J. Membrane Biol. 183, 15–23 (2001)
DOI: 10.1007/s00232-001-0049-6

The Journal of
Membrane
Biology

Effects of 8-cpt-cAMP on the Epithelial Sodium Channel Expressed in *Xenopus* Oocytes

A. Chraïbi[1], M. Schnizler[2], W. Clauss[2], J.-D. Horisberger[1]
[1]Institute of Pharmacology and Toxicology, University of Lausanne, Bugnon 27, 1005 Lausanne, Switzerland
[2]Institut für Tierphysiologie Justus-Liebig-Universität, Giessen, Wartweg 95, D-35392 Germany

Received: 19 January 2001/Revised: 27 April 2001

Abstract. Vasopressin stimulates the activity of the epithelial Na channel (ENaC) through the cAMP/PKA pathway in the cortical collecting tubule, or in similar amphibian epithelia, but the mechanism of this regulation is not yet understood. This stimulation by cAMP could not be reproduced with the rat or *Xenopus* ENaC expressed in *Xenopus* oocyte. Recently, it was shown that the α-subunit cloned from the guinea-pig colon (αgp) could confer the ability to be activated by the membrane-permeant cAMP analogue 8-chlorophenylthio-cAMP (cpt-cAMP) to channels produced by expression of αgp, βrat and γrat ENaC subunits. In this study we investigate the mechanism of this activation. Forskolin treatment, endogenous production of cAMP by activation of coexpressed β adrenergic receptors, or intracellular perfusion with cAMP did not increase the amiloride-sensitive Na current, even though these maneuvers stimulated CFTR (cystic fibrosis transmembrane conductance regulator)-mediated Cl currents. In contrast, extracellular 8-cpt-cAMP increased αgp, βrat and γrat ENaC activity but had no effect on CFTR. Swapping intracellular domains between the cpt-cAMP-sensitive αgp and the cpt-cAMP-resistant αrat-subunit showed that neither the N-terminal nor the C-terminal of α ENaC was responsible for the effect of cpt-cAMP. The mechanisms of activation of ENaC by cpt-cAMP and of CFTR by the cAMP/PKA pathway are clearly different. cpt-cAMP seems to increase the activity of ENaC formed by αgp and βγrat by interacting with the extracellular part of the protein.

Key words: ENaC — Amiloride — Protein kinase A — cAMP — cpt-cAMP — Cut-open oocyte

Correspondence to: J.-D. Horisberger
Jean-Daniel Horisberger@ioharm.unil.ch

Introduction

The epithelial Na channel (ENaC) is a heteromeric channel made of three subunits α, β and γ and is localized in the apical membrane of epithelial cells (Garty & Palmer, 1997). Sodium transport across the apical membrane through the Na channel is the rate-limiting step in sodium reabsorption by the epithelial cells of the distal nephron, distal colon, and in airways. ENaC thereby plays a key role in the regulation of the sodium balance, extracellular fluid volume and blood pressure by the kidney, and in the controlled fluid reabsorption in the airways. The activity of ENaC is regulated by several hormones such as aldosterone and vasopressin but the mechanisms of these regulations are not yet completely understood. Vasopressin evokes a rapid increase of Na reabsorption in amphibian epithelia (toad bladder, frog skin and A6 cells) (Krattenmacher & Clauss, 1988; Verrey, 1994) and in rat collecting tubule (Canessa & Schafer, 1992; Schafer & Hawk, 1992) but not in mammalian distal colon or urinary bladder (Garty & Palmer, 1997) and several of these studies have demonstrated that this activation is mimicked by permeable cAMP analogues and activators of adenylate cyclase. Two types of regulatory mechanisms have been proposed: an increase in the number of Na^+ channels in the apical membrane by recruitment of additional Na^+ channels from sub-apical membrane vesicles and/or the activation of quiescent channels already present in the apical membrane. The nature of these mechanism is not yet clear, even though a phosphorylation in the C-terminus of β or γ ENaC subunit has been proposed (Shimkets, Lifton & Canessa, 1998). In *Xenopus* oocyte, expressed rat or *Xenopus* ENaC could not be stimulated by cAMP and its analogues (Awayda et al., 1996). However Liebold et al. (1996) have shown that mRNA from distal colon of guinea pig injected into *Xenopus* oocyte, generated an amiloride-sensitive Na^+ conductance that could be con-

siderably increased by a membrane-permeable analogue of cAMP, 8-chlorophenyl-thio-cyclic-adenosine-monophosphate (cpt-cAMP). Recently, Schnizler et al. (2000) have cloned a cDNA encoding the α-subunit ENaC from guinea-pig colon, and showed that this subunit could confer the ability to be activated by cpt-cAMP to channels produced by coexpression of αgp, βrat and γrat ENaC subunits.

In this study, we have evaluated the mechanism of this activation by testing several procedures activating the cAMP/PKA pathway. We first recorded the effects of epinephrine on oocytes coexpressing the αgp-βrat-γrat ENaC with the human β_2 adrenergic receptor. To obtain a precise and rapid control of the intracellular medium to test the effects of intracellular factors, we used the "cut-open" oocyte technique. We also expressed chimeric constructs in which either the N-terminal, the C-terminal or both intracellular domains of the rat and guinea pig α subunit were swapped, and studied the effect of cpt-cAMP on these different constructs.

Our results indicate that the mechanisms of activation of ENaC and CFTR by the cAMP/PKA pathway are clearly different and they suggest the hypothesis that cpt-cAMP increases the activity of the channel formed by αgp and βγrat ENaC by interacting with the extracellular part of the protein.

Materials and Methods

Expression of ENaC in *Xenopus* Oocyte

In vitro-transcribed cRNA for the α-, β- and γ-subunits of rat ENaC (αr, βr, γr) and guinea pig αENaC (αgp) were injected into stage V/VI *Xenopus* oocytes (0.1 ng of cRNA of each subunit in a total volume of 50 nl) as described earlier (Canessa et al., 1993, 1994; Puoti et al., 1995). Electrophysiological experiments were performed one or two days after cRNA injection. In other experiments 6 ng of in vitro-transcribed human CFTR cRNA (clone generously provided by R. Boucher), or 6 ng of human β_2 adrenergic receptor cRNA (clone generously provided by S. Cotecchia) were injected into *Xenopus* oocytes (Chraïbi & Horisberger, 1999).

