

# Prostaglandin E<sub>2</sub> Inhibits Sodium Transport in Rabbit Cortical Collecting Duct by Increasing Intracellular Calcium

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## Abstract

The mechanism by which prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) inhibits sodium absorption ( $J_{Na}$ ) in the rabbit cortical collecting duct (CCD) was explored. PGE<sub>2</sub> activates at least three signaling mechanisms in the CCD: (a) by itself PGE<sub>2</sub> increases cAMP generation (b) PGE<sub>2</sub> also inhibits vasopressin-stimulated cAMP accumulation, and (c) PGE<sub>2</sub> raises intracellular calcium ( $[Ca^{++}]_i$ ). We tested the contribution of these signaling pathways to PGE<sub>2</sub>'s effect on Na<sup>+</sup> absorption, measuring <sup>22</sup>Na flux ( $J_{Na}$ ) and  $[Ca^{++}]_i$  (using fura-2) in microperfused rabbit CCDs. In control studies PGE<sub>2</sub> reduced  $J_{Na}$  from  $28.2 \pm 3.4$  to  $15.6 \pm 2.6$  pmol · mm<sup>-1</sup> · min<sup>-1</sup>. Lowering bath calcium from 2.4 to 45 nM did not by itself alter  $J_{Na}$  but in this setting PGE<sub>2</sub> failed to inhibit  $J_{Na}$  ( $28.6 \pm 5.4$  to  $38.5 \pm 4.0$ ). In separate tubules, PGE<sub>2</sub> raised  $[Ca^{++}]_i$  in a spike-like fashion followed by a sustained elevation. However, in 45 nM bath Ca<sup>++</sup>, PGE<sub>2</sub> failed to produce a sustained  $[Ca^{++}]_i$  elevation. While pretreatment of CCDs with pertussis toxin blocked PGE<sub>2</sub> inhibition of vasopressin-stimulated water permeability, it did not block the effect of PGE<sub>2</sub> on  $J_{Na}$ . To see if cAMP generation contributes to the effect of PGE<sub>2</sub> on  $J_{Na}$ , we tested the effect of exogenous cAMP, (8-chlorophenylthio)(CPT)cAMP on  $J_{Na}$ . 0.1 mM 8-CPTcAMP reduced  $J_{Na}$  from  $35.75 \pm 2.3$  to  $21.6 \pm 2.2$ . However, the addition of PGE<sub>2</sub> further blunted  $J_{Na}$  to  $15.9 \pm 1.3$ . In CCDs pretreated with indomethacin, 8-CPTcAMP did not significantly decrease  $J_{Na}$   $33.6 \pm 2.8$  vs.  $28.4 \pm 2$ . However, superimposed PGE<sub>2</sub> reduced  $J_{Na}$  to  $19.0 \pm 3.0$ . We conclude that PGE<sub>2</sub> inhibits sodium transport predominantly by increasing intracellular calcium. This action is not mediated by a pertussis toxin-sensitive G protein. Finally, cAMP, through a cyclooxygenase-dependent mechanism, also inhibits CCD  $J_{Na}$  and may contribute to the effects of PGE<sub>2</sub> on  $J_{Na}$  in the rabbit CCD. (*J. Clin. Invest.* 1991. 87:1992-1998.) Key words: prostaglandin E<sub>2</sub> • collecting duct • sodium • intracellular calcium

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## Introduction

Abundant evidence suggests that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>1</sup> is a major regulator of salt transport in the rabbit cortical collecting duct (CCD). PGE<sub>2</sub> infusion into the renal artery of the dog increases Na<sup>+</sup> and K<sup>+</sup> excretion (1, 2) without any major renal hemodynamic effect, suggesting a direct tubular action. Stokes et al. were the first to demonstrate that PGE<sub>2</sub> had a direct epithelial action by showing that PGE<sub>2</sub> decreased sodium transport in the rabbit (CCD) (3). Iino and Imai confirmed that PGE<sub>2</sub> reduced Na<sup>+</sup> absorption and the lumen negative voltage in rabbit CCD when added to the peritubular surface (4).

The cellular mechanism mediating the decrease in Na<sup>+</sup> absorption is unknown. Recently, Sonnenburg et al. (5) showed that by itself, high concentrations of PGE<sub>2</sub> increase cyclic AMP generation in immunodissected rabbit CCD cells. However, when administered together with AVP, PGE<sub>2</sub> inhibits AVP-stimulated cAMP production in this same preparation. This inhibitory action of PGE<sub>2</sub> was reversed by pertussis toxin. These latter findings are consistent with PGE<sub>2</sub>-mediated inhibition of adenylyl cyclase via a pertussis toxin-sensitive guanine nucleotide binding protein (G<sub>i</sub>) (5). These biochemical studies suggest that pertussis toxin pretreatment might reverse the inhibitory effect of PGE<sub>2</sub> on AVP-induced water flow and perhaps sodium transport.

We recently showed that PGE<sub>2</sub> also increases intracellular calcium ( $[Ca^{++}]_i$ ) in the rabbit CCD (6). Evidence from a number of different epithelia suggests that changes in  $[Ca^{++}]_i$  may regulate transepithelial sodium transport. Maneuvers that increase  $[Ca^{++}]_i$  inhibit sodium transport in frog skin (7) and the toad urinary bladder (8, 9). Taylor et al. have suggested that cell calcium might be involved in a feedback mechanism that links the rate of Na<sup>+</sup> entry across the luminal membrane with Na<sup>+</sup> extrusion across the basolateral membrane of isolated perfused proximal renal tubules (10). Thus, increased  $[Ca^{++}]_i$  may also contribute to PGE<sub>2</sub>'s inhibitory effects on sodium absorption.

The main purpose of this study is to determine if PGE<sub>2</sub>'s effects on cell calcium are required to inhibit sodium transport in isolated perfused rabbit cortical collecting duct. In addition, we wished to test if PGE<sub>2</sub>-mediated inhibition of Na<sup>+</sup> transport could be coupled to its demonstrated stimulatory and inhibitory effects on cyclic AMP generation in rabbit CCD.

1. Abbreviations used in this paper:  $[Ca^{++}]_i$ , intracellular calcium ion concentration; CCD, cortical collecting duct;  $J_{Na}$ , sodium transport;  $L_p$ , hydraulic conductivity; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>;  $R$ , fluorescence ratio;  $V_T$ , transepithelial voltage.

