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Apical Cl^- Channels in A6 Cells

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Abstract. Short-circuit current (I_{sc}), transepithelial conductance (G_t), electrical capacitance (C_T) and the fluctuation in I_{sc} were analyzed in polarized epithelial cells from the distal nephron of *Xenopus laevis* (A6 cell line). Tissues were incubated with Na^+- and Cl^--free solutions on the apical surface. Basolateral perfusate was NaCl-Ringer. Agents that increase cellular cAMP evoked increases in G_t, C_T, I_{sc} and generated a Lorentzian I_{sc}-noise. The responses could be related to active, electrogenic secretion of Cl^-. Arginine-vasotocin and oxytocin caused a typical peak-plateau response pattern. Stimulation with a membrane-permeant nonhydrolyzable cAMP analogue or forskolin showed stable increases in G_t with only moderate peaking of I_{sc}. Phosphodiesterase inhibitors also stimulated Cl^- secretion with peaking responses in G_t and I_{sc}. All stimulants elicited a spontaneous Lorentzian noise, originating from the activated apical Cl^- channel, with almost identical corner frequency (40–50 Hz). Repetitive challenge with the hormones led to a refractory behavior of all parameters. Activation of the cAMP route could overcome this refractoriness. All agents caused C_T, a measure of apical membrane area, to increase in a manner roughly synchronous with G_t. These results suggest that activation of the cAMP-messenger route may, at least partly, involve exocytosis of a vesicular Cl^- channel pool. Apical flufenamate depressed Cl^- current and conductance and apparently generated blocker-noise. However, blocking kinetics extracted from noise experiments could not be reconciled with those obtained from current inhibition, suggesting the drug does not act as simple open-channel inhibitor.

Key words: cAMP — Noise analysis — Hormones — Capacitance — Exocytosis

Introduction

Many tight epithelia establish active and electrogenic Na^+ absorption via apical, amiloride-blockable Na^+ channels that belong to the ENaC gene family (Garty and Palmer, 1997). In the case where Na^+ is not exchanged for K^+ or H^+, it will be accompanied by Cl^- (and, sometimes, H_2O). The route of this Cl^- movement (para-*vs.* transcellular) has been debated. More recently, it has been recognized that agents that stimulate cytosolic cAMP content, like forskolin (De Wolf, Van Driessche & Nagel, 1989), theophylline (Katz & Van Driessche, 1988) or primary hormonal messengers like oxytocin (De Wolf et al., 1989) open apical Cl^- channels in amphibian skin. Besides being possible candidates for the absorptive Cl^- route, they turned out to be the pathway for an active, electrogenic Cl^- secretion, powered by the basolateral Na/K/2Cl-symport. This mechanism has been found not only for amphibian skin but also in other tight epithelia including colon, stomach, airways or pancreatic glands (Greger, 1996). While distal kidney tubule cells are well known to classify as ''classical'' NaCl-absorbing tissues, their role in (active) Cl^- (and, thus, Na^+-) secretion has been recognized only recently (Simmons, 1993).

The amphibian distal kidney cell line A6 is a reliable model for tight epithelia displaying active and electrogenic Na^+ absorption (Yanase & Handler, 1986; Chalfant, Coupaye-Gerard & Kleyman, 1993). When stimulated by agents that generate cAMP as second messenger, A6 cells not only show increased Na^+ absorption but also active and electrogenic Cl^- secretion (Yanase & Handler, 1986; Chalfant et al., 1993). Since this cell line consists of a homogenous population, the very same epithelial cell must possess mechanisms for separate con-

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trol of electrogenic and active Na^+ absorption as well as Cl^- secretion.

A number of Cl^- channel types have been established, among which are species regulated by voltage, transmitters, cell Ca^{2+} or cAMP (Jentsch & Gunther, 1997). The chloride channel named CFTR (cystic fibrosis transmembrane conductance regulator) belongs to the family of so-called ''ATP-binding cassette'' transporters and is gated through ATP-splitting after phosphorylation by a cAMP-dependent kinase (Jentsch & Gunther, 1997). CFTR has become prominent due to a plethora of genetic defects that give rise to the cystic fibrosis syndrome where low NaCl and water secretion by mucous epithelia produces viscous and life-threatening lumenal conditions in intestine, airways, pancreatic ducts and sweat glands. Although the CFTR protein is abundantly expressed in the kidney, cystic fibrosis does not seem to be manifest in kidney malfunction (Simmons, 1993).

A6 cells have most recently been shown to possess the CFTR protein in the apical membrane as well as in intracellular vesicles (Ling et al., 1997). When incubated with antisense-oligonucleotides, the cAMP-induced active, electrogenic Cl^- secretion is severely blunted. Patch-clamp experiments on A6 cells demonstrated two types of apical Cl^- channels which are controlled by Ca^{2+} or/and cAMP (Marunaka & Eaton, 1990). We now report that the cAMP-regulated apical Cl^- channel in A6 cells gives rise to a ''spontaneous'' Lorentzian noise in the short-circuit current across the intact monolayer. This Cl^- channel signature depends on activation by the cAMP second messenger route as suggested by the stimulatory action of oxytocin, arginine vasotocin, caffeine, forskolin and a membrane-permeant cAMP analogue. The stimulus-induced rise in tissue conductance is mirrored by a synchronous increase in epithelial electrical capacitance which points to the involvement of exocytosis in Cl^- current activation. Short-circuit current and Lorentzian noise are strictly dependent on the presence of basolateral Cl^-. In the presence of the Cl^- channel blocker, flufenamate, I_{sc} can be suppressed whereas the noise appears to result from an interaction with the channel.