Construction of Chimeric α-Subunits

A BspE1 restriction site was introduced into the αgp cDNA sequence by PCR using sense (cggttccggagccgg) and antisense (ccggctccg-gaaccg) oligonucleotides at the amino acid position 585–589. SacII restriction sites were introduced (αgp cDNA sequence position 224–228; αrat cDNA: position 285–290) with sense (caaccgcggaagacg) and antisense (cgtcttcccgcggttg) oligonucleotides. All constructs were cloned into pSDEasy (Puoti et al., 1997).

Electrophysiological Measurements in Whole Oocytes

Amiloride-sensitive currents were measured as previously described (Canessa, Horisberger & Rossier, 1993) using the two-electrode voltage-clamp technique by means of a Dagan TEV voltage-clamp apparatus (Dagan, Minneapolis, MN), at room temperature (22–25°C) and at a holding potential of −60 mV in a solution containing (in mM) 100 Na gluconate, 0.4 $CaCl_2$, 10 Na-HEPES (pH 7.4), 5 $BaCl_2$ and 10 tetraethyl-ammonium chloride. The current signal was filtered at 20 Hz using the internal filter of the Dagan apparatus, and continuously recorded on a paper chart. Low chloride concentration and K^+ channel blockers were used to reduce the background membrane conductance. cAMP-stimulated Cl^- currents were measured between −60 and −80 mV.

Ion Current Measurement in the Cut-open Oocyte Technique

Amiloride-sensitive currents and cAMP-stimulated Cl^- currents were also measured by using the cut-open oocyte technique, as described earlier (Abriel & Horisberger, 1999). Briefly, a *Xenopus* oocyte was mounted in the cut-open oocyte chamber and the pipette for perfusion and voltage recording was inserted into the animal pole of the oocyte and advanced to just below (100–300 μm) the exposed membrane. The solution was perfused at a flow rate of about 1 μl/min by means of a precision syringe pump (Infors AG, Basel, Switzerland). The flow of solution removed almost all visible yolk platelets below the studied membrane. In order to minimize the dead space when the perfusion solution was changed, we introduced two thin capillaries by which test solutions were introduced into the perfusion pipette close to the tip. Membrane currents were recorded under voltage-clamp conditions by means of a Dagan cut-open oocyte voltage-clamp apparatus (Model CA-1 High Performance Oocyte Clamp). Data acquisition and analysis were performed using a TL1 DMA digital converter system and the pCLAMP software package (Axon Instruments, Foster City, CA, USA; version 5.5). For the measurement of amiloride-sensitive currents the holding potential was −60 mV and alternating between −40 and −80 mV for the measurement of the cAMP-stimulated Cl^- conductance.

Single-Channel Recordings

Before patch-clamp experiments the oocytes were placed for 3–5 min at room temperature in a hypertonic medium (475 mOsm) with the following composition (in mM): K-aspartate 200, KCl 20, $MgCl_2$ 1, EGTA 10, Na-HEPES 10, pH 7.4. The vitelline membrane could then be manually removed from the cell using fine forceps (Methfessel et al., 1986). The oocytes were then immediately transferred to the recording chamber.

Single-channel recordings were obtained in the cell-attached patch clamp configuration carried out according to the methods described by Hamill et al. (1981) with a List LM EPC 7 patch clamp amplifier (List Electronics, Darmstadt, Germany), displayed on an oscilloscope (Tektronix, Heerenveen, Netherlands) and stored on a digital tape recorder (Bio-Logic, Claix, France). Borosilicate glass (Corning, New York, New York, USA) patch pipettes were pulled in two stages with a PP-83 vertical puller (Narishige, Japan) and fire-polished. They had a resistance of 10–20 MΩ and were filled with 100 mM LiCl. The bath solution was 100 mM KCl buffered to pH 7.4 with 10 mM Na-HEPES.

Current signals were filtered at 200 Hz with an 8-pole Bessel filter (Frequency Devices Inc., Haverhill, Massachusetts, USA) and digitized at 1 KHz using a Labmaster analog-digital interface and Fetchex Software (Axon Instruments). The $N \cdot Po$ product (N = number of channel, Po = open probability) was calculated as

$$N \cdot Po = I_{ENaC}/i \quad (1)$$

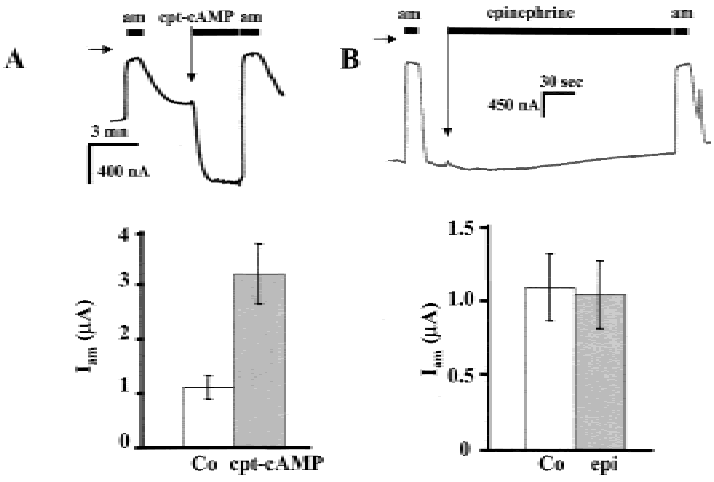

Fig. 1. Effect of cpt-cAMP and epinephrine on the amiloride-sensitive current in oocytes expressing guinea pig α+ rat βγ ENaC and human β_2 adrenergic receptor. (*A*) Original current recording in an oocyte expressing αgp-βr-γr ENaC. The current sensitive to 10 μM amiloride (*am*) was measured at −60 mV before and after a 3-min exposure to 100 μM cpt-cAMP. (*B*) Original current recording in an oocyte expressing αgp-βr-γr ENaC and human β_2 adrenergic receptor. The current sensitive to 10 μM amiloride was measured at −60 mV before and after a 10 min exposure to 1 μM epinephrine. Epinephrine has no detectable effect on the amiloride-sensitive current. The horizontal arrows indicate the zero-current level. The bottom panels present the mean amiloride-sensitive current I_{am} before (*Co*) and after perfusion of cpt-cAMP (*A*), $n = 15$, or epinephrine (*B*), $n = 10$. cpt-cAMP induced a significant increase in I_{am} ($P < 0.001$, paired *t*-test) while there was no significant effect of epinephrine.

where I_{ENaC} is the current due to ENaC (= total current minus current with no channel open) averaged over a 1-min recording and *i*, the average unitary current.