## Methods

### General microperfusion methods

In vitro microperfusion of isolated cortical collecting ducts was performed as previously described (11). Briefly, rabbits weighing 1.5–2.5 kg were killed by using an intramuscular injection of ketamine (44 mg/kg) and xylazine (11 mg/kg) for anesthesia followed by decapitation. The left kidney was quickly removed, and 1- to 2-mm coronal slices were placed in chilled dissection dishes for freehand dissection at 4°C. Tubules were perfused at 37°C at 2–3 nl/min to maximize sensitivity in measurement of changes in isotopic Na. Bath solution was continuously exchanged by an infusion pump (Sage, Orion Research Inc., Cambridge, MA) at 0.5 ml/min. Perfusate, containing  $^{22}\text{Na}$  (Dupont-NEN, Boston, MA) was collected in a constriction pipette of known volume (between 23 and 26 nl). Transepithelial voltage ( $V_T$ ) was measured via a Ringer's agarose bridge connected to the perfusion pipette and a calomel electrode. A similar bridge connected the bath to another calomel electrode and completed the circuit.  $V_T$ , in mV, was measured with an electrometer (model 602; Keithley Instruments, Inc., Cleveland, OH) and continuously recorded on a strip-chart recorder (Primeline model R-02; Soltec Co., Sun Valley, CA). The composition of standard bath medium, dissection medium, and isotonic perfusate were as follows (in millimolar): NaCl, 105;  $\text{NaHCO}_3$ , 25; Na acetate, 10;  $\text{NaH}_2\text{PO}_4$ , 2.3; KCl, 5;  $\text{CaCl}_2$ , 2.4;  $\text{MgSO}_4$ , 1.0 glucose, 8.3; and alanine, 5; (osmolality, 300 mosmol). The perfusate contained 0.2 mg/ml Food, Drug, and Cosmetic dye No. 3 (Aniline and Chemical Co., Chicago, IL) to detect cell damage and perfusate leak (12).

**Measurement of sodium transport in isolated perfused CCDs.** Experiments examining CCD sodium transport ( $J_{\text{Na}}$ ) were conducted according to the following time schedule: after a 30-min period of equilibration in isotonic bath and perfusate,  $^{22}\text{Na}$  (25  $\mu\text{Ci/ml}$ ), and  $^3\text{H}$ -inulin (75  $\mu\text{Ci/ml}$ ) were added to the lumen. After an additional 75 min of equilibration, four collections were made for determination of basal lumen-to-bath  $J_{\text{Na}}$ . Then in the experimental period,  $\text{PGE}_2$  or 8-*p*-chlorophenylthio (CPT) cAMP was added to the bath and after a 10-min equilibration period, four additional timed collections were made to determine sodium flux.  $J_{\text{Na}}$  was defined as the mean of the last three collections made during exposure to the different agonists used. Changes in lumen-to-bath  $J_{\text{Na}}$  likely reflect changes in net sodium absorption since bath-to-lumen  $J_{\text{Na}}$  is low and unaffected by most experimental maneuvers. In all experiments, a final period of  $J_{\text{Na}}$  measurements was taken with  $10^{-4}$  M ouabain in the bath. Ouabain reduces net sodium absorption to a value close to zero (13), thus the lumen-to-bath  $J_{\text{Na}}$  post-ouabain, is likely passive and equal to bath-to-lumen  $J_{\text{Na}}$ .

**Measurement of cell calcium  $[\text{Ca}^{++}]_i$  in isolated perfused CCD.** CCDs were perfused in vitro as described above with the following differences: the bath was a special low volume (0.150 ml) chamber to allow for rapid fluid exchange. The bath solution was preheated in a water-jacketed line and flow rate was maintained between 0.5 to 2.5  $\text{cm}^3/\text{min}$ . Tubules were bathed with 2.5  $\mu\text{M}$  acetoxymethyl ester of fura-2 (fura-2-AM) (Molecular Probes, Eugene, OR) for 45 min at 30°C. After tubules were loaded, the bath temperature was increased to 37°C, the flow rate was increased to 2.5  $\text{cm}^3/\text{min}$ , and tubules were allowed to equilibrate for 20–30 min. CCD fluorescence was measured using continuous rapidly alternating excitation (20 ms per reading) from dual monochromators set at 340 and 380 nm, respectively (Delta-scan; Photon Technology International, New Brunswick, NJ). The monochromator output was coupled to the inverted microscope using a 400-nm dichroic mirror and a 100 $\times$  lens (Nikon fluor oil immersion). Fluorescent emission of light greater than 435 nm was measured by photon counting. Before loading with fura-2, CCD autofluorescence and background light were measured (less than 10% of fluorescent emission in fura-2-loaded tubules) and this value was continuously subtracted from all measurements. The corrected emission intensity ratio, using 340- and 380-nm excitation (340/380 ratio,  $R$ ), was monitored continuously. After fura-2 loading and equilibration, a baseline

reading of 100 to 200 s was taken in standard bath medium. At the end of each experiment an in situ calibration of  $[\text{Ca}^{++}]_i$  was performed. The bath medium was changed to a  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ -free isotonic bath medium containing 2 mM ethyleneglycol-*bis*-B-aminoethylether  $\text{N,N,N',N'}$ -tetraacetic acid (EGTA) and 10  $\mu\text{M}$  4Br-A23187. After a stable 340/380 ratio (minimum fluorescence ratio,  $R_{\text{min}}$ ) was achieved, the bath was changed back to normal bath medium (2.4 mM  $\text{Ca}^{++}$ ) and 10  $\mu\text{M}$  of 4Br-A23187 and the ratio was again allowed to stabilize (maximum fluorescence ratio,  $R_{\text{max}}$ ).

**Measurement of hydroosmotic water flow (hydraulic conductivity  $[L_p]$ ).** Each experiment measuring osmotic water flow was conducted on an identical time schedule. All experiments were performed at 37°C. The perfusate, which contained  $^3\text{H}$  inulin (75  $\mu\text{Ci/ml}$ ) as a volume marker, was collected into a constriction pipette of known volume (between 90 and 130 nl) and counted for  $^3\text{H}$  (New England Nuclear). The perfusion rate was maintained between 12–20 nl/min by adjusting the hydrostatic pressure. At this perfusion rate osmotic equilibration between bath and lumen did not occur. During the first 45 min of equilibration all tubules were perfused with an isotonic solution similar to the bath. Subsequently, the perfusate was changed to hypotonic perfusate. In control studies, 30 min of further equilibration were allowed and then, three collections were made for determination of basal  $L_p$ . Tubules with a negative basal  $L_p$  were discarded. 10  $\mu\text{U/ml}$  AVP was then added to the bath, and after a 15 min equilibration period three to four timed collections were made to determine  $L_p$ . A stable  $L_p$  was usually observed 20–50 min after the addition of AVP. Subsequently, either  $\text{PGE}_2$  + AVP was added or AVP alone was continued. After a 15-min equilibration six more timed collections were made. In each period the three collections with the greatest calculated  $L_p$  were averaged to calculate mean  $L_p$  for this period.

