Indomethacin Alters the Na,K-ATPase Response to Protein Kinase C Activation in Cultured Rabbit Nonpigmented Ciliary Epithelium

Nicholas A. Delamere,* † James Parkerson,* and Yining Houf

Purpose. To test whether prostaglandin E₂ (PGE₂) is generated by cultured nonpigmented ciliary epithelial (NPE) cells treated with the phorbol ester, phorbol dibutyrate (PDBu), an activator of protein kinase C. In addition, the authors tested whether indomethacin, a cyclooxygenase inhibitor, influences the stimulation of active sodium–potassium transport observed in PDBu-treated cells.

Methods. A cell line derived from rabbit NPE was used in this study. PGE₂ was measured by an enzyme-linked immunosorbent assay technique. Ouabain-sensitive potassium (⁸⁶Rb) uptake was measured as an index of active sodium–potassium (Na,K-ATPase-mediated) transport. Ouabain-sensitive ATP hydrolysis (Na,K-ATPase activity) also was measured. Cell sodium and potassium content was determined by atomic absorption spectrophotometry.

Results. Marked PGE₂ generation was observed in PDBu-treated cells. Indomethacin abolished the PGE₂ response. Ouabain-sensitive potassium (⁸⁶Rb) uptake was stimulated ~40% in cells exposed to PDBu, but a stimulation of >100% was observed in cells exposed to PDBu in the presence of indomethacin. Added alone, indomethacin did not alter ouabain-sensitive potassium (⁸⁶Rb) uptake. Neither nordihydroguaiaretic acid (a lipoxygenase inhibitor) nor ethoxyresorufin (a cytochrome P450 inhibitor) altered the ⁸⁶Rb uptake response to PDBu. Sodium and cyclic adenosine monophosphate content was unchanged in cells treated with PDBu + indomethacin.

Conclusions. In PDBu-treated cells, there may be generation of cyclooxygenase metabolites of arachidonic acid that inhibit Na,K-ATPase activity, suppressing the stimulatory effect of PDBu on active sodium-potassium transport. Based on the observation that PGE₂ can inhibit Na,K-ATPase activity and also inhibit ouabain-sensitive potassium (⁸⁶Rb) uptake, the authors suggest PGE₂ may influence the Na,K-ATPase response to the activation of protein kinase C in NPE cells. Invest Ophthalmol Vis Sci. 1997;38:866–875.

The ciliary epithelium bilayer is responsible for the formation of aqueous humor, and it is widely believed that mechanisms exist by which hormones, neurotransmitters, and neuropeptides are able to alter the rate of fluid entry into the eye.¹ The transport of solutes across the nonpigmented layer of ciliary epithelium (NPE) is thought to contribute to this fluid movement by setting up an osmotic driving force that causes water to move across the highly invaginated basolateral NPE membrane toward the interior of the eye. Thus, one way for external signals to regulate the rate of aqueous humor formation is through alteration of solute-transport mechanisms at the basolateral membrane of the NPE.

Na,K-ATPase, the sodium pump, is the principal transport mechanism at the basolateral plasma membrane of the NPE. Na,K-ATPase antibodies bind densely to the NPE basolateral membrane,² and it has been shown that several different isoforms of Na,K-ATPase are expressed at that location.³ Sodium pumped actively into the clefts formed by the invaginations of the NPE basolateral membrane partially may drive the osmotic flow of water into the eye. Other solute transporters on the basolateral or apical NPE

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In a number of tissues, it has been suggested that specific changes in Na,K-ATPase activity can take place when external signals activate second-messenger pathways that hinge on protein kinase activation or cytoplasmic calcium mobilization. In addition, studies of the kidney tubule have illustrated the importance of eicosanoids in modulating Na,K-ATPase activity. Prostaglandin E₂ (PGE₂) in particular causes marked Na,K-ATPase inhibition in cells from the inner medul- lary collecting duct and cortical collecting duct. It has also been suggested that PGE₂ may be responsible for causing the sodium pump inhibition observed when renal tubule cells are exposed to endothelin or interleukin-1. Thus, it has been proposed that cytoplasmic generation of arachidonic acid metabolites such as PGE₂ by the cyclooxygenase pathway may be one of the events that leads to altered renal Na,K-ATPase activity after experimental maneuvers to activate cytoplasmic second-messenger pathways.