Solutions and Chemicals

After injection, the oocytes were kept at 19°C in a low-Na modified Barth's saline (MBS) containing (in mM): 10 NaCl, 90 NMDG-Cl, 5 KCl, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 0.82 $MgSO_4$ 10 NMDG-Hepes (pH 7.2) supplemented with penicillin (100 u/ml) and streptomycin (100 mg/ml). The extracellular solution for the cut-open oocyte experiment had the following composition (in mM): 80 Na-gluconate, 10 TEA-Cl, 5 $BaCl_2$, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES-NMDG (pH 7.5). The solution used for the intracellular perfusion in the "cut-open oocyte" experiments contained (in mM) 10 TEA-Cl, 5 $BaCl_2$, 1 $MgCl_2$, 0.5 Mg-ATP, 0.5 $CaCl_2$, 0.2 BAPTA, 10 HEPES-NMDG (pH 7.35), 90 or 50 K-gluconate and 10 or 50 KCl for the measurement of amiloride-sensitive or cAMP-stimulated Cl^- currents, respectively.

Experiments with chimeric α-subunit constructs were performed in a solution containing (in mM): 90 NaCl, 1 KCl, 2 $CaCl_2$ and 5 HEPES (pH 7.4). Amiloride, cAMP, cpt-cAMP and forskolin were purchased from Sigma (St. Louis, MO). Protein kinase A (PKA) and the protein kinase inhibitor PKI 6-22 were obtained from Calbiochem (La Jolla, CA).

Results

Effect of Permeable cAMP Analogues and Epinephrine on the Macroscopic Amiloride-sensitive Current in *Xenopus* Oocytes Expressing ENaC and CFTR

As shown in Fig. 1*A*, 100 μM cpt-cAMP stimulated the amiloride-sensitive current in oocytes expressing heteromeric channels composed of the α-subunit of guinea pig ENaC and the β and γ subunits of rat ENaC. I_{Na} increased from 1.14 ± 0.22 μA to 3.30 ± 0.58 μA ($n = 15$, $P < 0.001$). This effect was similar to that reported recently by Schnizler et al. (2000). In order to identify the pathway of this activation, we first tested the effect of epinephrine on oocytes coexpressing αgp-βr-γr ENaC and the human β_2 adrenergic receptor. The amiloride-sensitive current was measured before and after perfusion of epinephrine. In 10 measurements, exposure to 1 μM of epinephrine has no detectable effect on ENaC activity. The mean values of I_{Na} before and after epinephrine are shown in Fig. 1*B*. As a positive control, we co-expressed human CFTR and the human β_2 adrenergic receptor. As shown in Fig. 2, in these oocytes, 1 μM epinephrine induced a significant increase of the membrane conductance within 2 to 3 minutes. In contrast, we could not detect any effect of 100 μM cpt-cAMP on the whole-oocyte conductance even though epinephrine increased the CFTR-mediated conductance in the same oocytes (Fig. 2*B*). The effect of epinephrine on the CFTR-mediated conductance was clearly delayed by 30 to 60 seconds (*see* Fig. 2*A*) after the solution exchange, in contrast to the quasi instantaneous effect of cpt-cAMP on ENaC mediated current (*see* Fig. 1*A*).

We also examined the effects of isobutyl-methyl xanthine (IBMX) and forskolin on the amiloride-sensitive current and oocyte conductance in *Xenopus* oocytes expressing αgp-βr-γr ENaC or CFTR respectively.

As shown in Fig. 3*A*, no effect of 350 μM IBMX alone on the amiloride-sensitive current could be detected. In the presence of IBMX, addition of 20 μM of forskolin did not change significantly ENaC activity. Neither did addition of 200 μM cAMP in the extracellular solution modify the Na^+ current. In contrast, cpt-cAMP induced an increase in the amiloride-sensitive current of approximately three-fold. As a positive control, we measured the effect of IBMX and forskolin on the whole-oocyte conductance in *Xenopus* oocyte expressing human CFTR and human β_2 adrenergic receptor. No significant effect of IBMX alone on whole-oocyte conductance was detected. On the other hand, the

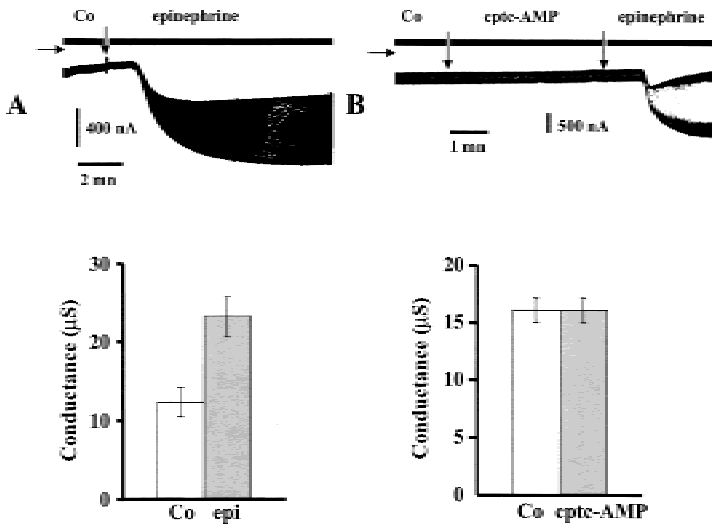

Fig. 2. Effect of 1 μM epinephrine and 100 μM cpt-cAMP on whole-oocyte conductance in oocytes expressing human CFTR and human β_2 adrenergic receptor. (*A*) Original recording in an oocyte expressing human CFTR and human β_2 adrenergic receptor. Whole-oocyte conductance was measured between −60 mV and −80 mV before and after exposure to 1 μM epinephrine. (*B*) Original recording in an oocyte expressing human CFTR and human β_2 adrenergic receptor. Whole-oocyte conductance was measured between −60 mV and −80 mV before and after exposure to 100 μM cpt-cAMP and 1 μM epinephrine. cpt-cAMP has no detectable effect on the whole-oocyte conductance. Epinephrine was used as positive control to demonstrate the expression of CFTR. The horizontal arrows indicate the zero-current level. The bottom panels present the mean results obtained before (*Co*) and after perfusion of cpt-cAMP (*A*), $n = 14$, or epinephrine (*B*), $n = 10$. Epinephrine induced a significant increase in membrane conductance ($P < 0.001$, paired *t*-test) while there was no significant effect of cpt-cAMP.