### Experimental protocols

#### 1. The role of intracellular calcium in $\text{PGE}_2$ 's action on CCD $\text{Na}^+$ transport:

**1a. Effect of  $\text{PGE}_2$  on  $J_{\text{Na}}$  in CCDs.** After four basal collections the CCD was exposed to  $10^{-7}$  M bath  $\text{PGE}_2$  and  $J_{\text{Na}}$  was determined. Afterwards,  $10^{-4}$  M ouabain was then added to the bath and  $J_{\text{Na}}$ , presumably equivalent to the passive component of the lumen-to-bath,  $^{22}\text{Na}$  flux was assessed.

**1b. Effect of  $\text{PGE}_2$  on  $[\text{Ca}^{++}]_i$  in CCDs pretreated with 45 nM bath calcium and TMB-8.** Measurements of cell calcium were determined with 2.4 mM bath calcium. The bath was then exchanged to 45 nM  $[\text{Ca}^{++}]$  +  $10^{-5}$  M TMB-8 and  $\text{PGE}_2$  was then added. Finally, the bath was changed to 2.4 mM calcium solution and  $[\text{Ca}^{++}]_i$  was measured in the continued presence of  $\text{PGE}_2$ .

**1c. Effect of  $\text{PGE}_2$  on  $J_{\text{Na}}$  in CCDs pretreated with 45 nM bath calcium and TMB-8.** We examined the effect of a 45 nM calcium buffer + 10  $\mu\text{M}$  TMB-8 on  $J_{\text{Na}}$ . The calcium ion concentration was calculated using a computerized ion affinity table taking into account temperature,  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$ , and EGTA concentration (14). We estimate that 1 mM  $\text{Ca}^{++}$  + 2 mM EGTA yields a free calcium activity of 45 nM. The basal  $J_{\text{Na}}$  collections were made using 2.4 mM calcium in the bath. The bath was then exchanged for 45 nM  $\text{Ca}^{++}$  +  $10^{-5}$  M TMB-8 and  $^{22}\text{Na}$  flux was measured. The CCDs were then exposed to  $\text{PGE}_2$  (in the continued presence of 45 nM  $\text{Ca}^{++}$  + TMB-8) and  $J_{\text{Na}}$  was again determined. Finally, ouabain was added to the bath and  $J_{\text{Na}}$  was measured. In these studies TMB-8 was used to blunt any rise in  $[\text{Ca}^{++}]_i$  induced by  $\text{PGE}_2$ . TMB-8 inhibits cell  $\text{Ca}^{++}$  released from intracellular stores by an, as yet, uncharacterized mechanism (15).

#### 2. Pertussis toxin sensitivity of $\text{PGE}_2$ 's effects on osmotic water flow, $J_{\text{Na}}$ , and $[\text{Ca}^{++}]_i$ :

**2a. Effect of pertussis toxin pretreatment on  $\text{PGE}_2$  inhibition of AVP-stimulated  $L_p$  in CCDs.** CCDs were pretreated with 0, 100, or 500 ng/ml pertussis toxin for 60 min before basal collections. After basal collections CCDs were exposed to 10  $\mu\text{U/ml}$  AVP and  $L_p$  was determined. Finally,  $\text{PGE}_2$  + AVP were added to the bath and peak  $L_p$  was determined.

2b. *Effect of PGE<sub>2</sub> on J<sub>Na</sub> in CCDs pretreated with pertussis toxin.* Pertussis toxin (500 ng/ml) was added to the bath medium for 60 min before basal J<sub>Na</sub> collections. After basal collections the CCD was exposed to PGE<sub>2</sub> and J<sub>Na</sub> was again determined. Finally, ouabain was added to the bath and J<sub>Na</sub> was determined.

2c. *Effect of PGE<sub>2</sub> on [Ca<sup>2+</sup>]<sub>i</sub> in CCDs pretreated with pertussis toxin.* The tubules were pretreated with pertussis toxin 500 ng/ml for 1 h at 37°C. A baseline reading of 100 to 150 s was taken in standard bath medium. The tubules were then exposed to 10<sup>-7</sup> M PGE<sub>2</sub> for 100 to 150 s.

3. *Role of 8-CPTcAMP in mediating PGE<sub>2</sub>'s effects on J<sub>Na</sub>:*

3a. *Effect of 8-CPTcAMP pretreatment on PGE<sub>2</sub> inhibition of J<sub>Na</sub> in CCDs.* Basal <sup>22</sup>Na lumen-to-bath flux was measured. After basal collections J<sub>Na</sub> was determined while tubules were exposed to 10<sup>-4</sup> M 8-CPTcAMP alone. This was followed by the addition of PGE<sub>2</sub> + 8-CPTcAMP and J<sub>Na</sub> was again determined. Finally, ouabain was added to the bath and J<sub>Na</sub> was measured.

3b. *Effect indomethacin pretreatment on 8-CPTcAMP + PGE<sub>2</sub> inhibition of J<sub>Na</sub> in CCDs.* The tubules were pretreated with 5 μM indomethacin for 15 min before basal collections. Then, the CCDs were exposed to 8-CPTcAMP and J<sub>Na</sub> was determined. PGE<sub>2</sub> was then added together with 8-CPTcAMP and J<sub>Na</sub> was calculated. Finally, ouabain was added to the bath and J<sub>Na</sub> was measured.

### Calculations

Lumen to bath <sup>22</sup>Na flux. Net volume flux (J<sub>v</sub>) was uniformly less than 1% of V<sub>0</sub>. This negligible J<sub>v</sub> discounted. The perfusion rate (V<sub>i</sub>) was thus equal to the collection rate (V<sub>0</sub>). J<sub>Na</sub> (l-b) was calculated from the rate of disappearance of tracer from the perfusate, using the following equation:

$$J_{Na}(l-b) = (1 - C_0^*/C_i^*) \times 145 \times V_0/L = \text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1},$$

where C<sub>i</sub><sup>\*</sup> and C<sub>0</sub><sup>\*</sup> are perfused and collected fluid concentrations of <sup>22</sup>Na (cpm/nl), assuming constant specific activity along the tubule length (L). Perfusion rates were adjusted so that only a small amount of perfusate tracer was lost along the tubule, ensuring relative axial uniformity of tracer specific activity.

