Epidermal growth factor inhibits the hydroosmotic effect of vasopressin in the isolated perfused rabbit cortical collecting tubule.

M D Breyer, … , H R Jacobson, J A Breyer

J Clin Invest. 1988;82(4):1313-1320. https://doi.org/10.1172/JCI113732.

Research Article

Epidermal growth factor (EGF) is a 53-amino acid polypeptide which is a potent mitogen for cultured cells. The kidney has recently been shown to be a major site of synthesis for the EGF precursor. EGF infusions in sheep result in a diuresis and natriuresis despite a fall in GFR, suggesting a direct tubular effect. Using in vitro microperfusion of rabbit cortical collecting tubules (CCTs) at 37 degrees C, we examined the effect of EGF on the transepithelial voltage (Vt) and arginine vasopressin (AVP)-stimulated hydraulic conductivity (Lp). Pretreatment with peritubular EGF at concentrations from 10(-8) to 10(-12) M resulted in a 50% inhibition of both AVP- and 8-chlorophenythio-cyclic AMP-stimulated peak Lp. This effect was reversed by the protein kinase C inhibitor, staurosporine, but unaffected by indomethacin. CCTs with an initially negative Vt, depolarized after exposure to bath EGF. 10(-8) M EGF applied from the lumen had no effect on either Lp or Vt. Specific binding of 20 nM 125I-EGF to microdissected CCTs was also demonstrated. These results suggest that EGF can modulate both salt and water transport in the CCT via a receptor linked to protein kinase C activation.

Epidermal Growth Factor Inhibits the Hydroosmotic Effect of Vasopressin in the Isolated Perfused Rabbit Cortical Collecting Tubule

Matthew D. Breyer, Harry R. Jacobson, and Julia A. Breyer
Division of Nephrology, Department of Internal Medicine, Veterans Administration Medical Center, and Vanderbilt University, Nashville, Tennessee 37212

Abstract

Epidermal growth factor (EGF) is a 53-amino acid polypetide which is a potent mitogen for cultured cells. The kidney has recently been shown to be a major site of synthesis for the EGF precursor. EGF infusions in sheep result in a diuresis and natriuresis despite a fall in GFR, suggesting a direct tubular effect. Using in vitro microperfusion of rabbit cortical collecting tubules (CCTs) at 37°C, we examined the effect of EGF on the transepithelial voltage (V_t) and arginine vasopressin (AVP)-stimulated hydraulic conductivity (Lp). Pretreatment with peritubular EGF at concentrations from 10^{-8} to 10^{-12} M resulted in a 50% inhibition of both AVP- and 8-chlorophenythio-cyclic AMP-stimulated peak Lp. This effect was reversed by the protein kinase C inhibitor, staurosporine, but unaffected by indomethacin. CCTs with an initially negative V_t, depolarized after exposure to bath EGF. 10^{-8} M EGF applied from the lumen had no effect on either Lp or V_t. Specific binding of 20 nM ^{125}I-EGF to microdissected CCTs was also demonstrated. These results suggest that EGF can modulate both salt and water transport in the CCT via a receptor linked to protein kinase C activation.

Introduction

Epidermal growth factor (EGF)[1] was originally isolated from extracts of mouse submaxillary gland as the active agent that induced precocious eyelid opening and early incisor eruption in newborn mice (1). Since then EGF has been demonstrated to be a potent mitogen in a wide variety of cultured epithelial and mesenchymal cells (2). Several intracellular events that precede or accompany this proliferative response, including activation of Na^+/H^+ exchange (3) and stimulation of tyrosine kinase activity (4), have also been described.

Although the cellular actions of EGF have been extensively studied, relatively little is known about the precise physiologic effects of EGF. EGF has been demonstrated to inhibit gastric acid secretion (5) and to be a potent vasodilator in the dog (6). These effects seem unrelated to its mitogenic capacity.

More recently, a role for EGF in the kidney has been suggested. EGF has been isolated from a variety of body fluids and, in fact, human EGF was originally purified from urine (5). The high levels of EGF in urine relative to blood suggested that the kidney itself may be the source of urinary EGF (7). In support of this, Rall and co-workers have recently demonstrated that the kidney is a major site of synthesis for the EGF precursor, preproEGF (8, 9). PreproEGF mRNA levels in the thick ascending limb of Henle and distal convoluted tubules of the kidney are orders of magnitude higher than in other tissues.

The function of the EGF precursor in the distal nephron remains a matter of speculation. Specific receptors for EGF in whole-kidney homogenates are well described (10), and more recently their existence has been demonstrated in numerous cultured kidney cells (11–13) and in suspensions of collecting duct cells. Scoggins et al. (14) found that EGF infusion in sheep caused a brisk naturesis and diuresis despite a fall in GFR. Taken together these findings suggest the possibility that EGF might have an effect on vasopressin-stimulated water reabsorption in the collecting duct. In the present studies, we examined the effects of EGF on the hydroosmotic response to vasopressin in the isolated microperfused rabbit cortical collecting tubule (CCT). We observed potent effects of EGF on both arginine vasopressin (AVP)-stimulated water flow and transepithelial voltage in the CCT.

Methods

Microperfusion experiments

Single CCTs (1.61±0.05 mm in length) were dissected from kidneys of female New Zealand White rabbits weighing between 1.5 and 2.0 kg. After isolation, the CCT was transferred to a thermostatically controlled, flowing bath chamber, cannulated, and perfused with an isotonic solution as previously described (15). An isotonic bath solution was also used and bath flow was adjusted to 0.5 ml/min using a perfusion pump (Sage Instruments, Inc., Cleveland, OH). The hydrostatic pressure of the perfusate was adjusted so luminal fluid was collected at a rate of 15–23 nl/min into a constriction pipette of known volume (four pipettes used, ranging from 65 to 107 nl). Transepithelial voltage (V_t) was continuously monitored by electometer (FD-223, World Precision Instruments, New Haven CT) and strip chart recorder (model R-02, Soltec Co., Sun Valley, Ca.).

Experimental design

All experiments were performed at 37°C. After an initial equilibration period of 45 min the isotonic perfusate was exchanged with a hypotonic solution (150 mosmol) containing exhaustively dialyzed tritiated inulin. The seven following protcols were conducted.

Portions of this study were presented at the Tenth International Congress of Nephrology, 1987, in London, England and the 1987 American Society of Nephrology meeting in Washington, D.C.

Address reprint requests to Dr. Matthew D. Breyer, Division of Nephrology, Veterans Administration Hospital and Vanderbilt University, Nashville, TN, 37232.

Received for publication 4 November 1987 and in revised form 10 May 1988.

1. *Abbreviations used in this paper:* AVP, arginine vasopressin; CcAMP, 8-(*p*-chlorophenylthio)-cyclic adenosine monophosphate; CCT cortical collecting tubule; EGF, epidermal growth factor; INDO, indomethacin; Lp, hydraulic conductivity; PMA, phorbol myristate acetate; STSP, staurosporine; V_t, transepithelial voltage.