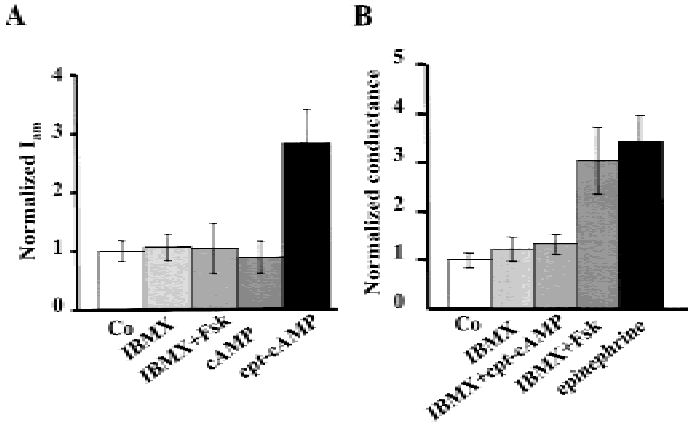

Fig. 3. Effect of IBMX or forskolin on amiloride-sensitive current and whole-oocyte conductance in oocytes expressing αgp-βr-γr ENaC or CFTR and human β_2. (*A*) The Na^+ current sensitive to 10 μM amiloride (I_{am}) was measured in oocytes expressing αgp βr γr ENaC before (white bar, *Co*) or after perfusion with 350 μM IBMX ($n = 6$), 10 μM forskolin and 100 μM IBMX ($n = 5$), 200 μM cAMP ($n = 4$), or 100 μM cpt-cAMP ($n = 10$). Results are expressed as relative to the control value *Co* measured in the same oocyte. Only cpt-cAMP induced a significant increase in I_{am} ($P < 0.002$, paired *t*-test). (*B*) Whole-oocyte conductance between −40 and −60 mV was measured in oocytes expressing human CFTR and human β_2 adrenergic receptor before (white bar) or after perfusion with 350 μM IBMX ($n = 7$), 100 μM cpt-cAMP + IBMX ($n = 7$), 10 μM forskolin + IBMX ($n = 7$) or 1 μM epinephrine ($n = 13$). Results are expressed as relative to the control value *Co* measured in the same oocyte. Forskolin + IBMX and epinephrine induced a large (>3-fold) and highly significant increase of the membrane conductance ($P < 0.01$ and $P < 0.001$, respectively). In the presence of IBMX and cpt-cAMP, the conductance was slightly larger (~40%) than in the control ($P < 0.05$), but not significantly increased compared to IBMX alone.

perfusion of 100 μM of forskolin in presence of IBMX increased the whole-oocyte conductance by about 3-fold (*see* Fig. 3*B*).

Effect of cpt-cAMP at the Single-Channel Level

The effects of cpt-cAMP on the single-channel current were examined in *Xenopus* oocyte expressing αgp βr γr ENaC using the cell-attached patch configuration. Currents in the patches were recorded with a 100 mM LiCl solution in the pipette, and the value of the single-channel conductance measured under these conditions was 7.9 ± 0.2 pS, a value similar to that published earlier (Schnizler et al., 2000).

The channel activity was recorded for 1 min under control conditions and then cpt-cAMP 100 μM was added to the bath solution surrounding the oocyte during 3 min and finally washed by control solution during 5 min. The $N \cdot Po$ was measured over a 1 min period before, during and after superfusing the oocyte with cpt-cAMP at $V_{pip} = +100$ mV. A highly variable rundown of channel activity ($N \cdot Po$) was observed with, in some cases, a decrease and, in other cases, an increase in $N \cdot Po$ after cAMP exposure. The mean $N \cdot Po$ values were 2.9 ± 0.7 before, 2.0 ± 0.5 during and 0.8 ± 0.3 ($n = 7$) after exposure to cpt-cAMP. Thus, no significant activation by cpt-cAMP could be observed although, because of the large variability of the $N \cdot Po$ values and the strong spontaneous rundown of channel activity, small

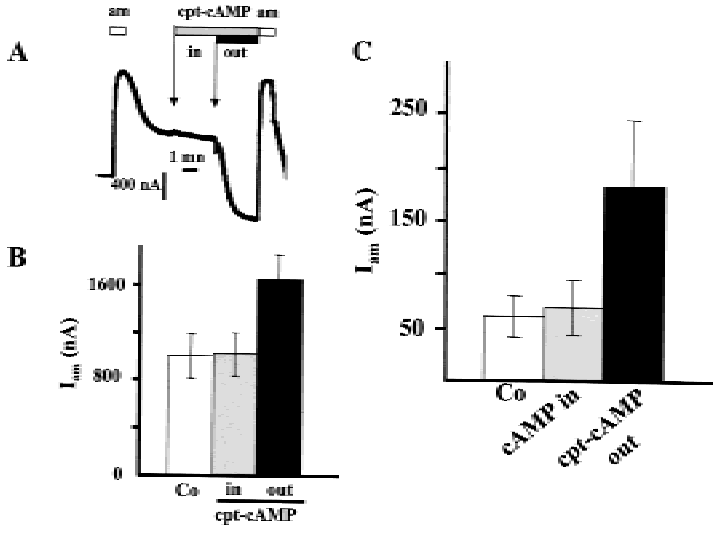

Fig. 4. Effect of intracellular perfusion of cpt-cAMP and cAMP on amiloride-sensitive current in oocytes expressing αgp-βr-γr ENaC. (*A*) Original recording of the membrane current at −60 mV in the "cut open oocyte" technique. The effect of amiloride (*am*), added to the extracellular side of the membrane, was tested in the control condition (*Co*) and after addition of cpt-cAMP first inside the oocyte (*in*) and then to the extracellular side (*out*). (*B*) Mean values of the amiloride-sensitive current measured before and after intracellular (*in*) and extracellular (*out*) perfusion of 100 μM cpt-cAMP. (*C*) In another set of experiments, the effect of intracellular cAMP (1 mM, *cAMP in*) was also tested and compared to the effect of extracellular cpt-cAMP (100 μM *cpt-cAMP out*). Extracellular cpt-cAMP induced a significant increase in I_{am} ($P < 0.05$, paired *t*-test), whereas intracellular cpt-cAMP or cAMP ($n = 6$, $n = 4$, respectively) had no significant effect.