*Intracellular calcium concentration.* Cell Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>), was calculated by: [Ca<sup>2+</sup>]<sub>i</sub> = K<sub>d</sub>(R - R<sub>min</sub>)/(R<sub>max</sub> - R) (380 min/380 max), assuming that the K<sub>d</sub> value for the fura-2-Ca<sup>2+</sup> complex is 224 nM at 37°C (16). Since this apparent K<sub>d</sub> might display shifts in the intracellular environment (17), the data is represented not only as [Ca<sup>2+</sup>]<sub>i</sub> but also as percentage increase in [Ca<sup>2+</sup>]<sub>i</sub> above basal levels, which is independent of K<sub>d</sub>.

*Hydraulic conductivity.* Net volume flux (J<sub>v</sub>) was calculated from J<sub>v</sub> = (V<sub>i</sub> - V<sub>0</sub>)/L where V<sub>i</sub> is the perfusion rate (nl/min), V<sub>0</sub> is the collection rate (nl/min), and L is the tubule length. V<sub>0</sub> was measured directly, and V<sub>i</sub> was calculated from V<sub>i</sub> = V<sub>0</sub>(cpmo/cpmi), where cpmo and cpmi are perfusate and collected fluid <sup>3</sup>H-counts × min<sup>-1</sup> × nl<sup>-1</sup>, respectively. Hydraulic conductivity (L<sub>p</sub>; cm × atm<sup>-1</sup> × s<sup>-1</sup>) was determined according to Dubois et al. (18).

$$L_p = (1/RTS) \cdot (1/O_b)^2 \cdot [O_b \cdot (V_i - V_0) + O_i \cdot V_i \cdot \ln\{(O_b - O_i) \cdot V_i / (O_b \cdot V_0 - O_i \cdot V_i)\}],$$

where R is the gas constant, T is °K, S is the tubule lumen surface area (assumed luminal diameter of 20 μm), and O<sub>b</sub> and O<sub>i</sub> represent the osmolality of the bath and perfusate respectively.

*Statistics.* Data are presented as mean ± SE and statistical analyses were made using paired t test or one way analysis of variance (ANOVA) whenever appropriate. Differences with P < 0.05 were considered statistically significant.

*Reagents.* AVP, EGTA, PGE<sub>2</sub>, 8-chloro-phenylthio-cyclicAMP, indomethacin, and pertussis toxin were purchased from Sigma Chemical Co., St. Louis, MO. FURA-2AM and 4Br-A23187 were purchased from Molecular Probes.

## Results

### 1. The role of intracellular calcium in PGE<sub>2</sub>'s action on CCD Na<sup>+</sup> transport:

1a. *PGE<sub>2</sub> decreases J<sub>Na</sub> in CCDs.* We first confirmed in the control group the effect of PGE<sub>2</sub> on sodium transport. In the control periods in the absence of PGE<sub>2</sub>, lumen-to-bath <sup>22</sup>Na flux was 28.2 ± 3.4. As expected, addition of 10<sup>-7</sup> M PGE<sub>2</sub> to the bath caused J<sub>Na</sub> fall to 15.6 ± 2.6 pmol/mm per min (P < 0.0005, n = 6) (Fig. 1 left). The addition of 10<sup>-4</sup> M of ouabain, as expected, further decreased J<sub>Na</sub> to 8.3 ± 2.5 pmol · mm<sup>-1</sup> · min<sup>-1</sup>. Thus, PGE<sub>2</sub> inhibits sodium transport in rabbit CCDs.

1b. *Effect of PGE<sub>2</sub> on [Ca<sup>2+</sup>]<sub>i</sub> in CCDs bath in 45 nM calcium + TMB-8.* In the control bath (Fig. 2 a), PGE<sub>2</sub> increases [Ca<sup>2+</sup>]<sub>i</sub>, resulting in an abrupt spike-like increase followed by a sustained elevation in [Ca<sup>2+</sup>]<sub>i</sub>. Fig. 2 b shows the effect of reducing ambient calcium concentration to 45 nM in the presence of 10<sup>-5</sup> M TMB-8, a substance that inhibits calcium release from the intracellular stores (15). It is clear that the effect of PGE<sub>2</sub> on [Ca<sup>2+</sup>]<sub>i</sub> is blunted. PGE<sub>2</sub> produces a small and transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, but there is no sustained increase. However, when bath calcium is returned to 2.4 mM in the continued presence of PGE<sub>2</sub>, a large and sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> is observed again.

1c. *Effect of PGE<sub>2</sub> on J<sub>Na</sub> in CCDs pretreated with 45 nM calcium + TMB-8.* We tested the effect of PGE<sub>2</sub> on J<sub>Na</sub> in the setting of the low Ca<sup>2+</sup> buffer + TMB-8. It can be seen that lowering extracellular calcium to 45 nM plus 10<sup>-5</sup> M TMB-8 by itself, had no effect on lumen-to-bath <sup>22</sup>Na flux (27.8 ± 2.6 vs. 28.6 ± 5.4 pmol/mm per min; Fig. 3). However, when PGE<sub>2</sub> was then added, a significant increase in J<sub>Na</sub> was seen to 36.4 ± 3.9 pmol/mm per min (n = 5). Subsequent administration of 10<sup>-4</sup> M ouabain decreased J<sub>Na</sub> to 12.8 ± 1.6 pmol/mm per min (Fig. 3). Thus, the low Ca<sup>2+</sup> bath + TMB-8 blocked PGE<sub>2</sub>'s capacity to produce a sustained increase in intracellular calcium, and reversed the inhibitory effect of PGE<sub>2</sub> on lumen-to-bath <sup>22</sup>Na flux.