Control studies with AVP or 8-chlorophenylthio-cyclic AMP (CcAMP).[2] 30 min after the perfusate exchange, three collections were taken for the determination of basal CCT hydraulic conductivity (Lp). Tubules with negative basal Lp values significantly less than zero ($< -4.0 \times 10^{-7}$ cm/atm per s) were presumed to have an inulin leak and were discarded. Immediately after basal measurements were taken, 10 μU/ml AVP or 0.1 mM CcAMP was added to the bath. We chose to use this dose of AVP since it is above the maximal physiologic concentration of AVP measured in the rabbit (16). This concentration might also allow us to see either suppression or stimulation of AVP-induced Lp (17).

A 10–15-min equilibration period was allowed and then six to eight collections were taken for the determination of peak Lp response to AVP or CcAMP. The peak Lp was defined as the mean of the three greatest contiguous values obtained after exposure to AVP or CcAMP. Preliminary studies showed this peak generally occurred 30–45 min after exposure to these agents.

Effect of peritubular EGF on AVP-stimulated Lp in the CCT. 15 min after the perfusate exchange, varying concentrations of EGF were added to the bath. Another 15-min equilibration period was allowed to elapse before the three collections for basal Lp were made. After basal collections, the 10 μU/ml AVP was added to the EGF-containing bath. The total elapsed time before the tubule was exposed to AVP was thus identical to that in the control group. A 10-min equilibration period was allowed and six to eight collections for peak Lp were made.

Effect of peritubular $3 \cdot 10^{-10}$ M EGF on CcAMP-stimulated Lp in the CCT. Since AVP stimulates osmotic water flow via a cAMP-dependent mechanism, we examined the effects of EGF on CcAMP-stimulated Lp. These studies were intended to distinguish an effect of EGF on cellular cAMP levels versus processes stimulated by cAMP-dependent mechanisms. The protocol was designed precisely as the previous experiment except that, after basal Lp collections, 0.1 mM CcAMP was added to the EGF-containing bath instead of AVP. Peak Lp was then determined.

Effect of luminal EGF on AVP-stimulated Lp. Because of evidence, reviewed in the discussion, that EGF is secreted into the urine, we examined the effect of luminal EGF on AVP-stimulated Lp. After a 45-min equilibration period, the isotonic perfusate was exchanged for hypotonic perfusate containing 0.1% albumin (to serve as a carrier) and $3 \cdot 10^{-8}$ M EGF. A 30-min equilibration period was allowed before basal Lp measurements were made. The tubule was then exposed to 10 μU/ml AVP and peak Lp was determined.

Effect of indomethacin (INDO) pretreatment on the EGF-mediated inhibition of AVP-stimulated Lp. 5 min after the hypotonic perfusate exchange was made, 5 μM INDO was added to the bath. This concentration of INDO was chosen since it has previously been shown to inhibit cyclooxygenase almost completely, have little effect on cAMP phosphodiesterase (18), and to reverse the inhibitory effects of both bradykinin (19) and the calcium ionophore A23187 (20) on AVP-stimulated Lp in the isolated perfused CCT. 15 min after the perfusate exchange, 10^{-10} M EGF was added to the bath. Basal and AVP-stimulated Lp collections were then taken as in previous protocols. In a separate group of control studies, tubules were pretreated with INDO and then exposed to AVP without exposure to EGF.

Effect of staurosporine (STSP) pretreatment on the EGF-mediated inhibition of AVP-stimulated Lp. STSP is a recently described potent inhibitor of protein kinase C (21). We have also recently demonstrated that 10^{-7} M STSP completely reverses the inhibitory effect of a maximal concentration of phorbol myristate acetate (PMA) on AVP-stimulated Lp in the isolated perfused rabbit CCT (20). 5 min after the hypotonic perfusate exchange was made, STSP (final concentration 10^{-7} M), in DMSO (final concentration 0.01% vol/vol), was added to the bath. 15 min after the perfusate exchange, 10^{-10} M EGF was added to the bath. Basal and AVP-stimulated Lp collections were then taken as in previous protocols. In a separate group of control studies, tubules were pretreated with STSP plus DMSO and then exposed to AVP, without exposure to EGF.

Effect of EGF on V_t. Although these studies were primarily designed to examine the effect of EGF on the response of the CCT to AVP, we noted striking changes in the V_t after the tubule was exposed to EGF. Comparison between the change in V_t (ΔV_t) for control tubules versus EGF-treated tubules were made at identical time points for all experiments. ΔV_t was defined as the change in V_t between two time points: 10 min after the perfusate exchange (i.e., 5 min before EGF was added to the bath in experimental tubules) and at the beginning of the first collection for basal Lp (i.e., 15 min after exposure to EGF in the experimental tubules). We also compared the peak hyperpolarization of V_t in response to AVP with and without EGF. Comparison was made by nonpaired t test.

Solutions

The composition of the bath medium, and isotonic perfusate was (in millimolar): NaCl 105, $NaHCO_3$ 25, Na acetate 10, $NaHPO_4$ 2.3, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 1.0, glucose 8.3, alanine 5 (300 mosmol/kg H_2O). 0.5% ablumin was added to the bath to serve as a carrier for EGF. The composition of the hypotonic perfusate was identical to that of the isotonic perfusate except the NaCl concentration was lowered to 30 mM to yield a 150 mosmol/kg H_2O solution and tritiated inulin (75 μCi/ml) was added to serve as a volume marker. In experiments where luminal EGF was added, 0.1% albumin was added to the perfusate to serve as a carrier. Before use, all solutions were bubbled at 37°C with a 95% O_2/5% CO_2 gas mixture to achieve equilibrium and a pH of $\approx$ 7.40.

Calculations

Hydraulic conductivity (Lp, 10^{-7} cm/atm per s was calculated according to Dubois et al. (22): $L_p = (1/RTS) \cdot (1/O_b)^2 \cdot [O_b \cdot (V_i - V_o) + O_i \cdot V_i \cdot \mathrm{Ln}\{(O_b - O_i) \cdot V_i/(O_b \cdot V_o - O_i \cdot V_i)\}]$, where R is the gas constant, T is the temperature of the solutions (°K), S is the luminal surface area of the tubule estimated from the directly measured tubular length and an assumed luminal diameter of 20 μm. O_b and O_i represent the osmolality of the bath and perfusate, respectively. V_i and V_o represent the perfusion rate and the collection rate, respectively. The difference between V_i and V_o represents the volume of water absorption. V_o is calculated from the volume of the constriction pipette divided by the time required to fill it to the constriction. V_i can be calculated by the ratio of counts per min/nl collectate (CPM_o) versus that of the perfusate (CPM_i) so that $V_i = [CPM_o/CPM_i] \cdot V_o$.