effects could not be excluded. In any case, the single-channel results observed with cpt-cAMP perfused around the patch-pipette cannot explain the effects of cpt-cAMP observed for the macroscopic amiloride-sensitive current.

Because the effects of cpt-cAMP were measured as Na currents in the two-electrode voltage-clamp experiments and as Li currents in the patch-clamp experiments, the apparent discrepancy between the two results could be related to the type of permeant cation. To check for this possibility, we also measured the effects of cpt-cAMP on the macroscopic Li current (extracellular solution with 100 mM Li instead of 100 mM Na) with the two-electrode voltage-clamp technique. In the presence of 100 mM LiCl, cpt-cAMP also induced an increase of the amiloride-sensitive current in oocytes expressing αgp-βr-γr ENaC. The amiloride-sensitive Li current increased from 1.75 μA ± 0.22 to 3.4 μA ± 0.50 ($P < 0.01$, $n = 8$) a stimulation similar to that observed in presence of Na (*see* Fig. 1*A*).

These results indicate that to have an effect on ENaC, cpt-cAMP needs to access the extracellular side of the membrane close to ENaC rather than activating a distant membrane receptor or reaching an intracellular site by diffusing into the oocyte.

Effect of Intracellular Perfusion of Permeable cAMP Analogues on the Macroscopic Amiloride-sensitive Sodium Current

To obtain a more direct and precise control of the intracellular cAMP level, we used the cut-open oocyte technique. After mounting an αgp-βr-γr ENaC-expressing oocyte in the cut-open oocyte chamber, the amiloride-sensitive current (I_{am}) was first measured while perfusing the inside of the oocyte without cyclic nucleotide. Then cpt-cAMP (100 μM) or cAMP (1 mM) was added to the intracellular solution and I_{am} was measured 3 min later. As shown in Fig. 4, no effect on the amiloride-sensitive current was detected. However, extracellular perfusion of 100 μM cpt-cAMP induced a large increase of I_{am}.

Under the same conditions, we then tested the effect of intracellular perfusion of the catalytic subunit of PKA on the amiloride-sensitive current. I_{am} was determined before and 20 min after intracellular perfusion of 40 u/ml of PKA. As shown in Fig. 5*A*, PKA had no significant effect on current carried by αgp-βr-γr ENaC ($n = 11$).

As positive control experiments, we tested the effect of intracellular perfusion of cAMP or PKA catalytic subunit on the Cl^- current in oocytes expression CFTR. As shown in Fig. 6, intracellular perfusion with 10 μM to 1 mM cAMP or with 40 u/ml PKA induced a strong stimulation of the membrane conductance in oocytes expressing CFTR. On average, *G* (μS) was increased from 0.58 ± 0.18 to 13.12 ± 1.91 ($n = 12$) and from 0.36 ± 0.07 ($n = 7$) to 5.19 ± 1.91 ($n = 7$) with intracellular cAMP or PKA, respectively.

Effect of PKA Inhibitor

Schnizler et al. (2000) observed that when a protein kinase A inhibitor was injected into αgp-βr-γr ENaC-expressing oocytes 20 min before electrophysiological measurements, the effect of cpt-cAMP on the amiloride-sensitive current was inhibited. We tested the effect of the PKA inhibitor PKI 6-22 on the stimulatory effect of cpt-cAMP using the cut-open oocyte technique. I_{am} was measured before and after intracellular perfusion with 0.5 or 1 mM PKI 6-22. As shown in Fig. 5*B*, PKI 6-22 has no effect on ENaC activity. The oocytes were then superfused with extracellular solution containing 100 μM cpt-cAMP and the amiloride-sensitive current was deter-

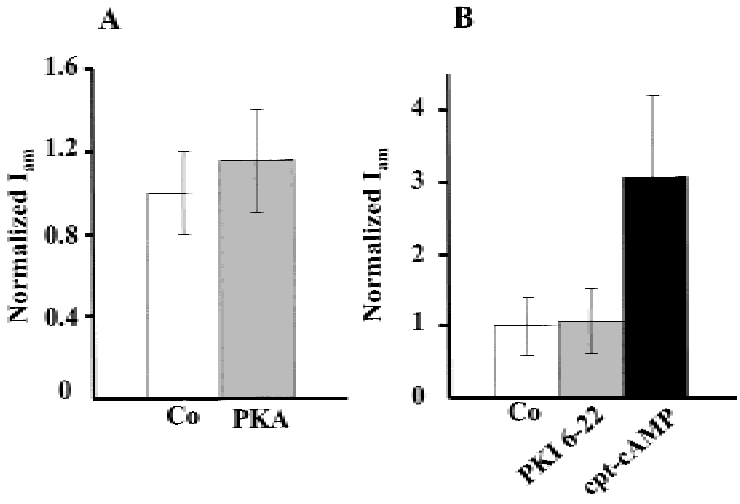

Fig. 5. Effect of intracellular perfusion of PKA and its inhibitor PKI 6-22 on amiloride-sensitive current in oocytes expressing αgp-βr-γr ENaC. (*A*) The amiloride-sensitive current (I_{am}) was measured before and after intracellular perfusion of 40 u/ml PKA. There was no significant effect of PKA (paired *t*-test, $n = 11$). (*B*) The amiloride-sensitive current (I_{am}) measured before and after intracellular perfusion of 500 μM PKI 6-22, and after subsequent exposure to 100 μM cpt-cAMP. PKI 6-22 had no significant effect on I_{am}, and did not prevent activation by extracellular cpt-cAMP. ($n = 5$, $P = 0.02$, paired *t*-test). I_{am} values are expressed as relative to the initial control value.