### 2. Pertussis toxin sensitivity of PGE<sub>2</sub>'s effects on J<sub>Na</sub> and hydraulic conductivity:

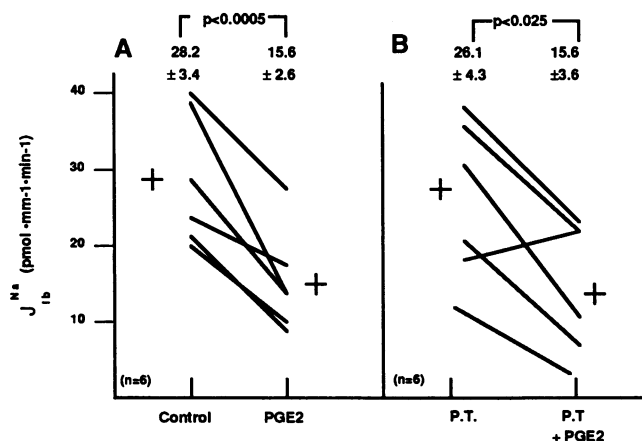


Figure 1. (A) 10<sup>-7</sup> M PGE<sub>2</sub> inhibits J<sub>Na</sub> (P < 0.0005 control compared with PGE<sub>2</sub>). (B) Pertussis toxin pretreatment does not block the inhibition of sodium transport J<sub>Na</sub> by 10<sup>-7</sup> M PGE<sub>2</sub> (P < 0.025 control compared with PGE<sub>2</sub>). n, Number of CCDs.

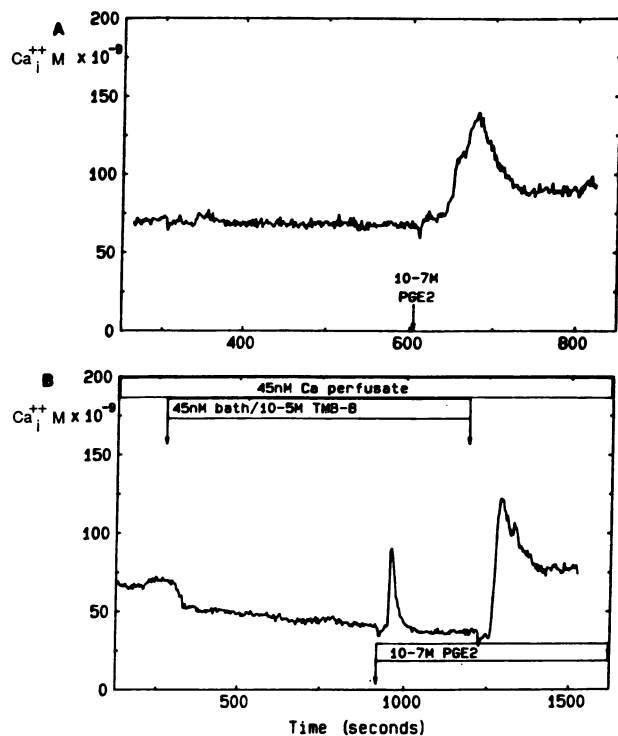


Figure 2. (A)  $10^{-7}$  M PGE<sub>2</sub> produces a peak followed by a lower but sustained increase in  $[Ca^{++}]_i$ . (B) In the presence of low bath calcium 45 nM + TMB-8, PGE<sub>2</sub> produces a small transient spike without any sustained elevation. Readdition of 2.4 mM calcium bath resulted in a sustained elevation in cell calcium.

**2a. Pertussis toxin reverses the inhibitory effect of PGE<sub>2</sub> on AVP-stimulated  $L_p$ .** To test the role of an inhibitory G protein ( $G_i$ ) in the action of PGE<sub>2</sub> to suppress AVP-mediated water transport, we pretreated CCDs with pertussis toxin for 1 h, pertussis toxin irreversibly ADP ribosylates  $G_i$  (19, 20). 100 ng/ml pertussis toxin partially reversed the inhibitory effect of  $10^{-7}$  M PGE<sub>2</sub> on AVP-induced  $L_p$  from  $91.4 \pm 14.1$  (PGE<sub>2</sub> alone) to  $134.5 \pm 19.2$  PT + PGE<sub>2</sub> (Fig. 4). This reversal of PGE<sub>2</sub> inhibition was more pronounced when the CCDs were pretreated with pertussis toxin 500 ng/ml in the bath: AVP-stimu-

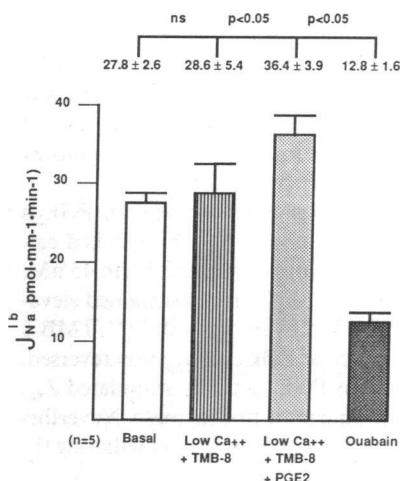


Figure 3. 45 nM calcium bath +  $10^{-5}$  M TMB-8 have no effect on  $J_{Na}$  compared with 2.4 mM calcium bath. In the presence of 45 nM Ca<sup>++</sup> +  $10^{-5}$  M TMB-8, PGE<sub>2</sub> increase  $J_{Na}$  ( $P < 0.05$  compared with 45 nM + TMB-8). Error bars = standard error. n, Number of experiments.

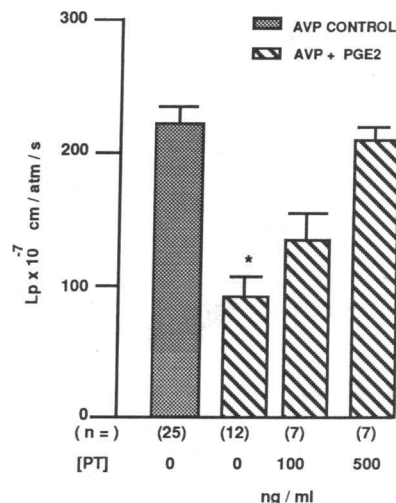


Figure 4. Pertussis toxin reverses the inhibitory effect of PGE<sub>2</sub> on  $10^{-7}$  M AVP-stimulated hydraulic conductivity. (\* $P < 0.0005$  compared with PGE<sub>2</sub> alone). Error bars = standard error. n, Number of experiments.

lated  $L_p$  was initially  $223.9 \pm 9.8$  (AVP alone) and fell only to  $204.3 \pm 11.0$  with PT + AVP + PGE<sub>2</sub> (NS,  $n = 7$ ), (Fig. 4). Thus, 500 ng/ml pertussis toxin potentially reversed the inhibitory effect of PGE<sub>2</sub> on AVP-stimulated  $L_p$ .