Microreceptor ^{125}I-EGF binding assay

Slices of rabbit kidney were microdissected in chilled (4°C) wash buffer (pH 7.4) containing (in millimolar): 12.0 Na_2HPO_4, 3.0 NaH_2PO_4, 90 NaCl, 5 KCl, 2 $MgSO_4$, 1 $CaCl_2$. In addition, bovine serum albumin (0.5% wt/wt), bacitracin (0.08%), and aprotinin (15 μM) were added to diminish proteolysis of the receptor and ^{125}I-EGF as well to diminish nonspecific binding. 36–45 CCTs per experiment were microdissected and their length determined by an eyepiece reticle. Tubules were arranged into six groups, each containing 9–13 mm of CCTs, and each group was transferred onto a small albumin-coated aluminum foil disc that was in a well of an aluminum plaque as described by Mujais et al. (23). Three wells were used to measure total binding and three wells for nonspecific binding. The microdissection solution was aspirated and replaced with 2 μl of chilled incubation solution which was identical except for the addition of the radiolabled and unlabeled EGF. Three additional wells containing foil but no tubules were also exposed to ^{125}I-EGF and counted as blanks.

In separate studies (24), experiments were performed in microdissected pars rectae to determine the optimal temperature, hormone concentration, and incubation time for the binding assay. These studies showed equilibrium binding on ice to occur within 4 h, specific

2. Since there is some modest variability in the mean control AVP-stimulated Lp during the year, it is important to perform controls concurrently with each experimental group. This practice was followed throughout this study.

^{125}I-EGF binding to be saturable (apparent K_d = 6.5 nM) and not competed off by other polypeptides. Based on these results, incubation of the microdissected CCTs was performed on ice (to prevent receptor internalization), for 4 h and using 20 nM ^{125}I-EGF with (nonspecific binding) or without (total binding) a 500-fold excess of noniodinated EGF. By means of a dissecting microscope, the incubation medium was then aspirated and the 5 μl of wash buffer was added. This wash procedure was repeated for each well four times. The foil discs were then counted for 10 min using an Isodyne model 1185 automatic gamma system (TM Analytic, Inc., Elk Grove Village, IL).

^{125}I-EGF binding was calculated in attomoles per centimeter as equal to: {[counts/well(total or nonspecific)-blk]/10 min/tubule length in cm} · (attomoles ^{125}I-EGF/cpm). Attomoles bound ^{125}I-EGF/cpm were determined using the counting efficiency of the counter (0.48 cpm/dpm), the specific activity of the ^{125}I-EGF ($\approx$ 170 μCi/μg), and the molecular weight of EGF. Specific binding was taken as the difference between total and nonspecific binding.

Statistics

Data are presented as mean±SE. Comparisons between groups were made by nonpaired Student's t test and P values ≤ 0.05 were considered significant.

Reagents

AVP, chlorophenylthio-cyclic AMP, and indomethacin, were purchased from Sigma Chemical Co., St. Louis, MO. Indomethacin was stored as a 5 mM stock in a 75 mM $NaCO_3$ solution. STSP was obtained from Kyowa Hakko (Tokyo, Japan and New York) and stored as a 1 mM stock in pure DMSO at −20°C. Mouse epidermal growth factor was the generous gift of Dr. Stanley Cohen and stored at −20°C as a 1 mg/ml stock solution in distilled water. The purification method for the EGF was devised by Savage and Cohen (25) and is the one used by commercial sources for EGF. ^{125}I-EGF and tritium-labeled inulin was purchased from DuPont NEN Products Co., Boston, MA.

Results

EGF inhibition of AVP-stimulated Lp. Pretreatment of isolated perfused CCTs with EGF resulted in a dose-dependent suppression of subsequent AVP (10 μU/ml)-stimulated Lp (10^{-7} · cm/atm/s) (Figs. 1 and 2). AVP control group (n = 15) had basal Lp values of 6.8±2.7, which increased to a peak value 232.4±9.6 after AVP addition. Tubules pretreated with doses of EGF ranging from 3×10^{-12} to 3×10^{-8} M demonstrated no significant difference in basal Lp (11.6±3.2) from the control group ($P > 0.1$); however, AVP-stimulated Lp in EGF-pretreated tubules was significantly lower over this dose range of EGF (e.g., 3×10^{-12} M EGF, n = 5, peak Lp = 142.9±20.27, $P < 0.0005$ vs. control). EGF also caused the AVP-stimulated peak Lp to occur significantly earlier than in the control group (AVP peak at 54.8±1.78 min after AVP and EGF/AVP peak 31.4±1.34 min after AVP, $P < 0.0005$). Nevertheless, at 31 min the Lp response to AVP was higher than in the EGF/AVP group (AVP 31 min = 178±10.65 vs. EGF/AVP 31 min = 117±8.88, $P < 0.0005$). When the dose of EGF was further decreased to 3×10^{-13} M, these effects were lost (n = 4, peak Lp = 221±19.4, $P > 0.1$ vs. control group).[3] Thus the inhibitory effect of EGF pretreatment on AVP-stimulated peak Lp is dose dependent and effective at picomolar concentrations.

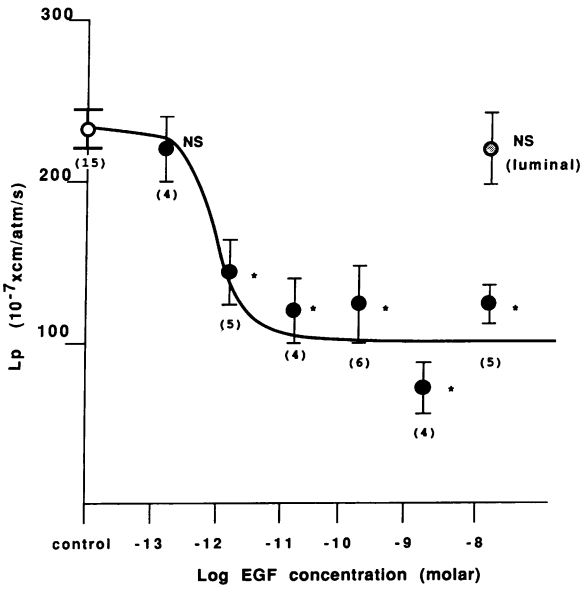

Figure 1. Inhibition of AVP-stimulated Lp in the CCT by EGF is dose dependent. Data are plotted as the mean±SE Lp for each dose of EGF. (○) Value obtained in tubules perfused without EGF; (●) Lp in tubules pretreated with the indicated dose of EGF in the bath. (○) Lp in tubules pretreated with the indicated dose of EGF in the lumen. Numbers in parentheses indicate the number of individual tubules perfused at each dose. *$P < 0.0005$; NS indicates $P > 0.05$ vs. control.