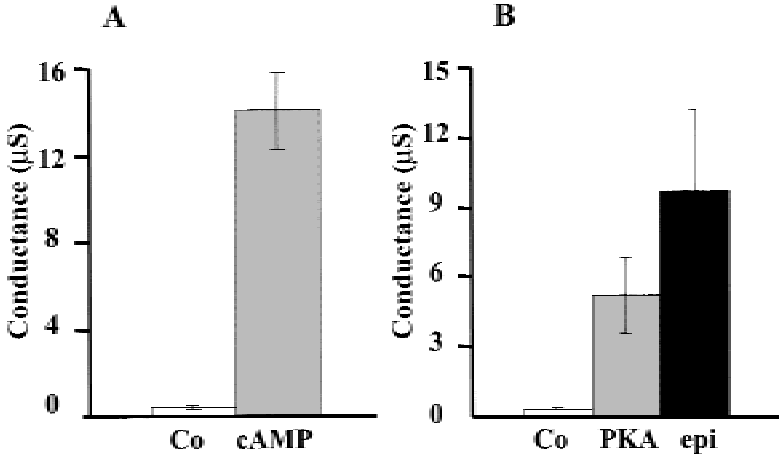

Fig. 6. Effect of intracellular perfusion of cAMP and PKA on whole-oocyte conductance in *Xenopus* oocytes expressing CFTR and human β_2 adrenergic receptor. The whole-oocyte membrane conductance between −40 and −80 mV was measured before and after intracellular perfusion of cAMP (0.01 to 1 mM, pooled values, $n = 12$) (*A*) or before and after intracellular perfusion of 40 u/ml PKA (*B*, $n = 7$). Both cAMP ($P < 0.001$, paired *t*-test) and PKA ($P < 0.01$, paired *t*-test) induced a significant increase in membrane conductance. Extracellular perfusion of 1 μM epinephrine was used as positive control.

mined. Figure 5*B* shows that we could not abolish the stimulatory effect of cpt-cAMP on αgp-βr-γr ENaC by intracellular perfusion of the PKA inhibitor.

To ascertain the effectiveness of the intracellular PKI perfusion, we tested the stimulatory effect of epinephrine on the CFTR-mediated conductance in presence of 0.5 mM intracellular perfusion of PKI. The membrane conductance was measured before and after intracellular perfusion with 1 μM epinephrine and then PKI was added to the intracellular perfusion. As shown in Fig. 7, epinephrine induced a increase of the Cl^- current and membrane conductance carried by CFTR. On average the membrane conductance increased from 6.25 ± 1.06 to 14.21 ± 3.34 μS ($n = 6$). The intracellular perfusion of 0.5 mM PKI abolished completely the stimulatory effect of epinephrine. The conductance decreased from 14.2 ± 3.3 to 6.9 ± 2.9 μS ($n = 6$).

Domain-swapping Experiments

To localize the cpt-cAMP-sensitive motif of the αgp-subunit we coexpressed chimeric constructs of the αgp and αr together with βr and γr ENaC-subunits. Either the N-terminus or the C-terminus of the αr-subunit were replaced by the corresponding domain of the αgp-subunit and conversely, either the N-terminus or the C-terminus of the αgp-subunit were replaced by the corresponding domain of the αr-subunit. The amiloride-sensitive current was measured at a holding potential of −60 mV before and 5 to 10 min after treatment with 100 μM cpt-cAMP and 1 mM IBMX. As shown in Fig. 8 replacement of both N-terminal and C-terminal cytosolic domains of the guinea pig α-subunit by the corresponding domains from the rat subunit did not abolish the effect of cpt-cAMP. The results of all the domain-swapping experiments, summarized in Fig. 8, show that the sensitivity to cpt-cAMP was not conferred by the N-terminal nor the C-terminal intracellular domains but rather by the parts of the protein that comprise the extracellular loop and both transmembrane segments.

Discussion

Vasopressin, acting through its V2 receptor and the cAMP-PKA pathway, stimulates sodium transport in

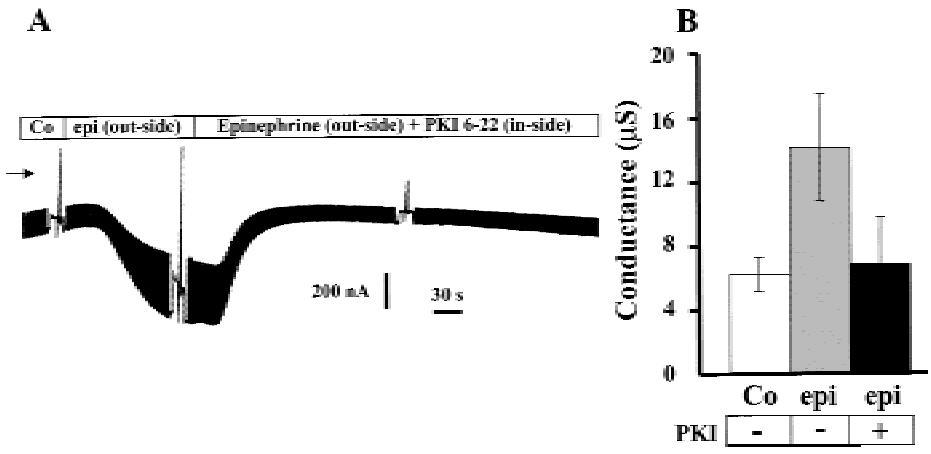

Fig. 7. Effect of intracellular perfusion of PKI 6-22 on stimulation of whole-oocyte conductance by epinephrine. (*A*) Original recording showing that intracellular perfusion of 500 μM PKI 6-22 abolishes the stimulation of the conductance of an oocyte expressing human CFTR and β_2 adrenergic receptor with 1 μM epinephrine. The holding potential was alternated between −40 and −80 mV. The horizontal arrow indicates the zero-current level. (*B*) Average values of whole-oocyte conductance in initial control condition (*Co*), with 1 μM extracellular epinephrine (*epi*) and with epinephrine + intracellular perfusion with PKI 6-22. Epinephrine induced a significant increase of the membrane conductance ($n = 6$, $P < 0.01$, paired *t*-test) and this effect was abolished by PKI 6-22.