**2b. Effect of PGE<sub>2</sub> on  $J_{Na}$  in CCDs pretreated with pertussis toxin.** To determine whether the inhibitory effect of PGE<sub>2</sub> on  $J_{Na}$  in the rabbit CCDs was mediated by a pertussis toxin-sensitive G protein, we examined the effect of PGE<sub>2</sub> on  $J_{Na}$  in tubules that had been pretreated with 500 ng/ml of pertussis toxin for 60 min. Pretreatment with pertussis toxin failed to reverse the inhibitory effect of PGE<sub>2</sub> on lumen-to-bath  $J_{Na}$ . Thus, sodium flux fell from  $26.1 \pm 4.3$  to  $15.6 \pm 3.6$  pmol/mm per min ( $P < 0.025$ ,  $n = 6$ ) (Fig. 1 right). Further addition of  $10^{-4}$  M of ouabain decreased  $J_{Na}$  to  $6.9 \pm 2.0$  pmol/mm per min. Pertussis toxin also failed to block the action of PGE<sub>2</sub> to depolarize lumen negative  $V_T$  in these tubules, ( $V_T = 16$  mV pre-PGE<sub>2</sub> and  $-5$  mV post-PGE<sub>2</sub>). These results argue against a significant role for a pertussis toxin sensitive G protein in mediating the inhibitory effect of PGE<sub>2</sub> on sodium transport in the rabbit CCD.

**2c. Effect of PGE<sub>2</sub> on  $[Ca^{++}]_i$  in CCD pretreated with pertussis toxin.** CCDs were exposed for 60 min to pertussis toxin 500 ng/ml. Subsequent addition of  $10^{-7}$  M PGE<sub>2</sub> to fura-2-loaded CCD resulted in a rapid increase in cell calcium.  $Ca^{++}$  transiently increased to  $244 \pm 17\%$  ( $n = 6$ ) of basal levels. Peak  $[Ca^{++}]_i$  occurred within 30–60 s after PGE<sub>2</sub> addition, and was followed by a fall to a new sustained  $[Ca^{++}]_i$  104% above base line levels. (Fig. 5). These results are no different from those observed in the absence of pertussis toxin. Thus, neither PGE<sub>2</sub>'s inhibition of  $J_{Na}$  nor the  $Ca^{++}$  spike were blocked by pertussis toxin.

### 3. Role of cyclic AMP:

**3a. PGE<sub>2</sub> reduces  $J_{Na}$  in 8-CPTcAMP pretreated CCDs.** We next examined the effect of the cell permeable cyclic AMP analogue  $10^{-4}$  M 8-CPTcAMP on sodium transport. We noted that while the first collection routinely showed 8-CPTcAMP slightly stimulated  $J_{Na}$ , subsequent collections revealed inhibition of  $J_{Na}$ . The last three collections were used to assess  $J_{Na}$  after 8-CPTcAMP. In the control period lumen-to-bath  $^{22}Na$  flux was  $35.8 \pm 2.3$  and decreased to  $21.6 \pm 2.2$  pmol/mm per min in the presence of 8-CPTcAMP ( $P < 0.0005$ ,  $n = 13$ ) (Fig.

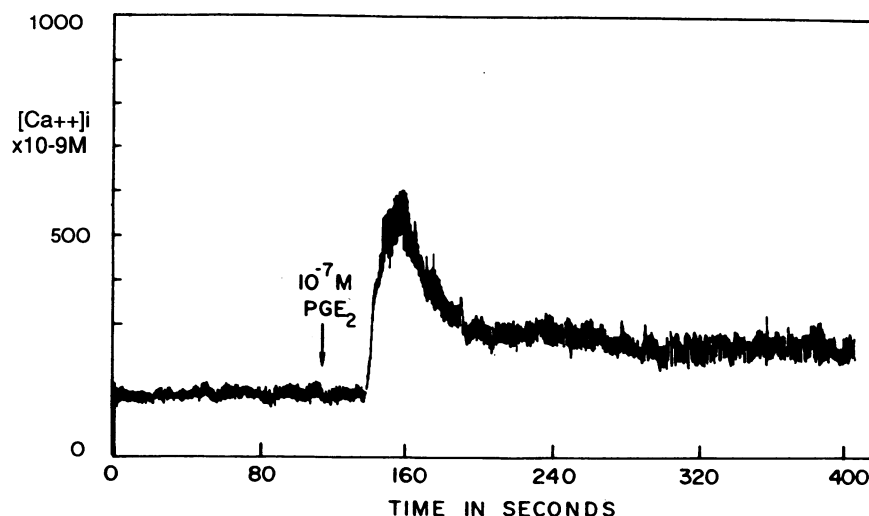


Figure 5. Pretreatment of pertussis toxin 500 ng/ml for 1 h does not block the effect of  $10^{-7}$  M PGE<sub>2</sub> on  $[Ca^{++}]_i$ .

6). Superimposition of PGE<sub>2</sub> in the presence of 8-CPTcAMP caused  $J_{Na}$  to fall further to  $15.9 \pm 1.3$  pmol/mm per min ( $P < 0.005$ ). These results demonstrate that while 8-CPTcAMP inhibits  $J_{Na}$ , PGE<sub>2</sub> further decreases sodium flux in the rabbit CCD.

**3b. Effect of 8-CPTcAMP on  $J_{Na}$  in CCDs pretreated with indomethacin.** Since Holt and Lechene (21) found that cyclooxygenase inhibition reversed the inhibitory effect of vasopressin on Na<sup>+</sup> absorption in rabbit CCDs, we tested the effect of 8-CPTcAMP on sodium transport in tubules that had been pretreated with 5  $\mu$ M indomethacin. Indomethacin pretreatment almost completely blocked the effect of 8-CPTcAMP on  $J_{Na}$  ( $33.6 \pm 2.85$  vs.  $28.4 \pm 2.8$  pmol/mm per min) ( $n = 7$ ) (Fig. 6). This suggests that the inhibition of  $J_{Na}$  by 8-CPTcAMP is mediated by cyclooxygenase metabolite(s). Furthermore, when PGE<sub>2</sub> was superimposed on 8-CPTcAMP in indomethacin-pretreated tubules, there was significant inhibition of  $J_{Na}$  from  $28.4 \pm 2.8$  to  $19.05 \pm 3.0$  pmol/mm per min ( $P < 0.025$ ,  $n = 7$ ) (Fig. 6). These results demonstrate PGE<sub>2</sub>'s capacity to inhibit

sodium transport in the rabbit CCDs is also independent of its capacity to raise cyclic AMP.