Using a cell line derived from NPE, we reported previously that sodium pump activity was stimulated approximately 40% in cells exposed to phorbol dibutyrate (PDBu), a recognized activator of protein kinase C. However, phorbol esters also are known to trigger the generation of PGE₂ by a mechanism that may not necessarily be related to protein kinase C activation. Because PGE₂ itself could have an effect on Na,K-ATPase activity, we conducted the current study to first test whether PGE₂ is generated by NPE cells exposed to PDBu. Second, we tested whether the sodium pump response to PDBu is altered by indomethacin, a cyclooxygenase inhibitor that blocks prostaglandin generation. These studies could have relevance to the eye because adrenergic agents cause prostaglandin synthesis, and cyclooxygenase inhibitors are well known to modify the intraocular pressure response to topically applied adrenergic agents.

MATERIALS AND METHODS

Chemicals

Phorbol12,13-dibutyrate (PDBu), indomethacin, oua- bain, ethoxyresorufin, and bumetanide were obtained from the Sigma Chemical Company (St. Louis, MO). Arachidonic acid, nordihydroguaiaretic acid (NDGA), and prostaglandin E₂ were purchased from Cayman Chemical (Ann Arbor, MI). 86RbCl was purchased from Amersham Corporation (Arlington Heights, IL). All other chemicals were obtained from the Fisher Scientific Company (Pittsburgh, PA). Water-insoluble substances were dissolved in a minimum volume of either ethanol or dimethylsulfoxide. Equal amounts of ethanol or dimethylsulfoxide were added to control solutions. The final concentration of dimethylsulfoxide or ethanol was less than 0.3%. Control experiments were conducted using reagent blanks to confirm that the dimethylsulfoxide or ethanol produced no detectable change in 86Rb uptake or ATP hydrolysis.

Cell Culture

These studies were conducted using confluent monolayers of simian virus 40 virus-transformed rabbit non-pigmented ciliary epithelial cells (NPE). The cell line, which was a gift from Dr. Miguel Coca-Prados (Yale University), was developed in accordance with ARVO guidelines for the use of animals in research. The cell line has been used previously in studies of the Na,K-ATPase, the Na–K–2Cl cotransporter, and the ascorbic acid transporter. The cell line and native rabbit ciliary epithelium appear to express similar Na,K-ATPase isoforms. The cells were grown in Dulbecco’s minimum essential medium (Sigma Chemical) supplemented with 10% fetal bovine serum at 37°C under a humidified atmosphere of 95% air–5% CO₂. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were used as antibacterial agents. The cells were passaged at a split ratio of 1:2 to 1:4. At this split ratio, the cultures became confluent after 3 to 5 days. Experiments were conducted using confluent monolayers of cells on 8-mm diameter 24-well culture plates (Costar, Cambridge, MA). Cell monolayers were used within 1 day of reaching confluence. There was no detectable difference in 86Rb uptake rate between batches of cells with different passage numbers.

Before each experiment, the cell culture medium was removed and the cells were equilibrated for 1 hour with Krebs solution, which contained 119 mM sodium chloride, 4.7 mM potassium chloride, 1.2 mM KH₂PO₄, 25 mM sodium bicarbonate, 2.5 mM calcium chloride, 1 mM magnesium chloride, and 5.5 mM glucose at pH 7.4. The Krebs solution was preequilibrated with 95% air–5% CO₂ before use. The experiments were conducted in a 37°C tissue culture incubator under a humidified atmosphere with 5% CO₂–95% air.

86Rb Uptake Studies

The ouabain-sensitive rate of 86Rb uptake was used as an index of active ion transport mediated by the Na,K-ATPase. It was assumed that 86Rb is transported similarly to potassium; this is the case in other cell types. 86Rb uptake was determined either in the presence or absence of 1 mM ouabain. Cell monolayers in 24-well culture plates were exposed to ouabain for 10 minutes, and 86Rb (approximately 1 µCi/ml) was added for a further 10 or 20 minutes. In separate experiments, we have verified that total 86Rb uptake and 86Rb uptake in the presence of ouabain is approximately linear for
at least 20 minutes (data not shown). The $^{86}$Rb uptake was terminated by tipping off the radioactive solution and immersing the culture plate in 1 l ice-cold nonradioactive Krebs solution for 2 minutes to wash out extracellular $^{86}$Rb. Separate experiments were conducted to verify that no significant loss of intracellular $^{86}$Rb could be detected over a wash period of up to 5 minutes. The cells then were lysed by 0.5 N sodium hydroxide, and radioactivity in the lysate was measured by scintillation counting (LS8801; Beckman, Irvine, CA). The protein content in each well was measured using a Bio-Rad assay (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. Knowing the specific activity of $^{86}$Rb in the Krebs solution, the uptake data were expressed as nanomoles of potassium accumulated per milligram of protein per minute.