both mammalian and amphibian tight epithelia by increasing the activity of the epithelial Na channel (Krattenmacher et al., 1988; Canessa & Schafer, 1992; Schafer & Hawk, 1992; Verrey, 1994). The complete mechanism of this stimulation is, however, not yet well understood, maybe in part because this stimulation could not be reproduced with ENaC expressed in *Xenopus* oocytes (Awayda et al., 1996). Recently Schnizler et al. (2000) showed that the α subunit of the guinea pig ENaC, when coexpressed with rat β- and γ-subunits, resulted in the expression of functional Na channels that could be strongly stimulated by the membrane-permeant and phosphodiesterase-resistant cAMP analogue, cpt-cAMP. In the present work we have investigated the mechanism by which this stimulation may occur. Using endogenous production of cAMP by stimulation of a coexpressed β adrenergic receptor or agents known to increase the cAMP level, we failed to observe an increase of the current carried by αgp-βr-γr ENaC, even though our results do not allow us to exclude a small stimulation that might have been masked by the spontaneous rundown of ENaC activity. The same treatments produced a large activation of the chloride conductance due to expressed human CFTR, our positive control, similar to what has been described in several experimental models (Gadsby & Nairn, 1999). In addition, extracellular treatment with 100 μM cpt-cAMP consistently activated αgp-βr-γr ENaC as reported earlier, while this treatment failed to activate CFTR.

We first thought that these results may indicate that different isoforms of PKA with largely different sensibilities to various analogues of cAMP would be responsible for the activation of ENaC and CFTR: cpt-cAMP would enter the cell through the membrane and specifically activate the PKA isoform responsible for the effect on ENaC, while it would have little effect on another PKA isoform associated with CFTR. This hypothesis was not confirmed by two types of experiments. First, no significant activation of ENaC resulted from the application of cpt-cAMP around a patch pipette in the "cell attached" configuration. Second, using the "cut-open" oocyte technique which allows control of the composition on both sides of the oocyte membrane, we showed that activation of ENaC was specifically produced by extracellular but not by intracellular perfusion with cpt-cAMP. Intracellular perfusion with cAMP was very efficient to activate CFTR under these conditions.

From these experiments, we conclude that cpt-cAMP but not cAMP itself is able to activate αgp-βr-γr ENaC by some interaction with a receptor site located in the extracellular side of the oocyte membrane.

Because PKA is an intracellular enzyme, we do not expect that it is involved in this response. Indeed, intracellular perfusion with a PKA inhibitor failed to prevent the effect of extracellular cpt-cAMP, while it prevented the activation of CFTR by maneuvers known to increase intracellular cAMP. These last results differ from those reported earlier in which injection of oocytes, with high doses of the PKA inhibitor inhibited ENaC activity and prevented its activation by cpt-cAMP (Schnizler et al., 2000). It is possible that other effects of the PKA inhibitor were responsible for a strong inhibition of ENaC. For instance, the PKA inhibitor may have affected turnover processes in the oocyte membrane and produced a large decrease of ENaC surface expression. This would explain the reported slow but total downregulation of ENaC activity, because no αgp-βr-γr ENaC would be left in the membrane to be stimulated by extracellular 8-cpt-cAMP.

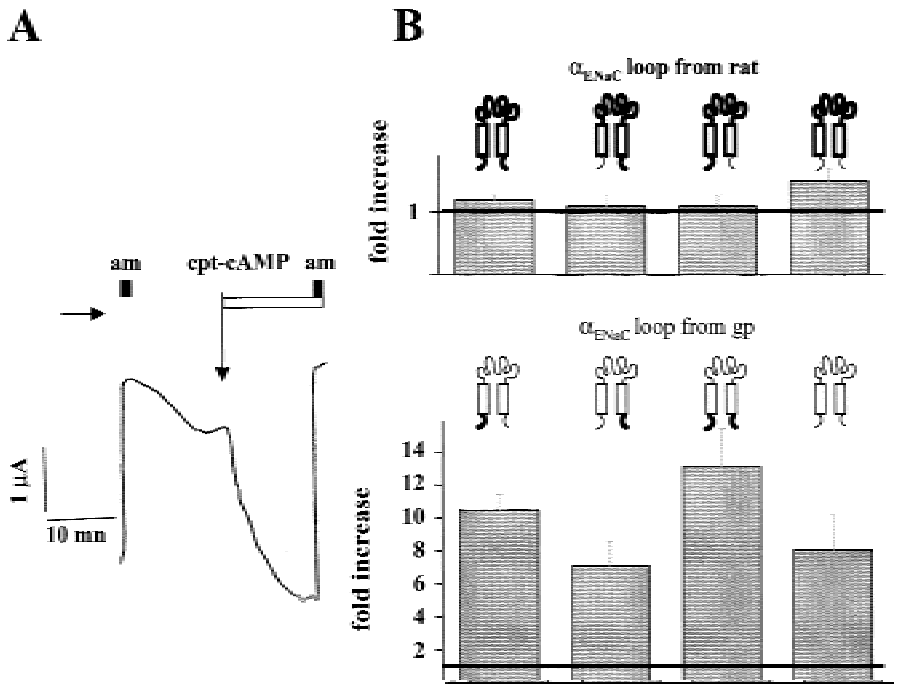

Fig. 8. Effect of cpt-cAMP on sodium channels produced by the expression of guinea pig α-subunit, rat α-subunit or guinea pig/rat chimeric α-subunit constructs. (*A*) Original current recording from an oocyte coexpressing the guinea pig ENaC α-subunit in which both the intracellular N- and C-terminal domains had been replaced by the corresponding domains of the rat ENaC subunit. The membrane potential was clamped at −60 mV. The current sensitive to 10 μM amiloride was measured before and after addition of 100 μM cAMP in combination with 1 mM IBMX to the superfusing solution. The horizontal arrow indicates the zero-current level. (*B*) Mean cpt-cAMP-induced increase in amiloride-sensitive current in the guinea pig α-, rat α- or guinea pig/rat chimeric α-subunit constructs. The thick horizontal line indicates the level of the control values. The parts of the protein belonging to the rat subunit are indicated by a thick line while the guinea pig parts are indicated by a thin line in the schemes above each column. In all cases, wild-type or chimeric α-subunits were coexpressed with wild-type rat β- and γ-subunits. All currents were measured at a holding potential of −60 mV and normalized to the current measured before cpt-cAMP treatment ($n = 3$ to 5).