Materials and Methods

Cell Culture

A6 cells obtained from Dr. J.P. Johnson (Univ. of Pittsburgh, PA) were cultured as described before (De Smet et al., 1995). Tissues were grown on permeable culture inserts (Anopore, Nunc Intermed, Roskilde, Denmark) and used after 15 to 30 days of growth (passages 103–113) in culture medium.

Electrical Measurements

Transepithelial DC-Measurements

Monolayers attached to the filter support were mounted in a modified Ussing-type chamber as already described (Smets et al., 1998) and continuously perfused on both sides. Voltage-clamp was performed with a low-noise equipment (Smets et al., 1998) using the 4-electrode constellation with KCl-agar bridges. Short-circuit current (I_{sc}) and transepithelial conductance (G_t) were registered (G_t as ratio of current deflection in response to a small voltage pulse).

Noise Analysis

The fluctuations in I_{sc} were subjected to Fourier analysis and the respective power spectra were normalized to the membrane area. Details may be found elsewhere (Smets et al., 1998). In case of the appearance of Lorentzian noise, the data were fitted by a sum of a linear background plus a single Lorentzian component, yielding as fit parameters the Lorentzian plateau value (S_o) and the corner frequency (f_c). The latter parameter ($2\pi f_c$) represents the sum of opening and closing rates for ion channel fluctuations, while S_o is a function (Van Driessche, 1994) of several parameters:

$$S_o = 2\,Mi^2\,P_o\,(1 - P_o)/\pi f_c \tag{1}$$

where M is the channel density, i the single-channel current and P_o the channel open-probability.

For channel blockers (like flufenamate, FFNM) with first-order kinetics, a Lorentzian noise in addition to that from spontaneous channel fluctuations can be obtained. The latter will, however, be much depressed in the presence of the blocker so that a sufficiently clear analysis of the former is mostly possible (Van Driessche, 1994; Smets et al., 1998). Then, the corner frequency of the blocker-induced noise should increase linearly with the drug concentration.

$$2\pi f_c = k_{on}\,[\mathrm{FFNM}] + k_{off} \tag{2}$$

where k_{on} and k_{off} are the respective rate constants for the blocker association with, and dissociation from, the open channel. Their ratio, k_{off}/k_{on}, equals the Michaelis constant of current inhibition, K_F.

Capacitance Measurements

Epithelial capacitance (Van Driessche, 1994) was measured with 5 sine waves that were repetitively imposed to the epithelium. The frequencies used for these measurements were: 2.0, 2.7, 4.1, 5.4 and 8.2 kHz. In this frequency range, the AC characteristics of the epithelium can be described by a lumped RC network consisting of the parallel arrangement of a capacitor (C_T) and a resistor (R_T) in series with a resistance (R_s) attributed to the bathing solution between the voltage electrodes. R_s was determined as the extrapolated intercept of the impedance function with the real axis in the Nyquist diagram. In this frequency range C_T equals the equivalent capacitance of the series arrangement of the apical (C_a) and basolateral (C_b) membrane:]

$$C_T = C_a/(1 + C_a/C_b) \tag{3}$$

Since the basolateral membrane capacitance in A6 epithelia is about 10–12 times larger than those of the apical membrane (Erlij, De Smet & Van Driessche, 1994), C_T is mainly determined by the apical membrane capacitance. Within this configuration, increases of C_T in the order of 10% as found in this study can only be attributed to the apical border. Within the frequency range used (2–8.2 kHz) C_T did not noticeably change with frequency. This observation validates the use of membrane capacitance as index for membrane area.

