process adenylate cyclase to various activators*

<table>
<thead>
<tr>
<th>Activators</th>
<th>With rabbit ciliary process PKC added (15 units/tube, n = 3)</th>
<th>With rat brain PKC added (150 units/tube, n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (basal)‡</td>
<td>—</td>
<td>1.20 ± 0.06</td>
</tr>
<tr>
<td>Isoproterenol (10 μM)</td>
<td>1.64 ± 0.15‡</td>
<td>1.47 ± 0.11‡</td>
</tr>
<tr>
<td>VIP (1 μM)</td>
<td>1.19 ± 0.18</td>
<td>1.48 ± 0.13‡</td>
</tr>
<tr>
<td>AlF (0.1/2 mM)</td>
<td>1.18 ± 0.23</td>
<td>1.38 ± 0.10‡</td>
</tr>
<tr>
<td>Forskolin (60 μM)</td>
<td>1.18 ± 0.06</td>
<td>1.14 ± 0.05‡</td>
</tr>
</tbody>
</table>

* Reconstituted incubations contained 1 mM CaCl₂ with 1 μM PMA + PKC added. Control incubations contained 1 mM CaCl₂ with 1 μM PMA + buffer A added.
† These concentrations of PKC are estimated to be approximately 10-20% and 100-200% respectively of the endogenous concentration in ciliary processes.
‡ Basal adenylate cyclase activity ranged from 72.5-106.7 pmoles/mg/min with 1 mM CaCl₂ in the medium.

protein kinase C (Table 4). When PMA was omitted, a smaller degree of potentiation was observed, and the presence of Ca²⁺ was required for the potentiation of adenylate cyclase activation by protein kinase C + PMA (data not shown).

Discussion

Protein kinase C, first discovered by Nishizuka and coworkers in 1977,²⁵⁻²⁶ plays important physiological roles in many organs (see reviews).⁶⁻⁸ Not much is known, however, about protein kinase C in relation to the control of water and electrolyte transport by ciliary processes. The only report on PMA noted that it causes desensitization of the adenylate cyclase system in the rabbit iris/ciliary body in vitro and in the SV 40 transformed human ciliary epithelial cultured cell line.²⁷

We began this study by demonstrating the presence of the PMA receptor protein, protein kinase C itself, in rabbit ciliary processes. As shown in Figure 1, protein kinase C is the major kinase activity in the rabbit ciliary processes when H1 histone is used as a substrate. In many organs and cells, protein kinase C activity is greater than that of cyclic-AMP-dependent protein kinase,²⁸⁻²⁹ and our results on rabbit ciliary processes are consistent with these findings. Phorbol esters are specific activators of protein kinase C.²⁻⁵ In ciliary processes PMA also activates protein kinase C but not cyclic-AMP-dependent protein kinase (Fig. 2).

This specific property of PMA was used to explore possible roles of protein kinase C in the ciliary processes. Intravitreal administration of low doses of PMA (50-100 pmoles/eye) caused a substantial fall of IOP in rabbit eyes, while injection of the analogue PDD, which is inactive on protein kinase C (Fig. 2), caused no intraocular pressure change (Fig. 3). The apparent threshold dose of PMA to affect IOP determined by intravitreal injection was about 10 pmoles per eye. The inflammatory response as measured by aqueous humor protein caused by doses of PMA less than 100 pmoles was significant but not as pronounced as with PMA at higher doses (0.5–10 nmoles per eye), which caused a very strong protein response (10–30 mg/ml). The reduction of IOP by 50–100 pmoles PMA appeared to be only partly related to the inflammatory response because a pressure response, though significantly smaller, was still present in indomethacin-pretreated animals where the inflammatory protein response was suppressed (Table 1).

A mechanism for the PMA effect on IOP involving protein kinase C is not easy to demonstrate. Translocation of protein kinase C from the cytoplasm to the cell membrane after PMA treatment and the phosphorylation of target membrane proteins are thought to mediate the cellular responses to PMA treatment.³⁰⁻³¹ We have attempted to demonstrate the shift of enzyme activity in vivo by comparing protein kinase C activities recovered in soluble fractions and in membrane fractions from PMA-injected and vehicle-injected eyes at 72 hr after treatment. However, we did not find any significant differences in protein kinase C distribution in five separate experiments to indicate that there had been translocation of protein kinase C from soluble to particulate, or from particulate to soluble fractions (data not shown). However, negative experiments of this kind may not be definitive because we could not control for the possibility that the enzyme might dissociate from membranes during the preparation of the tissue fractions, or for other artifacts that may obscure measurement of the translocation (see discussion below).

Adenylate cyclase is generally believed to be a key regulatory enzyme in ciliary processes because the level of cyclic AMP appears to modify water and electrolyte transport.⁹⁻¹⁰ Even before the discovery of its specific protein kinase C-activating capacity, PMA had been known to affect adenylate cyclase ac-
tivities in some cell types and organs (see review).\textsuperscript{1} We tested for direct effects of in vitro PMA on adenylate cyclase by treating whole isolated processes or washed membrane preparations, but found no effects either with or without added Ca\textsuperscript{2+} (Tables 2, 3). However, these results are subject to artifact in a way similar to the protein kinase C translocation experiments. In the case of treating isolated ciliary processes with PMA, the effects on adenylate cyclase might have been reversed during subsequent membrane preparation, which is done with Ca\textsuperscript{2+} chelating buffers. When only the membranes are used for treatment with PMA, most of the target protein of the drug is absent since the membranes contain only about 30\% of the total tissue protein kinase C.\textsuperscript{24} Nevertheless, the experiments on membranes treated with 1 \textmu M PMA showed that this treatment did not have non-specific effects on the adenylate cyclase system either alone or in the presence of Ca\textsuperscript{2+}.