Luminal EGF has no effect on AVP-stimulated Lp. Because of the possibility that EGF is secreted into the urine and is in

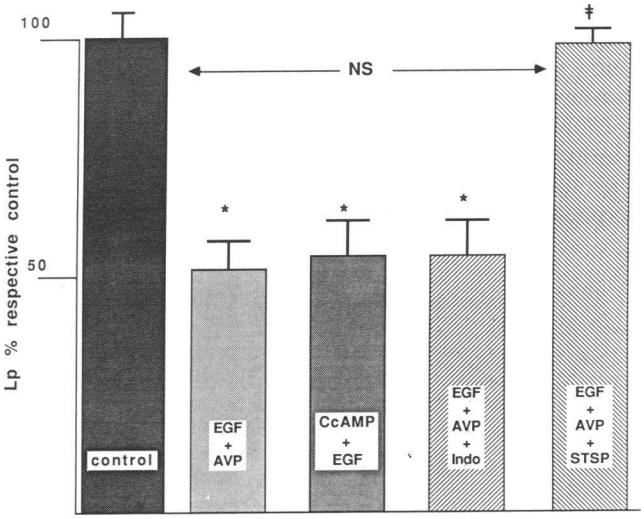

Figure 2. Mechanism of peritubular EGF-mediated inhibition of AVP- or CcAMP-stimulated peak Lp. Data are plotted as a percentage of the mean Lp (±SE) for each experimental group vs. its appropriate respective control group Lp. Thus the control group for EGF + AVP + STSP was AVP + STSP, whereas the control group for CcAMP + EGF was CcAMP alone. Where indicated the concentrations of compounds used were: AVP 10 μU/ml, 0.1 mM CcAMP, EGF 10^{-10} M, INDO $5 \cdot 10^{-6}$ M, and STSP 10^{-7} M. See text of Results for absolute values of Lp in each group. *$P < 0.01$, ‡$P < 0.005$ vs. EGF + AVP, NS indicates $P > 0.05$ vs. control.

3. The steep dose-response relationship seen with EGF and its effects on AVP-stimulated Lp in the CCT is typical of other regulatory peptides. The effect of AVP itself goes from undetectable to maximal stimulation of Lp over a similar concentration range (i.e., 10^{-12} to 10^{-11} M) (49).

contact with the tubular cell luminal membrane, we examined the effects of luminal EGF on AVP-stimulated Lp. Pretreatment with 3×10^{-8} M luminal EGF had no effect on either basal or AVP-stimulated peak Lp (−1.18±.98 and 239.8±31.1, respectively, $p > 0.1$ vs. control) (Fig. 1).

Inhibitory effect of peritubular EGF on CcAMP-stimulated Lp. Pretreatment of CCTs with peritubular EGF (10^{-10} M) resulted in a 42% reduction in CcAMP-stimulated Lp (132.2±17.43, $n = 6$, vs. 225.8±25.2 in CcAMP control group, $n = 6$, $P < 0.01$). The magnitude of this inhibition of CcAMP-stimulated Lp was comparable to that observed for the AVP-stimulated water flow (42% vs. 50% inhibition) (Fig. 2).

INDO fails to reverse the effects of EGF on AVP-stimulated Lp. Pretreatment of the CCT with 5 μM INDO failed to reverse EGF-mediated inhibition of vasopressin-stimulated osmotic water flow (INDO/EGF/AVP mean Lp = 145.8±20.0, $n = 8$ vs. EGF/AVP mean Lp = 127.9±13.6, $P > 0.1$) (Fig. 2). Furthermore, INDO/EGF/AVP Lp was significantly less than AVP alone (Lp = 232±9.58, $n = 15$, $P < 0.0005$) or AVP plus INDO (258.8±27.5, $n = 3$, $P < 0.01$). There was no significant difference between the two control groups AVP alone or the concurrent control group of AVP plus INDO ($P > 0.1$).

STSP reverses the effect of EGF on AVP-stimulated Lp. CCTs were pretreated with the protein kinase C inhibitor, STSP (10^{-7} M), and 0.01% DMSO and were then subsequently exposed to AVP alone or 10^{-10} M EGF then AVP. Lp in CCTs pretreated with STSP plus 10^{-10} M EGF and AVP was significantly greater than the Lp in tubules exposed to EGF and AVP alone (STSP/EGF/AVP mean Lp = 182.9±8.5, $n = 5$ vs. EGF/AVP mean Lp = 127.9±13.6, $n = 6$, $P < 0.005$, Fig. 2). Furthermore, there was no significant difference in the mean Lp of the STSP/EGF/AVP group and STSP/AVP without EGF (mean Lp = 187±9.7, $n = 5$, $P > 0.375$). Thus STSP completely reversed the effects of EGF on AVP-stimulated Lp as compared to its concurrent control group.

Effect of EGF on V_t. Isolated perfused rabbit CCTs frequently exhibit a spontaneous lumen-negative V_t, which correlates with net cation transport (27). Within 5 min of exposing CCTs (used in the previously described water flux studies) to peritubular EGF, we observed a reproducible depolarization of the lumen-negative V_t (Fig. 3). No deterioration in tubule morphology was noted. Using the parameter ΔV_t as defined in Methods we compared the change in V_t between EGF-treated and nontreated tubules over comparable time points. ΔV_t in control tubules was −1.7±0.66 mV in control tubules ($n = 23$) and +8.9±1.9 mV in tubules exposed to EGF over the dose range seen to inhibit AVP-stimulated Lp (i.e., 10^{-8} to 10^{-12} M EGF, $n = 24$, $P < 0.0005$). Luminal addition of EGF had no effect on V_t. Thus there is a highly significant and rapid effect of peritubular EGF on ΔV_t in the CCT.

AVP is known to cause a transient hyperpolarization in the CCT (26). Control tubules hyperpolarized by −6.8±1.14 mV in response to AVP. The AVP-induced hyperpolarization peak was not prevented by EGF (mean voltage hyperpolarization −4.52±0.94, P = ns vs. controls). Thus the depolarizing effect of EGF did not prevent or diminish the voltage response to AVP.

Neither INDO nor STSP prevented the depolarizing effect of EGF on V_t (ΔV_t INDO/EGF = 11.5±3.2, $n = 8$, ΔV_t STSP/EGF = 6.4±1.52, $n = 5$). Interestingly, however STSP, itself, resulted in a significant depolarization of V_t (V_t before STSP = −0.8±2.22 vs. after STSP = +3.0±1.9, $n = 5$, $P < 0.05$). This effect of STSP, however, did not blunt the subsequent hyperpolarization in response to AVP in the control group (mean Δ = −5±1 mV).

Specific binding of ^{125}I-EGF to microdissected rabbit CCTs. Binding of 20 nM ^{125}I-EGF to non–collagenase-treated microdissected rabbit CCTs from 5 separate animals, revealed 198.8±33.3 amol/cm total binding, and 70.4±13.4 nonspecific binding (35%), yielding 128.0±44.8 amol/cm of specific EGF binding (Fig. 4).