**Measurement of Na,K-ATPase Activity**

As described above, cell monolayers in 24-well culture plates were preequilibrated in Krebs solution for 1 hour before use. ATP hydrolysis rates then were determined in cell monolayers permeabilized by a combination of osmotic shock and freeze-thaw. Specifically, the Krebs solution was removed and 100 $\mu$l water was added to each well. The culture plate was placed in liquid nitrogen for 5 seconds to freeze the cell monolayers, which subsequently were thawed by adding 200 $\mu$l of a buffer mixture made up to give a final composition of histidine (40 mM), potassium chloride (10 mM), magnesium chloride (5 mM), ethylene glycol-bis(β-aminoethyl ether) NNN'-tetraacetic acid (EGTA) (1 mM), alamethicin (1 $\mu$g/ml), and sodium chloride (100 mM) at pH 7.4. Alamethicin was included in the solution to ensure permeability of the cell membranes to ions and ATP. As specified, ouabain or other test agents were added to some wells. The final ouabain concentration was 1 mM. After preincubation for 5 minutes, adenosine triphosphate (ATP) was added to each well (final concentration 1 mM), and the culture plates were incubated at 37°C for 30 minutes. The ATP hydrolysis reaction was terminated by adding 150 $\mu$l of 50% trichloroacetic acid to each well and placing the culture plate on ice. The contents of each well was removed and placed in a centrifuge for 10 minutes at 3000 revolutions per minute (rpm). An aliquot of the supernatant was collected and added to a tube containing 400 $\mu$l 4% ferrous sulfate in ammonium molybdate reagent (Sigma Chemical). PO$_4$ release resulting from ATP hydrolysis was determined by measuring the absorbance at a wavelength of 750 nm using KH$_2$PO$_4$ as a standard. Na,K-ATPase activity was calculated as the difference ATPase activity in the presence and absence of ouabain. The Na,K-ATPase data were expressed as nanomoles ATP hydrolyzed/milligram per protein per 30 minutes.

**Measurements of Sodium and Potassium**

Cellular sodium and potassium content was determined using atomic absorption spectrophotometry. As described above, monolayers of cells grown in 24-well culture plates were preequilibrated for 1 hour in Krebs solution. Some cell monolayers then were exposed to PDBu for 20 minutes in the presence or absence of 1 $\mu$M indomethacin added 15 minutes before PDBu addition. After this, the Krebs solution was tipped off, and the cells were washed by immersing the 24-well culture plate for 5 minutes in 1 l ice-cold isotonic magnesium chloride (pH adjusted to 7.4 using TRIS) and then lysed in 200 $\mu$l 30% nitric acid. The lysates were diluted with deionized water, and sodium and potassium in the mixture was measured using an atomic absorption spectrophotometer (Perkin-Elmer 372, Norwalk, CT).

**Measurement of Prostaglandin E$_2$**

As described above, monolayers of cells grown in 24-well culture plates were preequilibrated for 1 hour in Krebs solution. Some cell monolayers then were exposed to PDBu for 20 minutes in the presence or absence of 1 $\mu$M indomethacin added 15 minutes before PDBu addition. After this, the Krebs solution was removed and placed in a tube containing 1 ml chloroform–methanol (2:1). Then 250 $\mu$l methanol was added to the monolayer of cells remaining in the culture well. After 30 minutes, the cells were scraped from the well. Then the cell–methanol mixture was removed and combined with the Krebs solution samples in the chloroform–methanol mixture. The tubes were shaken vigorously for 2 hours and then allowed to settle. The lower layer was removed and dried under a stream of nitrogen. This then was suspended in buffer and assayed for PGE$_2$ with an enzyme-linked immunosorbent assay kit (ELISA Technologies, Lexington, KY) using authentic PGE$_2$ standards in the range of 0.1 to 10 ng/ml.

**Measurements of Cyclic Adenosine Monophosphate**

Monolayers of cells grown in 24-well culture plates were preequilibrated for 1 hour in Krebs solution. Some cell monolayers then were exposed to PDBu for 20 minutes in the presence or absence of 1 $\mu$M indomethacin added 15 minutes before PDBu solution. After this, the Krebs solution was tipped off and the cells scraped from the well and boiled 5 minutes in 4 mM EDTA. Then the samples were placed in a centrifuge for 5 minutes at 1000 rpm. The concentration of cyclic adenosine monophosphate (cAMP) in
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FIGURE 1. The influence of phorbol dibutyrate (PDBu) and indomethacin on prostaglandin E<sub>2</sub> production. One group of cultured rabbit nonpigmented ciliary epithelial cells (PDBu) was exposed to PDBu (10 μM) for 20 minutes before the measurement of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Another group of cells (PDBu after INDO) received 1 μM indomethacin 15 minutes before the addition of PDBu. A different group of cells (INDO alone) received only 1 μM indomethacin. Control cells (CTRL) received neither indomethacin nor PDBu. For each group, PGE<sub>2</sub> was measured as described in Materials and Methods. The values are the mean of results from at least 12 monolayers taken from several different batches of cells. ** indicates a significant difference (P < 0.01) from control.