Because the membrane preparation lacks the soluble component of ciliary process protein kinase C, we investigated the effect of PMA on adenylate cyclase in membranes reconstituted with rat brain protein kinase C to an estimated activity approximately equivalent to that of the enzyme in intact ciliary processes.\textsuperscript{24} The reconstitution experiments showed that exogenously added activated protein kinase C can within 3–6 min augment adenylate cyclase activation by ISO, VIP, and A1/F (Table 4), all of which require the stimulatory Gs-protein to activate the enzyme. (A suggestion of this effect on the VIP response was also seen in two experiments in Table 3, first data column, where isolated ciliary processes in physiological buffer were treated with PMA in vitro). Although the PKC preparations used are not pure enzyme and contain other proteins, the Ca\textsuperscript{2+} dependency and increased effect of PMA in the reconstitution experiments strongly suggest that protein kinase C is responsible for the potentiation of responsiveness to adenylate cyclase activators. Reconstitution with smaller amounts of protein kinase C isolated from ciliary processes did not give the effects noted with ten-fold greater amounts of the rat brain enzyme. It is possible that the effectiveness of the rat brain enzyme is due to the properties of this enzyme and not just to the greater activity used in experiments, because three forms of protein kinase C have been found in the rabbit of which the \gamma form is ubiquitous in peripheral tissues while the \alpha and \beta forms predominate in the brain.\textsuperscript{33}

It seems likely that the effect of protein kinase C on adenylate cyclase is reversible during membrane preparation in view of the negative results obtained after PMA treatment of rabbit eyes in vivo (Fig. 4) or ciliary processes in vitro followed by membrane preparation (Table 3). However, the difference between our results showing increased adenylate cyclase responsiveness and those of Wax et al,\textsuperscript{27} who reported decreased responsiveness, is not easily explained. Although PMA potentiates adenylate cyclase responses in some cell types and desensitizes in others (see discussion below), both ours and Wax et al’s experiments are directed at the same tissue, ciliary processes. Differences in the tissue preparation (Wax et al used cultured SV40 transformed ciliary epithelial cells or whole dissected iris/ciliary body) and in the adenylate cyclase analysis method (Wax et al used the indirect adenosine prelabelling method) may be important factors.

Recently, several papers have reported on the cross-over of two different signal transduction systems, the cyclic AMP system and the Ca\textsuperscript{2+}/protein kinase C system. For example, protein kinase C is known to cause a potentiation of adenylate cyclase responses in pinealocytes,\textsuperscript{34,35} pituitary cells,\textsuperscript{36} and S49 lymphoma cells.\textsuperscript{37} Other cell types show different responses of the adenylate cyclase to activated protein kinase C. In avian erythrocytes,\textsuperscript{38,39} Leydig cells,\textsuperscript{40} epidermis,\textsuperscript{41} glioma C6 cells\textsuperscript{42} or hepatocytes\textsuperscript{43} PMA treatment desensitizes adenylate cyclase responsiveness. There seems to be no general rule in the cross-over effect of the protein kinase C system on the cyclic AMP-dependent protein kinase system. According to the recent classification proposed by Nishizuka,\textsuperscript{8} our results show that rabbit ciliary processes fall into the group of monodirectional control systems in which one receptor class acting through one signal transduction system potentiates the signals of another transduction system.\textsuperscript{8}

The molecular mechanism(s) to explain the potentiation of adenylate cyclase activation by protein kinase C needs to be elucidated. Our data suggest that sensitization of the G\textsubscript{i}-protein, and not effects on receptors or the cyclase catalytic units, may be involved because the G\textsubscript{i}-dependent responses are potentiated whereas basal activity and the direct activation of adenylate cyclase by forskolin was not significantly affected. Our findings also fit with reported phorbol ester effects on S49 lymphoma cell adenylate cyclase.\textsuperscript{37} In this cell type, Katada et al\textsuperscript{44} have described phosphorylation by protein kinase C of the \alpha-subunit of G\textsubscript{i}, a GTP-binding protein which inhibits the adenylate cyclase enzyme. The phosphorylation leads to inactivation of the biological activity of G\textsubscript{i}, and after protein kinase C treatment, G\textsubscript{i} is no longer inhibitory to the catalytic moiety of adenylate cyclase in fat cells or in platelets.\textsuperscript{45} These findings provide an alternative hypothesis for the results on ciliary processes. The potentiation of adenylate cyclase activation we have observed suggests that when membranes are as-
sayed under the specific conditions used in these experiments, the ciliary process adenylate cyclase may be partly under G
, control. Phosphorylation by protein kinase C inactivates the G
, and this is reflected by an increase in the apparent responsiveness to agents acting via G
. Both hypotheses will require further direct biochemical evidence for the presence of the G
 and G
 proteins in rabbit ciliary process membranes and for their phosphorylation by protein kinase C.

Key words: rabbit, phorbol ester, intraocular pressure, protein kinase C, adenylate cyclase, ciliary process

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