Discussion

The present studies represent the first demonstration of a direct renal tubular action of EGF. These studies are of particu-

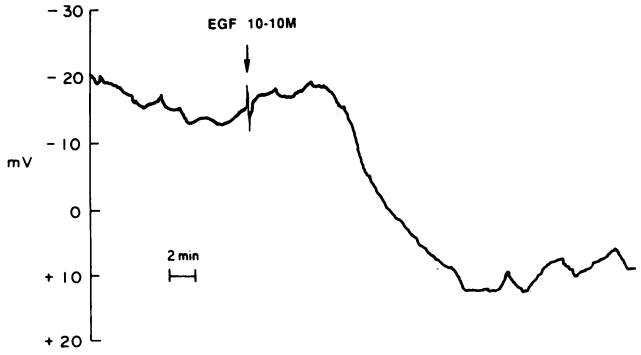

Figure 3. EGF-induced depolarization of the transepithelial voltage in the CCT. A representative tracing of the V_t in the CCT during an experiment in which 10^{-10} M EGF was added to the bath (at the arrow). Within 5 min of addition of EGF the V_t began to progressively drop towards zero and then become positive. The voltage remained positive with the continued presence of EGF for over 2 h.

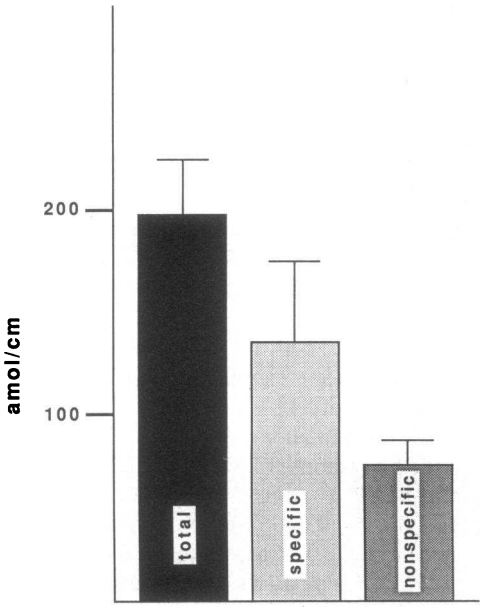

Figure 4. ^{125}I-EGF binding to microdissected rabbit CCTs. Incubations were carried out on ice for 4 h, with 20 nM ^{125}I-EGF in the presence (nonspecific binding) or absence (total binding) of a 500-fold excess of unlabeled EGF. Total binding and nonspecific binding was carried out in triplicate on each rabbit kidney. The data represents the mean±SE of binding to CCTs from five different rabbits. Binding is expressed in attomoles per centimeter tubule length.

lar significance since the kidney appears to be a major site of synthesis for the EGF precursor prepro-EGF. EGF has been shown to be mitogenic in a variety of cultured cells including renal epithelial cells (11, 13, 28). However, the relevance of the mitogenic effects of EGF on cultured cells to its role in the intact animal is uncertain. For example insulin, bradykinin, and AVP are also mitogenic in cultured cells (28, 29) yet in vivo their primary roles are not as growth factors. The demonstration of acute functional effects of EGF on the CCT, freshly obtained from the in vivo setting, provides important new information. Since this tissue is not actively proliferating, these findings raise the possibility that, in a manner analogous to AVP or insulin, EGF might function not only as a growth factor, but may play a dual role in the intact animal, modulating both transport and growth.

The first evidence that EGF might modulate renal tubular transport was obtained in conscious sheep, where intravascular infusions of EGF resulted in an acute natriuresis and diuresis despite a fall in GFR (14). Infusion of EGF directly into the renal artery (12.5 μg/h) increased urine flow without an increase in Na^+ excretion. Intravenous EGF infusion (125 μg/h) resulted in a 500% increase in urine flow and doubled Na^+ excretion, while ^{51}Cr EDTA-estimated GFR and renal plasma flow (^{125}paraaminohippurate clearance) both gradually fell. These effects of EGF infusion in the unanesthetized animal suggest that the EGF-induced diuresis (and natriuresis) is due to a tubular and not a hemodynamic effect of EGF. Our studies support this inference and suggest the CCT is at least one of the segments involved in this diuretic response of the intact animal to EGF. Whether blood EGF concentrations vary in response to changes in water balance or volume status is unknown, so a physiologic role for EGF in modulating water flow in vivo remains to be determined.

In order to further define the role of EGF in the kidney, one must determine the intrarenal segmental distribution of EGF receptors, the cellular effects of EGF in the segments bearing specific EGF receptors, and the physiologic parameters that regulate delivery of EGF to these segments. The present studies help address the first two points. We have demonstrated specific ^{125}I-EGF binding to microdissected CCTs and a functional effect of EGF in this segment. Pretreatment of the CCT with picomolar concentrations of EGF results in rapid depolarization of the CCT and blunts the subsequent hydroosmotic response to vasopressin. It is unlikely that these effects are nonspecific or due to a toxic effect of the EGF preparation for several reasons. First, this preparation of EGF is highly pure as determined by amino acid analysis.[4] In addition, application of EGF from the lumen, at a concentration equivalent to the highest concentration of EGF tested in the bath (25 nM EGF), had no effect on either V_t or Lp. If the effects of EGF were not receptor mediated but mediated by some nonspecific toxic effect of EGF, one would expect equal activity from the lumen as from the bath. Together with the demonstration of specific EGF binding, these results are highly suggestive that these effects are mediated by a specific EGF receptor.

The high-potency, dose-dependent effects of EGF on the CCT are also consistent with a receptor-mediated process. Although the presence of a specific glycoprotein EGF receptor in whole kidney has been demonstrated (10), the intrarenal distribution of this receptor is unknown. EGF receptors have also been demonstrated in cultured cells derived from the proximal tubule, inner medullary collecting duct, and papillary collecting duct (11–13). The present studies suggest that specific EGF receptors also exist in the CCT. Furthermore, to our knowledge, these results represent the first demonstration of function-linked EGF receptors in a noncultured renal epithelium.

To characterize the mechanism of the EGF-mediated inhibition of AVP-stimulated water transport in the CCT, we examined its effect on CcAMP-induced water reabsorption. The binding of vasopressin to its receptor in the CCT is linked to stimulation of adenylate cyclase and cAMP accumulation via a process modulated by guanine nucleotide binding proteins (30–33). This cascade can be bypassed by the addition of cell permeable cAMP analogues, such as CcAMP. This allows one to distinguish events modulating cAMP accumulation from events resulting from these high intracellular cAMP levels. In the CCT 0.1 mM CcAMP results in a comparable increase in Lp as that seen with 10 μU/ml AVP. The present studies demonstrate that EGF suppressed both AVP- and CcAMP-stimulated Lp to an equal extent. This suggests that its inhibitory effect is predominantly at a step distal to cAMP generation. These results do not exclude additional effects of EGF, such as downregulation of the AVP receptor, alteration of the AVP receptor affinity for its ligand, or decreased cAMP production per molecule of AVP bound to the CCT. However, the fact that EGF blunts the hydroosmotic effect of maximal concentrations of the phosphodiesterase resistant cAMP analogue, CcAMP, precludes examining these possibilities using Lp as a functional assay. The biochemical events that occur after cAMP generation are less well characterized than those involved in its generation but appear to involve alterations in cytoskeletal elements which results in exocytosis of the putative water channel into the apical cell membrane (32).