Data Analysis

Unless otherwise noted, the data are expressed as the mean ± standard error (SE) with an n value signifying the number of cell monolayers tested.

RESULTS

Generation of PGE<sub>2</sub> by PDBu-Treated Cells

As measured by an enzyme-linked immunosorbent assay technique, cultured rabbit NPE cells maintained in control Krebs solution for 20 minutes generated only a small amount of PGE<sub>2</sub> (~60 pg/monolayer). In marked contrast, cells exposed to PDBu (10 μM) for 20 minutes generated >600 ng PGE<sub>2</sub>/monolayer (Fig. 1). In the presence of indomethacin (1 μM), a cyclooxygenase inhibitor, the stimulatory effect of PDBu on PGE<sub>2</sub> generation was abolished.

Indomethacin Alters the Size of the Na,K-ATPase Response to PDBu

We know from earlier studies that PDBu increases the rate of ouabain-sensitive potassium (<sup>86</sup>Rb) uptake. In the current study, monolayers of cultured rabbit NPE cells were exposed for 10 minutes to PDBu, and then potassium (<sup>86</sup>Rb) uptake was measured over a period of 20 minutes, still in the presence of PDBu. At concentrations of 3 and 10 μM, PDBu increased the ouabain-sensitive potassium (<sup>86</sup>Rb) uptake rate by 35% and 40%, respectively. However, a significantly greater increase in the ouabain-sensitive potassium (<sup>86</sup>Rb) uptake rate was observed when the cells were exposed to PDBu in the presence of the cyclooxygenase inhibitor indomethacin (1 μM) added 15 minutes before the addition of PDBu. Thus, cells challenged with 10 μM PDBu in the presence of indomethacin had a ouabain-sensitive potassium (<sup>86</sup>Rb) uptake rate of 262 ± 40 nmol/mg protein per 10 minutes (mean ± SE, n = 12), whereas a significantly different (P < 0.05) rate of 140 ± 9 was observed in cells challenged with 10 μM PDBu alone (Fig. 2); both these values were significantly higher (P < 0.01) than the

the supernatant was measured using a competitive binding assay kit (Sigma Chemical).

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ouabain-sensitive potassium ($^{86}$Rb) uptake rate of 103 ± 9 measured in control cells that received neither PDBu nor indomethacin. Similarly, indomethacin-treated cells challenged with $3 \mu M$ PDBu had an ouabain-sensitive uptake rate that was 61% greater (significant at $P < 0.05$) than the rate observed with $3 \mu M$ PDBu but no indomethacin. At $1 \mu M$ or lesser concentrations of PDBu, the presence of indomethacin did not significantly alter the ouabain-sensitive potassium ($^{86}$Rb) uptake response. However, small (<30%) changes in ouabain-sensitive potassium ($^{86}$Rb) uptake could go undetected.

Indomethacin inhibits the cyclooxygenase pathway for the metabolism of arachidonic acid. In the presence of piroxicam (5 $\mu M$), a different cyclooxygenase inhibitor, added 15 minutes before the addition of PDBu, a ouabain-sensitive potassium ($^{86}$Rb) uptake rate of 229 ± 12 nmol/mg protein per 10 minutes (mean ± SE, $n = 8$) was observed in cells challenged with $10 \mu M$ PDBu (Fig. 2); this value was significantly different ($P < 0.01$) from the rate measured in cells with $10 \mu M$ PDBu alone but not significantly different from the rate measured in cells challenged with $10 \mu M$ PDBu in the presence of $1 \mu M$ indomethacin. Added alone, $5 \mu M$ piroxicam did not significantly alter the rate of ouabain-sensitive potassium ($^{86}$Rb) uptake.