## Discussion

These studies are the first to investigate the mechanism by which PGE<sub>2</sub> inhibits sodium reabsorption ( $J_{Na}$ ) in the rabbit cortical collecting duct. Based on the data from separate in vitro perfusion studies of Stokes et al. and Iino et al. (3, 4), it is clear that PGE<sub>2</sub> inhibits sodium transport in the rabbit CCD in addition to its well known modulation of vasopressin-stimulated water permeability (6, 22, 23). Studies examining the interaction of PGE<sub>2</sub> with vasopressin and cyclic AMP-stimulated water permeability, suggest that PGE<sub>2</sub> interacts with at least three different signaling mechanisms in the rabbit CCD: (a) a pathway linked to stimulation of the hydraulic conductivity via increased cAMP accumulation; (b) a second pathway coupled through G<sub>i</sub> by which PGE<sub>2</sub> inhibits hydrosmotic water flow in response to vasopressin by decreasing cAMP accumulation; (c) and finally, a third pathway by which PGE<sub>2</sub> can also release cell calcium from intracellular stores. The purpose of these studies was, therefore, to characterize which of these three signaling mechanisms, if any, is used by PGE<sub>2</sub> to inhibit sodium transport. First, the ambient calcium concentration was lowered into the nanomolar range to block the effect of sustained increases in intracellular calcium as a mechanism for PGE<sub>2</sub> action. Second, a role for G<sub>i</sub> in the action of PGE<sub>2</sub> was examined using pertussis toxin. Finally, the possible role of increased cell cyclic AMP was examined using exogenous 8-CPTcAMP alone and in the presence of PGE<sub>2</sub>.

Initial studies examined the relationship between PGE<sub>2</sub>'s capacity to increase  $[Ca^{++}]_i$  and inhibit  $J_{Na}$ . We blocked calcium influx by lowering bath calcium concentration to 45 nM. We found that not only was the PGE<sub>2</sub>-induced sustained elevation in  $[Ca^{++}]_i$  completely blocked by the 45-nM Ca<sup>++</sup>/TMB-8 buffer, but the inhibitory effect of PGE<sub>2</sub> on  $J_{Na}$  was reversed. Indeed, under these conditions PGE<sub>2</sub> actually stimulated  $J_{Na}$ . We did not pursue the mechanism of this increase. Nevertheless, it is clear that calcium plays a critical role in mediating the inhibitory effect of PGE<sub>2</sub>.

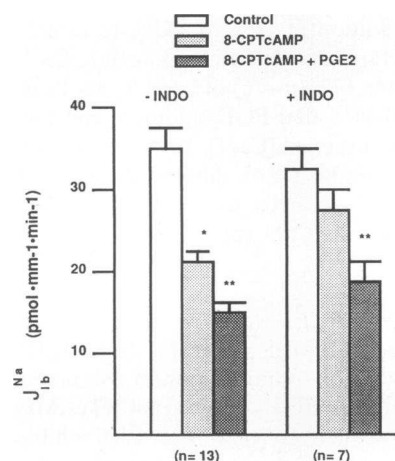


Figure 6.  $10^{-4}$  M 8-CPTcAMP inhibits  $J_{Na}$  ( $*P < 0.0005$  control compared with 8-CPTcAMP). Superimposition of PGE<sub>2</sub> in the continued presence of 8-CPTcAMP further reduces  $J_{Na}$  ( $**P < 0.005$  compared with 8-CPTcAMP alone). Pretreatment with 5  $\mu$ M indomethacin reverses the inhibition of 8-CPTcAMP on  $J_{Na}$ . However, in the presence of 8-CPTcAMP, PGE<sub>2</sub> still inhibits  $J_{Na}$ , ( $**P < 0.025$  compared

with 8-CPTcAMP alone). Error bars = standard error.  $n$ , Number of experiments.

The mechanism by which increased  $[Ca^{++}]_i$  inhibits  $J_{Na}$  in the CCD is only partially characterized. Inhibition of  $J_{Na}$  by increased  $[Ca^{++}]_i$  could occur via inhibition of the apical sodium entry step or the basolateral  $Na^+/K^+$  ATPase (24–28). Frindt and Windhager suggested that calcium inhibits  $Na^+$  entry through the apical amiloride-sensitive sodium channel (27). Their data argue against inhibition of  $J_{Na}$  by a direct action of  $Ca^{++}$  on the basolateral  $Na^+/K^+$ -ATPase because ionomycin-induced inhibition of  $J_{Na}$  was reversed by amphotericin. There is also evidence that  $Ca^{++}$  inhibits apical sodium permeability indirectly through activating protein kinase C (28). Because we have recently shown that  $PGE_2$  inhibits water flow in rabbit CCD, in part through the activation of protein kinase C (PKC) (6), we tested whether PKC inhibition by staurosporine (SSP) or H-7 had any effect on  $PGE_2$ 's action to inhibit sodium transport. However, both SSP and H-7, by themselves, inhibited  $J_{Na}$  in rabbit CCD (data not shown). Thus, whether  $PGE_2$ 's inhibition of  $J_{Na}$  occurs directly or indirectly through PKC activation still remains to be tested. However, we have clearly shown that preventing a sustained increase in  $[Ca^{++}]_i$  blocks the effect of  $PGE_2$  on sodium transport.

We next examined whether pertussis toxin reverses the inhibitory effect of  $PGE_2$  on  $Na^+$  transport as well as vasopressin-stimulated water flow. Studies on freshly isolated CCD cells show that  $PGE_2$  inhibits cAMP accumulation in a manner that is sensitive to pertussis toxin. Treatment of rabbit CCD with pertussis toxin results in ADP ribosylation of a 41-kD protein (20, 29) consistent with the presence of  $G_i$  in the collecting duct. Similarly, studies by Watanabe showed copurification of a pertussis toxin sensitive GTP binding protein with a  $PGE_2$  receptor from renal medulla (30). Our observation that pertussis toxin reverses the effect of  $PGE_2$  on AVP-mediated hydroosmotic water flow supports the notion that  $PGE_2$  blocks AVP-stimulated cAMP accumulation through an action involving  $G_i$ . However, similar treatment failed to block  $PGE_2$  inhibition of  $J_{Na}$  or its capacity to increase  $[Ca^{++}]_i$ . Thus,  $PGE_2$  inhibition of sodium transport may not be mediated by a pertussis toxin sensitive G protein, such as  $G_i$ .