By means of in vitro microperfusion several endogenous inhibitors of vasopressin action in the CCT have been described. These include prostaglandin E_2, alpha-2 adrenergic agents, bradykinin, and atrial natriuretic factor (33–36). None of these agents inhibits both AVP- and cAMP-stimulated Lp. They therefore appear to act by interfering with cell cAMP accumulation. This is in marked contrast to our results with EGF which also inhibits CcAMP-stimulated Lp. EGF appears to be the first peptide modulator of AVP-stimulated water flow, which acts predominantly at a post-cAMP step.

Multiple cellular actions of EGF have been described (2, 37). These can be separated into early effects (seen in minutes, including tyrosine kinase activity, increased prostaglandin production, increased cell calcium influx, inositol phosphate production, and stimulation of Na^+/H^+ exchange) and late effects (may require exposure to EGF for up to 8 h, including increased proto-oncogene transcription, including *c-fos,* and *c-myc,* and stimulation of DNA synthesis and cell proliferation). Recently, it has been demonstrated that both the immediate and late cellular effects of EGF are dependent upon the receptor-associated tyrosine kinase activity (37). Cells transfected with EGF receptors, containing a point mutation which abolishes the receptor tyrosine kinase activity, display normal EGF binding but no calcium transient, stimulation of *c-fos* transcription, or mitogensis—findings which can be demonstrated in cells transfected with the normal receptor. Thus it

4. Mouse EGF is devoid of alanine, phenylalanine, and lysine residues (25). Each preparation is extensively characterized by amino acid analysis and shown free of these amino acid residues before use.

appears that tyrosine kinase activity is required for normal transduction of the other cellular effects of EGF. These other cellular effects of EGF are less consistently demonstrable. For example, while it seems likely that EGF stimulates phosphatidyl inositol turnover in the human epidermoid carcinoma cell line, A-431, similar findings have not been observed in either Chinese hamster lung or NIH 3T3 fibroblasts (29, 38, 39).

To further characterize the signal transduction pathway mediating the action of EGF on vasopressin-stimulated osmotic water flow, we used the cyclooxygenase inhibitor, INDO, and the protein kinase C inhibitor, STSP. In the both the isolated perfused stomach preparation and Madin-Darby canine kidney cells, EGF increases PGE_2 production (40, 41). Since PGE_2 has been shown to decrease V_t and inhibit the hydroosmotic effect of AVP in the CCT (26, 33, 42) it is plausible that prostaglandins might mediate the effects of EGF on the CCT. Pretreatment with 5 μM INDO failed to reverse the inhibitory effects of EGF on AVP-stimulated Lp. Since this concentration of INDO is known to reverse the inhibition of Lp by either bradykinin or the calcium ionophore A23187 (20, 34), this lack of reversal argues against a role for prostaglandins or other cyclooxygenase metabolites in mediating these effects of EGF.

EGF-mediated inhibition of AVP-stimulated Lp bears some similarities to the effects of exogenous activators of protein kinase C (PMA or dioctanoylglycerol). Both EGF and PMA inhibit either AVP-or CcAMP-stimulated Lp, and each also depolarizes the CCT (20, 43). The effects of both EGF and PMA are also insensitive to INDO. In contrast, pretreatment with the protein kinase C inhibitor STSP completely reversed the inhibitory effects EGF on AVP-stimulated Lp.

In vitro, STSP inhibits protein kinase C with a K_i of 2.7 nM. At higher concentrations in vitro, STSP also inhibits cAMP-dependent kinase, cGMP-dependent kinase and myosin light chain kinase but the degree of selectivity for protein kinase C is 10 times greater that that of H-7 (21, 44). We have previously reported that STSP completely reverses the inhibitory effect of 10^{-7} M PMA on AVP-stimulated Lp in the CCT (20). This concentration produces complete inhibition of the CCT response to both AVP and CcAMP. This is effect of STSP was specific since it did not reverse the inhibitory action of A23187 in the same system. In addition, STSP did not affect the hydroosmotic response of the CCT to concentrations vasopressin ranging from 0.5 to $230 \cdot 10^{-12}$ M (17). This concentration range encompasses the threshold of the maximal response in the in vitro perfused CCT to AVP. Since it is felt that AVP stimulates water flow in the collecting duct via cAMP-mediated stimulation of A kinase (32), the normal hydroosmotic effect of AVP, in the setting of STSP argues against STSP-mediated inhibition of A kinase. Given the persistence of an effect of EGF on V_t (*vide infra*) in the setting of STSP, an effect on EGF receptor binding or receptor tyrosine-kinase activity seems less likely. Thus although we cannot definitively exclude other possible effects of STSP, given the fact that EGF has been shown to increase phosphatidylinositol bisphosphate breakdown in other systems (38), and that STSP reverses the effect of PMA in the present system as well as in other systems, we would suggest that the simplest interpretation of the current results is that EGF stimulates protein kinase C in the CCT.

It also seems likely that EGF alters ion transport in the CCT. The spontaneous negative voltage of this segment is mainly generated by electrogenic Na^+ absorption. The Na^+ current is shunted by paracellular Cl^- movement as well as transcellular electrogenic K^+ secretion and H^+ secretion (28, 45). The observed depolarization of the CCT could be accounted for by diminished Na^+ absorption, increased K^+ secretion, increased H^+ secretion, or an increased paracellular Cl^- shunt. Recently, our results have been confirmed and extended by Vehaskari et al. (46). These investigators found that nanomolar EGF in the bath but not the lumen reduced the lumen-negative V_t in the isolated perfused rabbit CCT. These investigators also demonstrated that EGF inhibits net sodium absorption in the CCT. It will be important to determine the effects of EGF on other ion transport rates in this segment as well.

Interestingly, we found the effects of EGF on ΔV_t were not prevented by STSP. This is in marked contrast to the effect STSP on the EGF/AVP interaction with Lp. The failure of STSP to prevent the effects of EGF on V_t suggests that EGF may affect CCT V_t and AVP-stimulated L_p by separate signal transduction mechanisms. Caution must be applied in interpreting this data since an independent effect of STSP (leading to slight depolarization of the CCT) was also noted. Further studies examining the interaction between STSP and EGF on ion transport in the CCT must be performed to validate this observation.

Given the fact that urinary EGF levels are orders of magnitude greater than those in blood, we examined the effect of luminal EGF on V_t and AVP-stimulated Lp in the CCT (7). We chose a luminal concentration of EGF comparable to that measured in the urine (i.e., $3 \cdot 10^{-8}$ M). In contrast to the effects of peritubular EGF, luminal EGF had no effect on either AVP stimulated Lp or V_t. Previous studies have suggested that urinary EGF may be derived from the kidney itself. Using in situ hybridization, Bell et al. (9) charactarized the thick ascending limb of Henle and distal convoluted tubule as the major site for preproEGF mRNA synthesis. Salido et al. (47) localized intrarenal EGF immunoreactivity to the apical cell membrane of the thick ascending limb of Henle and distal convoluted tubule (47). Thus it is conceivable that the thick ascending limb of Henle or distal convoluted tubule could actively secrete EGF or prepro-EGF into the urine. The lack of an effect of luminal EGF on V_t or Lp in the CCT, argues against a "downstream" role for secreted EGF. At least in the CCT, peritubular EGF is much more important in modulating these two functions.