Indomethacin alone caused no detectable change of potassium ($^{86}$Rb) uptake. In a paired experiment, a ouabain-sensitive potassium ($^{86}$Rb) uptake rate of 112 ± 12 nmol potassium/mg protein per 10 minutes (mean ± SE, $n = 12$) was observed in cells exposed to $1 \mu M$ indomethacin, whereas a value of 115 ± 23 was observed in control (no indomethacin) cells. In plain contrast, indomethacin added 15 minutes after the addition of $10 \mu M$ PDBu caused a pronounced stimulation of ouabain-sensitive potassium ($^{86}$Rb) uptake rate to a value of 263 ± 40 nmol/mg potassium per milligram protein per 10 minutes (significantly greater than control $P < 0.01$). The addition of $1 \mu M$ indomethacin did not increase ouabain-sensitive potassium ($^{86}$Rb) uptake in cells exposed to $10 \mu M$ 4α-phorbol 12,13-didecanoate (4αPDD), a phorbol ester that does not activate protein kinase C (Fig. 3). Added alone, $10 \mu M$ 4αPDD did not cause a significant change of ouabain-sensitive potassium ($^{86}$Rb) uptake. However, when cells were exposed to $10 \mu M$ 1,2-dioc-tanoylglycerol (DOG), an exogenous diacylglycerol analog that can stimulate PKC, the subsequent addition of $1 \mu M$ indomethacin significantly increased ouabain-sensitive potassium ($^{86}$Rb) uptake almost twofold (Fig. 3).

A significant component of the inward potassium movement is insensitive to ouabain but can be inhibited by bumetanide, an inhibitor of the Na–K–2Cl cotransporter.10 The response of the ouabain-insensi-

![Figure 3. Changes of ouabain-sensitive potassium ($^{86}$Rb) uptake rate caused by indomethacin (1 $\mu M$). One group of cultured rabbit nonpigmented ciliary epithelial cells (4α-phorbol 12,13-didecanoate [PDD] pretreated) was exposed to $10 \mu M$ 4αPDD for 30 minutes before the start of the $^{86}$Rb uptake period; for half the cells, this was followed by $1 \mu M$ indomethacin 15 minutes before the start of the $^{86}$Rb uptake period. A different group of cells (phorbol dibutyrate [PDBu] pretreated) received 10 $\mu M$ PDBu 30 minutes before the start of $^{86}$Rb uptake; for half the cells, this was followed by $1 \mu M$ indomethacin 15 minutes before the start of $^{86}$Rb uptake. A different group of cells (1,2-dioctanoylglycerol [DOG] pretreated) received 10 $\mu M$ DOG 5 minutes before the start of $^{86}$Rb uptake; for half the cells, this was followed by $1 \mu M$ indomethacin 1 minute before the start of $^{86}$Rb uptake. One group of cells (no pretreatment, but half these cells received indomethacin added 15 minutes before the start of $^{86}$Rb uptake. For each set of cells, $^{86}$Rb uptake was measured for 10 minutes in the presence or absence of $10^{-5} M$ ouabain. The data are presented as the percent change of ouabain-sensitive potassium ($^{86}$Rb) uptake rate caused by the addition of $1 \mu M$ indomethacin. In the several different experiments used to accumulate these data, the control (no indomethacin) rate of ouabain-sensitive potassium ($^{86}$Rb) uptake ranged from 90 to 120 nmol potassium/mg protein per 10 minutes; the rate was similar in 4α PDD-pretreated cells but higher in cells pretreated with PDBu or DOG. The values are the mean ± standard error of results from at least 12 monolayers taken from several different batches of cells. ** Significant difference ($P < .01$) from control. * Significant difference ($P < .05$) from control.}
in PDBu-treated cells exposed to 1 μM indomethacin added 15 minutes before PDBu addition.

Inhibition of Other Pathways for Arachidonic Acid Metabolism

In cells pretreated with a relatively high concentration (1 μM) of the recognized lipoxygenase inhibitor NDGA 15 minutes before the addition of 10 μM PDBu, the rate of ouabain-sensitive potassium (86Rb) uptake was 130 ± 18 nmol potassium/mg protein per 10 minutes (mean ± SE, n = 12), which was not significantly different from the value of 147 ± 9 observed in cells treated with PDBu alone. A similar ouabain-sensitive potassium (86Rb) uptake rate (165 ± 33 nmol potassium/mg protein per 10 minutes) was observed in cells treated with a cytochrome P450 inhibitor ethoxyresorufin (10⁻⁷ M) 15 minutes before the addition of 10 μM PDBu. Added alone, neither 10 μM NDGA nor 10⁻⁷ M ethoxyresorufin altered the rate of ouabain-sensitive potassium (86Rb) uptake. A similar concentration of ethoxyresorufin completely inhibited CAMP-mediated Na,K-ATPase activity changes in rat kidney cortical collecting duct cells.16