We have previously shown that  $PGE_2$  increased  $[Ca^{++}]_i$  in rabbit cortical collecting duct consistent with stimulation of  $IP_3$  production (6).  $PGE_2$  recently has been shown to raise  $[Ca^{++}]_i$  in UMR-106 cells and MDCK cells (31, 32). In both these cell types  $PGE_2$  increased  $IP_3$  production confirming stimulation of phospholipase C (PLC) by  $PGE_2$ . Agonist-induced PLC activation has been shown to be mediated by both pertussis toxin-sensitive (33) and pertussis toxin-insensitive G proteins in a variety of tissues. In chromaffin cells a  $PGE$  receptor is thought to be linked to phospholipase C via a pertussis toxin insensitive G protein (34, 35). In Swiss 3T3 cells, bombesin has been shown to cause a rapid transient rise in  $[Ca^{++}]_i$  as a consequence of inositol 1,3,4-trisphosphate generation (36, 37), and this is also pertussis toxin insensitive. Under the conditions of our experiments, there is no evidence for a pertussis toxin-sensitive G protein involved in calcium signaling by  $PGE_2$ . Indeed, in data not shown, extension of the incubation period with pertussis toxin to 3 h did not prevent  $PGE_2$ -induced  $Ca^{++}$  changes.

We next examined the possible role of cAMP in mediating  $PGE_2$ 's effect. Several biochemical studies have shown that in purified freshly isolated cortical collecting duct cells,  $PGE_2$  (0.1–10  $\mu$ M) stimulates cyclic AMP accumulation, suggesting

that in these cells there is a  $PGE_2$  receptor coupled to the activation of adenyl cyclase (5). Functional studies by Grantham et al., Nadler et al., and Hébert et al. have confirmed that  $PGE_1$  or  $PGE_2$  by themselves increase CCD hydraulic conductivity presumably through stimulation of cAMP production (6, 22, 23). To test whether  $PGE_2$  might inhibit  $J_{Na}$  via its capacity to increase cyclic AMP, we examined the effect of the cell permeable cyclic AMP analogue,  $10^{-4}$  M 8-CPTcAMP on sodium transport in the rabbit cortical collecting duct. In these studies, we found a major inhibitory effect of 8-CPTcAMP on sodium transport. Schuster had previously found that using hypotonic perfusate (55 mM), 8-BrcAMP depolarized the tubules but had no effect on lumen-to-bath sodium flux (13). In contrast, using isotonic perfusate, we found that both 8-BrcAMP and 8-CPTcAMP decrease sodium transport. These data are in perfect agreement with those of Kimmel et al. who showed that 8-CPTcAMP inhibited lumen-to-bath  $Na$  transport with isotonic perfusate (38), but when they used hypotonic perfusate, 8-CPTcAMP had no effect on lumen-to-bath sodium flux. While cAMP could potentially mediate the effect of  $PGE_2$  on  $J_{Na}$ , we found that  $PGE_2$  further significantly decreased  $J_{Na}$  in CCDs already treated with 8-CPTcAMP. This clearly shows an inhibitory effect of  $PGE_2$  on  $J_{Na}$  independent of cAMP generation.

The effect of cAMP on  $J_{Na}$  is similar to the effect of AVP on  $J_{Na}$  in the rabbit CCD. Whereas AVP produces a persistent increase in  $J_{Na}$  in the rat (39, 40), it only transiently stimulates  $J_{Na}$  and  $V_T$  in the rabbit (21, 27, 41). The biphasic effect of vasopressin action on  $V_T$  and  $J_{Na}$  in the rabbit CCD has been attributed to prostaglandin production in the rabbit CCD (21). Holt and Lechene found that pretreatment of rabbit collecting tubules with meclofenamate prevented the inhibitory effect of vasopressin on  $J_{Na}$ . They speculated that endogenous prostaglandin synthesis, stimulated by vasopressin, inhibits  $J_{Na}$  (21). Because of the possibility that cyclic AMP might also inhibit sodium transport through an effect involving stimulation of endogenous prostaglandin synthesis, we tested the effect of 8-CPTcAMP on sodium flux in tubules that had been pretreated with the cyclooxygenase inhibitor, indomethacin. Inhibition of lumen-to-bath sodium flux by cyclic AMP was diminished in CCDs pretreated with indomethacin, suggesting an important role for product(s) of the cyclooxygenase pathway. However, when  $PGE_2$  was superimposed on cAMP in the presence of indomethacin, there was further significant inhibition of sodium transport. This strongly supports the previous findings obtained with  $PGE_2$  addition to 8-CPTcAMP in the absence of indomethacin.

The mechanism by which endogenous prostaglandin synthesis could be stimulated by cAMP is unclear. However, because cAMP raises CCD  $[Ca^{++}]_i$  (42), this could stimulate phospholipase activity generating free arachidonic acid leading to prostaglandin production (43). Alternative mechanisms for cAMP inhibition of  $J_{Na}$  could exist, including direct activation of phospholipases by cAMP (28). Finally, some comments should be made regarding the possible contribution of cAMP to the calcium response of CCD to  $PGE_2$ . This is pertinent because it has been shown recently that cAMP increases  $[Ca^{++}]_i$  in rabbit CCD (42). The rise in cell calcium induced by cAMP requires extracellular calcium, whereas  $PGE_2$  clearly raises cell calcium by both promoting release from intracellular stores and by promoting entry from the extracellular fluid. It is pres-

ently unknown what contribution cAMP generation makes to calcium entry stimulated by PGE<sub>2</sub>. Nevertheless, both the inhibition of  $J_{Na}$  and the PGE<sub>2</sub>-mediated increase in  $[Ca^{++}]_i$  can clearly occur by cAMP-independent mechanisms (6).

In summary, these studies have shown for the first time that PGE<sub>2</sub>-mediated increases in cytosolic calcium are responsible for the inhibitory action of PGE<sub>2</sub> on sodium transport. While stimulation of cAMP production by PGE<sub>2</sub> may contribute to the inhibition of Na<sup>+</sup> transport, it is not required since in the presence of 8-CPTcAMP, PGE<sub>2</sub> still decreases sodium transport in the rabbit cortical collecting duct. Finally, the effect of PGE<sub>2</sub> on  $J_{Na}$  is pertussis toxin insensitive and it is thus unlikely to be mediated by G<sub>i</sub>. We conclude that PGE<sub>2</sub> inhibits sodium transport in rabbit cortical collecting ducts predominantly by increasing intracellular calcium.

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