In another approach to identify the domain of the αgp protein responsible for the effect of cpt-cAMP, we used rat/guinea pig chimeric constructs. Swapping either the N-terminal or the C-terminal intracellular domains between rat and guinea pig ENaC α subunit had no effect, indicating that neither the N-terminal nor the C-terminal of αgp ENaC confers the ability to be activated by cpt-cAMP. These results confirm the hypothesis that the effect of cpt-cAMP is not transmitted to ENaC by an interaction with an intracellular part of the ENaC protein.

We have therefore reached the rather surprising conclusion that cpt-cAMP, or the down stream element of the cascade responsible for its effect, interacts with an extracellular domain of ENaC. The mechanism of activation is most certainly different from that responsible for the activation of ENaC in tight epithelia, which is known to involve PKA (Garty & Palmer, 1997; Verrey, 1994).

Although these results do not help to understand the mechanism of the regulation of Na transport by the vasopressin V2 receptor and the cAMP/PKA pathway, they provide evidence for the existence of a regulatory site located in the extracellular part of the protein. Proteases are also known to activate ENaC by an interaction with the extracellular part of the protein (Chraïbi et al., 1998) and we have recently provided some evidence for the existence of another regulatory site located on the extracellular side of the channel, namely the effect of glibenclamide on ENaC activity (Chraïbi & Horisberger, 1999). Further studies using these extracellular agents may help to understand the physiological role of the large extracellular domain of ENaC and the other channels of this family.

We are grateful to Laurent Schild for reading the manuscript and providing useful suggestions and to Margret Hündt for excellent technical assistance. This work was supported by the Human Science Frontier Programm, grant RG 0261/2000 to J.-D.H. and by the Deutsche Forschungsgemeinschaft grant Cl 63/12-3 to W.C.

References

Abriel, H., Horisberger, J.-D. 1999. Feedback inhibition of amiloride-sensitive epithelial sodium channel in *Xenopus leavis* oocyte. *J. Physiol.* **516:**31–43

Awayda, M.S., Ismailov, I.I., Berdiev, B.K., Fuller, C.M., Benos, D.J.

1996. Protein kinase regulation of a cloned epithelial Na^+ channel. *J. Gen. Physiol.* **108:**49–65

Canessa, C.M., Horisberger, J.-D., Rossier, B.C. 1993. Functional cloning of the epithelial sodium channel: relation with genes involved in neurodegeneration. *Nature* **361:**467–470

Canessa, C.M., Schafer, J.A. 1992. AVP stimulates Na^+ transport in primary cultures of rabbit cortical collecting duct cells. *Am. J. Physiol.* **262:**F454–F461

Canessa, C.M., Schild, L., Buell, G., Thorens, B., Gautshi, Y., Horisberger, J.-D., Rossier, B.C. 1994. The amiloride-sensitive epithelial sodium channel is made of three homologous subunits. *Nature* **367:**463–467

Chraïbi, A., Horisberger, J.-D. 1999. Stimulation of epithelial sodium channel activity by the sulfonylurea glibenclamide. *J. Pharmacol. Exp. Ther.* **290:**341–347

Chraïbi, A., Vallet, V., Firsov, D., Kharoubi-Hess, S., Horisberger, J.-D. 1998. Protease modulation of the activity of the epithelial sodium channel expressed in *Xenopus* oocyte. *J. Gen. Physiol.* **111:**1–12

Gadsby, D.C., Nairn, A.C. 1999. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol. Rev.* **79:**S77–S107

Garty, H., Palmer, L.G. 1997. Epithelial sodium channels—function, structure, and regulation. *Physiol. Rev.* **77:**359–396

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp technique for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Krattenmacher, R., Clauss, W. 1988. Electrophysiological analysis of sodium-transport in the colon of the frog (Rana esculenta). *Pfluegers Arch.* **411:**606–612

Liebold, K.M., Reifarth, F.W., Clauss, W., Weber, W.M. 1996. cAMP-activation of amiloride-sensitive Na^+ channels from guinea-pig colon expressed in *Xenopus* oocytes. *Pfluegers Arch.* **431:**913–922

Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S., Sakmann, B. 1986. Patch-clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. *Pfluegers Arch.* **407:**577–588

Puoti, A., May, A., Canessa, C.M., Horisberger, J.-D., Schild, L., Rossier, B.C. 1995. The highly selective low conductance epithelial Na channel of *Xenopus laevis* A6 kidney cells. *Am. J. Physiol.* **38:**C188–C197

Puoti, A., May, A., Rossier, B.C., Horisberger, J.-D. 1997. Novel isoforms of the beta and gamma subunits of the *Xenopus* epithelial Na channel provide information about the amiloride binding site and extracellular sodium sensing. *Proc. Natl. Acad. Sci. U.S.A.* **94:**5949–5954

Schafer, J.A., Hawk, C.T. 1992. Regulation of Na^+ channels in the cortical collecting duct by AVP and mineralocorticoids. *Kidney Int.* **41:**255–268

Schnizler, M., Mastroberardino, L., Reifarth, F.W., Weber, W.-M., Verrey, F., Clauss, W. 2000. cAMP sensitivity conferred to the epithelial Na^+ channel by α-subunit cloned from guinea-pig colon. *Pfluegers Arch.* **439:**579–587

Shimkets, R.A., Lifton, R., Canessa, C.M. 1998. In vivo phosphorylation of the epithelial sodium channel. *Proc. Natl. Acad. Sci. USA.* **95:**3301–3305

Verrey, F. 1994. Antidiuretic hormone action in A6 cells: Effect on apical Cl and Na conductances and synergism with aldosterone for NaCl reabsorption. *J. Membrane Biol.* **138:**65–76