Measurements of Cell Sodium, Potassium, and Cyclic Adenosine Monophosphate

Because high concentrations of cAMP can influence ion channels in ciliary epithelium23 and because the Na,K-ATPase can be stimulated by elevation of cytoplasmic sodium concentration, we tested whether the cell sodium and potassium content was altered when cells were exposed to a combination of PDBu and indomethacin. Sodium and potassium levels were measured in control (no PDBu) cells, cells treated with 1 μM indomethacin alone, cells treated with 10 μM PDBu, and cells treated with PDBu in the presence of indomethacin added 15 minutes before PDBu addition. The PDBu exposure time was 20 minutes. Cell sodium content was the same in each case (Table 1), and the ratio of cell sodium to potassium (approximately 1:8.5) was similar to that reported by Bowler et al23 who measured ion content by X-ray microanalysis. For comparison, cell sodium content was increased to 217 ± 25% of the control value (mean ± SE, n = 6; significantly different from control, P < 0.01) in cells that were exposed to 1 mM ouabain for 20 minutes. Similarly, the cell sodium content was increased to 261 ± 27% of the control value (mean ± SE, n = 6; significantly different from control, P < 0.01) in cells that were exposed to 1 μM nigericin for 20 minutes.

Because an elevation cytoplasmic cAMP can influence the Na,K-ATPase activity24 and because exogenous PGE2 can stimulate cAMP production, we tested whether cell cAMP content was altered when cells exposed to PDBu for 20 minutes in the presence or absence of indomethacin added 15 minutes before PDBu addition. No detectable change of cAMP content was observed (Table 1).

Influence of PGE2 on the Sodium Pump

There have been several reports that adding exogenous PGE2 can inhibit sodium pump activity.25,26 This also was the case for cultured rabbit NPE cells, as judged by measurements of ouabain-sensitive potassium (86Rb) uptake. In the presence of 1 μM PGE2 added 10 minutes before the start of the 86Rb uptake period, the rate of ouabain-sensitive potassium (86Rb) uptake was approximately 50% of the value measured in control (no PGE2) cells. At higher PGE2 concentrations, ouabain-sensitive potassium (86Rb) uptake was almost fully inhibited (Fig. 4).

In a different experiment, we tested the direct influence of PGE2 on Na,K-ATPase activity (ouabain-sensitive ATP hydrolysis). Cells were permeabilized by an osmotic shock–freeze-thaw maneuver to enable substances in the external buffer solution to enter. In control cells (no PGE2), the rate of ouabain-sensitive ATP hydrolysis was 137 ± 43 nmol/mg protein per 30 minutes (mean ± SE, n = 12). In the presence of 1

### Table 1. Sodium, Potassium, and cAMP Content of Cultured Rabbit Nonpigmented Ciliary Epithelial Cells

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Sodium (μmol/mg protein)</th>
<th>Potassium (μmol/mg protein)</th>
<th>cAMP (pmol cAMP/monolayer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.30 ± 0.08</td>
<td>3.47 ± 0.07</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>PDBu 10 μM</td>
<td>0.38 ± 0.11</td>
<td>3.58 ± 0.14</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>Indomethacin 1 μM</td>
<td>0.42 ± 0.07</td>
<td>3.40 ± 0.07</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>Indomethacin and PDBu</td>
<td>0.45 ± 0.10</td>
<td>3.53 ± 0.22</td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

Sodium, potassium, and cAMP levels were measured in cells exposed to 10 μM PDBu for 20 minutes. Some cells were pretreated with 1 μM indomethacin added 15 minutes before PDBu, and some cells received 1 μM indomethacin alone for 35 minutes. Control cells received neither indomethacin nor PDBu. Sodium and potassium data are presented as mean ± SE (n = 12 cell monolayers) in units of mol × 10⁻⁹/mg protein. cAMP data are presented as mean ± SE (n = 6 cell monolayers) in units of pmol cAMP/monolayer. cAMP = cyclic adenosine monophosphate; NPE = nonpigmented ciliary epithelium; PDBu = phorbol dibutyrate.
FIGURE 4. Changes of ouabain-sensitive potassium (86Rb) uptake rate caused by prostaglandin E2. Different groups of cultured rabbit nonpigmented ciliary epithelial cells were exposed to prostaglandin E2 [PGE2] 10 minutes before the start of the 86Rb uptake period. For each set of cells, 86Rb uptake was measured for 10 minutes in the presence or absence of 10^-3 M ouabain. The data are presented as the percent change of ouabain-sensitive potassium (86Rb) uptake rate caused by the addition of PGE2. The control (no PGE2) rate of ouabain-sensitive potassium (86Rb) uptake was 78.1 ± 9.5 nmol potassium/mg protein per 10 minutes. The values are the mean ± standard error of results from at least six monolayers taken from several different batches of cells. * Significant difference (P < 0.05) from control.