The factors regulating peritubular EGF levels are unknown. In humans, circulating blood levels of EGF have been determined to be $\sim 10^{-11}$ M. However, Oka et al. (48) found that most of blood borne EGF is associated with platelets. Thus platelets might be the source of immunoreactive EGF (or its antigenic and functional homologue transforming growth factor-alpha).[5] Free circulating EGF concentration in platelet poor human plasma is < 15 pg/ml (2.5 pM/liter), i.e., below the measurable limits of current assays. It should be noted however that if EGF is a regulatory hormone, blood concentrations would be expected to be on the steep part of our dose response curve (i.e., below 3 pM/liter). Thus current assays may not be sensitive enough to detect free circulating EGF

5. Transforming growth factor-alpha has been shown to be released by activated platelets. This homologous molecule binds to the EGF receptor and has similar cellular effects as does EGF (50, 51).

concentrations under normal or pathophysiologic circumstances.

It is also possible that EGF levels in renal peritubular vessels or renal interstitium are higher than levels in systemic venous blood. This would require that renal prepro-EGF be processed to EGF and released into the peritubular capillaries or renal interstitium. Measurement of renal arterial, venous, and interstitial (lymphatic) EGF concentrations would be required to document this possibility.

In conclusion, we have described potent effects of picomolar EGF on the isolated perfused rabbit CCT. EGF inhibits both AVP- and cAMP-stimulated water flow. It also causes the spontaneously negative V_t of the CCT to depolarize towards zero or become positive, suggesting effects on ion transport. These findings are observed only with EGF in the bath but not the lumen. These results coupled with the demonstration of specific EGF binding sites argue for the presence of specific basolateral EGF receptors in the CCT. Finally, the EGF-mediated effects on AVP-stimulated osmotic water flow can be reversed by STSP, suggesting that protein kinase C may be involved. The potent and rapid effects of EGF in the CCT raise the possibility that in addition to its role as a mitogen EGF could play a role in regulating renal function.

Acknowledgments

The authors thank Drs. Stanley Cohen and Terry Guigni for their thoughtful discussion and Ms. Melinda Mosely, Debra Asbelle-Gillespie, and Carrie Lopez for their expert technical assistance.

Dr. Matthew Breyer is the recipient of a Veterans Administration Associate Investigator award 151-JAI-733 and Dr. Julie Breyer is the recipient of National Institutes of Health Physician Scientist award 1K11DK01660-01. This work was also funded in part by National Institutes of Health grants AM-37097 and 1P50DK38667-01.

References

1. Cohen, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. *J. Biol. Chem.* 237:1555–1562.

2. Carpenter, G., and S. Cohen. 1979. Epidermal growth factor. *Annu. Rev. Biochem.* 48:193–216.

3. Moolenaar, W. H., R. Y. Tsien, P. T. VanderSaag, and S. W. deLaat. 1983. Na^+/H^+ exchange and cytoplasmic pH in action of growth factors in human fibroblasts. *Nature (Lond.).* 304:645–650.

4. Hunter, T., and J. A. Cooper, 1981. Epidermal growth factor induces rapid tyrosine phosophorylation of proteins in A431 human tumor cells. *Cell.* 24:741–752.

5. Gregory, H. 1975. Isolation and structure of urogastorone and its relationship to epidermal growth factor. *Nature (Lond.).* 257:325–327.

6. Gan, B. S., K. L. MacCarrell, and M. D. Hollenberg. 1987. Epidermal growth factor-urogastrone causes vasodilitaion in the anesthetized dog. *J. Clin. Invest.* 80:199–206.

7. Olsen, P. S., E. Nexo, S. S. Poulsen, H. F. Hansen, and P. Kierkegaard. 1984. Renal origin rat urinary epidermal growth factor. *Regul. Peptides.* 10:37–43.

8. Rall, L. B., J. Scott, and G. I. Bell. 1985. Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues. *Nature (Lond.).* 313:228–231.

9. Bell, G. I., N. M. Fong, M. M. Stempien, M. A. Wormsted, D. Caput, L. Ku, M. S. Urdea, L. B. Rall, and R. Sanchez-Pescador. 1986. Human epidermal growth factor precursor: cDNA sequnence, expression in vitro and gene organization. *Nucleic Acids Res.* 14:8427–8446.

10. Cohen, S., R. A. Fava, and S. T. Sawyer. 1982. Purification and characterization of epidermal growth factor receptor/protein kinase from normal mouse liver. *Proc. Natl. Acad. Sci. USA.* 79:6237–6241.

11. Norman, J., B. Badie-Dezfooly, E. P. Nord, I. Kurtz, J. Schlosser, A. Chaudhari, and L. G. Fine. 1987. EGF-induced mitogenesis in proximal tubular cells: potentiation by angiotensin II. *Am. J. Physiol.* 253:F299–309.

12. Boxer, D. C., R. C. Stanton, and J. L. Seifter. 1987. Growth rate of rat inner medullary collecting duct (IMCD) cells is stimulated by low potassium (LK) and by epidermal growth factor (EGF) and determines the expression of Na^+/H^+ exchange and H^+ ATPase. *Kidney Int.* 31:161. (abstr.)

13. Harris, R. C., and J. Breyer. 1987. Rat renal papillary collecting duct cells possess specific epidermal growth factor (EGF) receptors. Tenth International Congress of Nephrology. Alden Press, Oxford, England. p. 261. (abstr.)

14. Scoggins, B. A., A. Butkus, J. P. Coghlan, D. T. W. Fei, J. G. McDougall, H. D. Niall, and X.-M. Wang. 1984. In-vivo cardiovascular renal and endocrine effects of epidermal growth factor in sheep. *In* Endocrinology. F. Labrie and L. Proulx, editors. Elsevier Science Publishers, B. V., Amsterdam. 573–576.

15. Breyer, M. D., J. P. Kokko, and H. R. Jacobson, 1986. Regulation of net bicarbonate transport in rabbit cortical collecting tubule by peritubular pH, carbondioxide tension, and bicarbonate concentration. *J. Clin. Invest.* 77:1650–1660.

16. Husain, K. H., N. Fernando, M. Shapiro, A. Kagan, and S. M. Glick. 1973. Radioimmunoassay of arginine vasopressin in human plasma. *J. Clin. Endocrinol. Metab.* 37:616–626.

17. Ando, Y., M. D. Breyer, and H. R. Jacobson. 1988. Functional evidence against a physiological role for V1 receptor linked modulation of osmotic water permeablity in rabbit cortical collecting tubules. *Clin. Res.* 36:592A. (Abstr.)

18. Flores, A. G. A., and G. W. G. Sharp. 1972. Endogenous prostaglandins and osmotic water flow in the toad baldder. *Am. J. Physiol.* 223:1392–1397.

19. Schuster, V. L., J. P. Kokko, and H. R. Jacobson. 1984. Interactions of Lysyl-badykinin and antidiuretic hormone in the rabbit cortical collecting tubule. *J. Clin. Invest.* 73:1659–1667.

20. Ando, Y., H. R. Jacobson, and M. D. Breyer. 1988. Phorbol ester and A23187 have additive but mechanistically separate effects on vasopressin action in rabbit collecting tubule. *J. Clin. Invest.* 87:1578–1584.

21. Tamaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem. Biophys. Res. Commun.* 135:397–402.

22. DuBois, R., A. Verniory, and M. Abramow. 1976. Computaion of the osmotic water permeability of perfused tubule segments. *Kidney Int.* 10:478–479.

23. Mujais, S. K., S. Kaufmann, and A. I. Katz. 1986. Angiotensin II binding sites in individual segments of the rat nephron. *J. Clin. Invest.* 77:315–318.

24. Breyer, M. D., J. A. Breyer, C. Lopez, and H. R. Jacobson. 1988. Characterization and distribution of 1251 epidermal growth factor (I-EGF) binding sites in microdissected rabbit nephron segments. *Clin. Res.* 36:514A. (Abstr.)

25. Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative: rapid isolation procedureas and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.

26. Holt, W. F., and C. Lechene. 1981. ADH-PGE2 interactions in cortical collecting tubule. Depression of sodium transport. *Am. J. Physiol.* 241:F452–460.

27. O'Neil, R. G., and S. C. Sansom. 1984. Characterization of apical cell membrane Na^+ and K^+ conductances of cortical collecting duct using microelectorde techniques. *Am. J. Physiol.* 247:F14–24.

28. Taub, M., P. E. Devis, and S. H. Grohol. 1984. PGE_1-independent MDCK cells have elevated intracellular cyclic AMP but retain the growth stimulatory effects of glucagon and epidermal growth factor in serum-free medium. *J. Cell. Physiol.* 120:19–28.

29. Wakelam, M. J. O., S. A. Davies, M. D. Houslay, I. McKay, C. J. Marshall, and A. Hall. 1986. Normal $p21^{n\text{-}ras}$ couples bombesin

and other growth factor receptors to inositol phosphate production. *Nature (Lond.).* 323:173–175.

30. Ausiello, D. A., K. L. Skorecki, A. S. Verkman, and J. V. Bonventre. 1987. Vasopressin signaling in kidney cells. *Kidney Int.* 31:521–529.

31. Dillingham, M. A., J. K. Kim, M. F. Horster, and R. J. Anderson. 1984. Forskolin Increases osmotic water permeability of the rabbit cortical collecting tubule. *J. Membr. Biol.* 80:243–248.

32. Abramow, M., R. Beauwens, and E. Cogan. 1987. Cellular events in vasopressin action. *Kidney Int.* 32(Suppl. 21):56–66.

33. Nadler, S. P., S. C. Hebert, and B. M. Brenner. 1985. PGE_2, forskolin, and cholera toxin interactions in rabbit cortical collecting tubule. *Am. J. Physiol.* 250:F127–135.

34. Schuster, V. L. 1985. Mechanism of bradykinin, ADH, and cAMP interactions in rabbit cortical collecting duct. *Am. J. Physiol.* 249:F645–653.

35. Ribeiro, C. P., F. Ribeiro-Neto, J. B. Field, and W. N. Suki. 1987. Prevention of alpha-2 adrenergic inhibition on ADH action by pertussis toxin in rabbit CCT. *Am. J. Physiol.* 253:C105–112.

36. Dillingham, M. A., and R. J. Anderson. 1986. Inhibiton of vasopressin action by atrial natriuretic factor. *Science (Wash. DC).* 231:1572–1573.

37. Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsein, G. N. Gill, and M. G. Rosenfeld. 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature (Lond.).* 328:820–823.

38. Wahl, M. I., J. D. Sweatt, and G. Carpenter. 1987. Epidermal growth factor (EGF) stimulates inositol trisphosphate formation in cells which overexpress the EGF receptor. *Biochem. Biophys. Res. Commun.* 142:688–695.

39. L'Allemain, G., and J. Pouyssegur. 1986. EGF and insulin action in fibroblasts: evidence that phosphoinositide hydrolysis is not an essential mitogenic signalling pathway. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 197:344–348.

40. Chiba, T., Y. Hirata, T. Taminato, S. Kadowaki, S. Matsukura, and T. Fujita. Epidermal growth factor stimulates prostaglandin E release form isolated perfused rat stomach. *Biochem. Biophys. Res. Commun.* 105:370–374.

41. Levine, L., and A. Hassid. 1977. Epidermal growth factor stimulates prostaglandin biosynthesis by canine kidney (MDCK) cells. *Biochem. Biophys. Res. Commun.* 76:1181–1187.

42. Stokes, J. B., and J. P. Kokko. 1977. Inhibition of sodium transport by prostaglandin E_2 across the isolated, perfused rabbit collecting tubule. *J. Clin. Invest.* 59:1099–1104.

43. Hays, S. R., M. Baum, and J. P. Kokko. 1987. Effects of protein kinase C activation on sodium, potassium, chloride, and total CO_2 transport in the rabbit cortical collecting tubule. *J. Clin. Invest.* 80:1561–1570.

44. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. 1984. Isoquioline-sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry.* 23:5036–5041.

45. Sansom, S. C., E. J. Weinman, and R. G. O'Neil. 1984. Microelectrode assessment of chloride-conductive properties of cortical collecting duct. *Am. J. Physiol.* 147:F291–302.

46. Vehaskari, V. M., K. Hering-Smith, D. Moskowitz, and L. Hamm. 1988. Epidermal growth factor (EGF) inhibits sodium transport in the rabbit cortical collecting tubule. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:A708. (Abstr.)

47. Salido, E. C., L. Barajas, J. Lechago, N. P. Laborde, and D. A. Fisher. 1986. Immunocytochemical localization of epidermal growth factor in mouse kidney. *J. Histochem. Cytochem.* 34:1155–1160.

48. Oka, Y., and D. N. Orth. 1983. Human plasma epidermal growth factor/β-urogastrone is associated with blood platelets. *J. Clin. Invest.* 72:249–259.

49. Reif, M. C., S. L. Troutman, and J. A. Schafer. 1984. Sustained response to vasopressin in the isolated rat cortical collecting tubule. *Kidney Int.* 26:275–732.

50. Marquardt, H., M. W. Hunkapiller, L. E. Hood, and G. J. Todaro. 1984. Rat transforming growth factor type 1: structure and relationship to epidermal growth factor. *Science (Wash. DC).* 223:1079–82.

51. Assoian, R. K., G. R. Grotendorst, D. M. Miller, and M. B. Sporn. 1984. Cellular transformation by coordinated action of three peptide growth factors form human platelets. *Nature (Lond.).* 309:804–806.