DISCUSSION

The aim of these experiments was first to test whether PGE2 is generated in cultured NPE cells treated with PDBu and second to test whether indomethacin, a cyclooxygenase inhibitor, influences stimulation of the Na,K-ATPase observed in PDBu-treated cells. In earlier studies, we have established that phorbol esters stimulate the Na,K-ATPase as evidenced by increased ouabain-sensitive potassium (86Rb) uptake in cultured cells derived from human nonpigmented ciliary epithelium.8 However, other investigators have shown that in many cell types, phorbol esters also initiate the release of arachidonic acid and subsequent eicosanoid biosynthesis.10,27-29 In ciliary epithelium, arachidonic acid can be metabolized to generate a broad range of products, including PGE2, PGF2α, 5-HETE, and 12-HETE.10 and it is possible that these metabolites could influence cell function. More important, some arachidonic acid metabolites can act as Na,K-ATPase inhibitors.10,15,31 Indeed, it has been suggested recently that arachidonic acid metabolites may have a functional role in downregulating Na,K-ATPase activity in cells lining the medullary thick-ascending limb of the Loop of Henle (MTAL), perhaps to reduce the metabolic demands on the cell because of the sodium pump.32 Thus, we hypothesized that in cells challenged with PDBu, arachidonic acid metabolites may inhibit Na,K-ATPase activity to suppress the extent of sodium pump stimulation that otherwise would occur. In keeping with this idea, cells challenged with PDBu in the presence of indomethacin or piroxicam, cyclooxygenase inhibitors, responded with a significantly greater stimulation of ouabain-sensitive potassium (86Rb) uptake (>100% stimulation versus ~40% stimulation with no indomethacin).

The cyclooxygenase pathway is just one route of arachidonic acid metabolism; the lipoxygenase and cytochrome P450 pathways also generate arachidonic acid metabolites within the cell. One particular cytochrome P450 metabolite of arachidonic acid, 12-R-HETE, inhibits Na,K-ATPase in rabbit ciliary epithelium33 as well as corneal epithelium34 and Satoh et al.15 have built a convincing case for the cytochrome P450 pathway of arachidonic acid metabolism in modulating the kidney sodium pump. However, we found no evidence to suggest that under the conditions of our

μM PGE2, a significantly (P < 0.05) lower rate of 55 ± 30 nmol/mg protein per 30 minutes was observed.
experiments, lipoxygenase or cytochrome P₄₅₀ metabolites of arachidonic acid alter the sodium pump response to PDBu in cultured ciliary epithelium. Although marked changes in the sodium pump response to PDBu were observed in the presence of indomethacin, neither NDGA, a lipoxygenase inhibitor, nor ethoxyresorufin, an inhibitor of the cytochrome P₄₅₀ pathway, caused detectable alteration of the response.

The ability of indomethacin to increase ouabain-sensitive potassium (²⁶⁸Rb) uptake in PDBu-treated cells needs to be interpreted with caution. PDBu is a recognized activator of protein kinase C, but nevertheless, it is difficult to discriminate with confidence between an effect due to protein kinase C activation or a direct response to the phorbol ester, especially because a relatively high phorbol ester concentration is needed to elicit responses in this cell line. In several tissues, it is well known that the phospholipase A₂-mediated release, then subsequent metabolism of arachidonic acid can be initiated by protein kinase C activation after the application of phorbol esters. However, in studies of cultured rat astrocytes, activation of arachidonic acid metabolism seemed to be a direct phorbol ester effect that did not require protein kinase C activation. In our previous studies with this rabbit NPE cell line, we observed that ion transport activity was not altered by 4αPDD, a phorbol ester that does not activate protein kinase C. In addition, in previous studies, we found that the influence of PDBu on ion transport could be inhibited by H7 and staurosporine (protein kinase inhibitors) and also could be prevented by maneuvers to downregulate protein kinase C. Taken together, these observations led us to conclude that the responses of this cell line to PDBu likely are to be related to PKC activation. Consistent with this notion, we report here that indomethacin did not increase the rate of ouabain-sensitive potassium (²⁶⁸Rb) uptake in cells treated with 4αPDD. The finding that indomethacin does not change potassium (²⁶⁸Rb) uptake in 4αPDD-treated cells leads us to favor the idea that the cyclooxygenase pathway of arachidonic acid metabolism may generate Na,K-ATPase inhibitors in PDBu-treated cells by a mechanism that is linked at least partially to protein kinase C activation as opposed to being entirely a nonspecific phorbol ester effect. This idea is further supported by the observation that the increase of ouabain-sensitive potassium (²⁶⁸Rb) uptake caused by indomethacin also was observed in cells exposed to PDBu is greater in the presence of DOG, a different activator of protein kinase C.

In this cell line derived from rabbit NPE, PDBu inhibits the Na–K–2Cl cotransporter responsible for most of the ouabain-insensitive inward potassium movement. Here we report that indomethacin causes only a small change in the magnitude of the PDBu-induced decrease in ouabain-insensitive potassium (²⁶⁸Rb) uptake. This suggests that cyclooxygenase metabolites of arachidonic acid may not have an important role in the mechanism responsible for PDBu-induced inhibition of Na–K–2Cl cotransporter activity. NDGA and ethoxyresorufin equally were without influence on the ouabain-insensitive component of potassium (²⁶⁸Rb) uptake in either control cells or PDBu-treated cells, even though it has been suggested that in some cells, specific cytochrome P₄₅₀ metabolites of arachidonic acid are capable of causing marked changes in Na–K–2Cl cotransporter function.

In a recent study, Cantley et al detected a Na,K-ATPase inhibitor released by rabbit kidney MTAL cells, the production of which could be inhibited by indomethacin. Similarly, we suggest that cyclooxygenase metabolites of arachidonic acid could inhibit Na,K-ATPase activity in PDBu-treated cells. Although the identity of the putative arachidonic acid metabolites cannot be confirmed, earlier studies have identified PGE₂ as the principal indomethacin-inhibitable arachidonic acid metabolite generated by rabbit ciliary epithelium. Here we report that PGE₂ production was indeed initiated by exposing the cells to PDBu and that indomethacin effectively abolished the PDBu-induced production at PGE₂. Moreover, PGE₂ added exogenously to monolayers of cultured NPE cells was found to cause sodium pump inhibition as judged by reduction of ouabain-sensitive potassium (²⁶⁸Rb) uptake and also by inhibition of ouabain-sensitive ATP hydrolysis. There are several cell types in which PGE₂ is known to be an inhibitor of sodium pump activity.

The sodium pump rate is dependent on the concentration of sodium in the cytoplasm, and some arachidonic acid metabolites are capable of causing changes in active sodium-potassium transport by a mechanism that involves changes in cell sodium entry. However, our results are consistent with the generation of a putative arachidonic acid metabolite that acts to cause inhibition of Na,K-ATPase because there was no evidence of a change of cell sodium in PDBu-treated cells either in the presence or absence of indomethacin. Elevation of cAMP also can lead to Na,K-ATPase inhibition in ciliary epithelium. For this reason, we considered the possibility that PGE₂ generated by PDBu-treated cells could perhaps act to cause cAMP elevation, perhaps by activating EP receptors as described by Jumblatt et al. Added to this, in bovine iris sphincter, PDBu is known to stimulate adenylate cyclase and so cause increased cAMP. However, no detectable PDBu-induced change of cAMP was observed during the relatively brief time course of the current experiments.
It is possible that PGE₂ generated by PDBu-treated cells has an inhibitory influence on Na,K-ATPase activity. In keeping with this idea, we observed that Na,K-ATPase activity measured in this NPE cell line was inhibited significantly in the presence of 1 μM PGE₂. Of course, it is also possible that other cyclooxygenase metabolites of arachidonic acid could act as Na,K-ATPase inhibitors and the relative potency of PGE₂ and other arachidonic acid metabolites as Na,K-ATPase inhibitors awaits further investigation.

Although there likely are to be important differences between the responses of native tissues and the responses of a cell line, the current study does raise the possibility that arachidonic acid metabolites could influence Na,K-ATPase activity in the NPE as is the case in other cell types. Specifically, PDBu seems to cause the generation of cyclooxygenase metabolites of arachidonic acid that inhibit Na,K-ATPase activity. Because it is well known that adrenergic agonists also can initiate the metabolism of arachidonic acid, the current findings raise the interesting possibility that cyclooxygenase metabolites of arachidonic acid may act as cytoplasmic messengers, contributing to the short-term modulation of Na,K-ATPase activity in the NPE as in other cell types. This idea is consistent with reports that in both humans and rabbits, epi-

Key Words

ciliary epithelium, eicosanoids, Na,K-ATPase, phorbol ester, protein kinase C

References


Indomethacin Alters Response to Protein Kinase C Activation