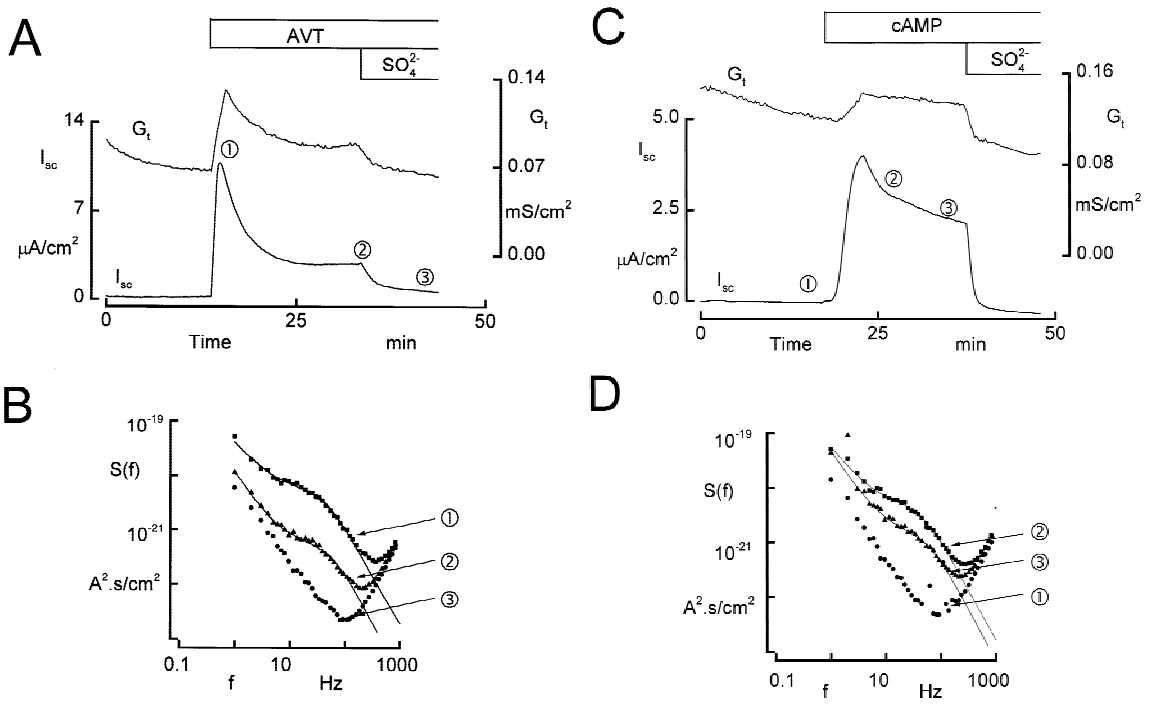

Fig. 1. Activation of Cl^- secretion by AVT or cAMP. (*A*) Time course of I_{sc} and G_t after addition of basolateral AVT (0.1 mM). At the end, basolateral NaCl was replaced by Na_2SO_4-Ringer. Apical solution: $NMDG_2SO_4$ Ringer. (*B*) Power spectra of the current noise at times indicated in *A*. Lorentzian components: ① $S_o = 4.89 \cdot 10^{-21}$ A^2 sec/cm^2, $f_c = 46.8$ Hz. ② $S_o = 0.54 \cdot 10^{-21}$ A^2 sec/cm^2, $f_c = 57.7$ Hz. (*C*) Time course of I_{sc} and G_t after addition of basolateral CPT-cAMP (1 mM). Solution conditions as in *A*. (*D*) Power spectra of the current noise at times indicated in *C*. ② $S_o = 4.69 \cdot 10^{-21}$ A^2 sec/cm^2, $f_c = 47.7$ Hz. ③ $S_o = 1.39 \cdot 10^{-21}$ A^2 sec/cm^2, $f_c = 61.4$ Hz.

Solutions and Chemicals

The basolateral solution contained (in mM 135 NaCl, 2.5 $KHCO_3$, 1 $CaCl_2$ (pH 8, 260 mOsm/kg H_2O). When Cl^- was substituted by sulfate, the osmolality was adjusted with sucrose. The apical solution was NaCl-free and contained instead 69 mM N-methyl-D-glucamine-sulfate, [$(NMDG)_2SO_4$; 180 mOsm/kg H_2O]. Inorganic chemicals were obtained from MERCK, all others were purchased from SIGMA.

Basolateral drugs: arginine vasotocin (AVT) at 100 μM; oxytocin (Oxy) at 0.1 I.U./ml; caffeine (Caf) at 1 or 10 mM; forskolin (Fsk) at 5 μM; 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) at 1 mM. Apical drug: flufenamate (FFNM; stock solution in dimethyl-sulfoxide) in concentrations up to 150 μM.

Statistics

For pooled data, mean values (±SEM) were calculated for n independent experiments, i.e., different tissues. Statistical significance was evaluated using Student's *t*-test.

Results

Hormonal Stimulations of Short-Circuit Current, Transepithelial Conductance and Noise

It had been reported (Yanase & Handler, 1986; Chalfant et al., 1993) that, in the presence of NaCl-Ringer on both sides, A6-cell treatment with antidiuretic hormones, or their major second messenger, cAMP, elicited a biphasic response in short-circuit current: a very fast initial peak was followed by a current decline which subsequently developed into a slower increase. The initial transient phase was shown (Yanase & Handler, 1986) to represent active, electrogenic Cl^- secretion whereas the slower increase was due to a rise in active Na^+ uptake. In the absence of apical NaCl (replaced by $(NMDG)_2SO_4$), the addition of the ''natural'' amphibian antidiuretic hormone, arginine vasotocin (AVT), to the basolateral NaCl-Ringer (Fig. 1*A*) caused I_{sc} to rise rapidly from zero to a high peak, which was followed within a few minutes by a sharp and more than 70% decline to a plateau. This I_{sc} was abolished when basolateral Cl^- was replaced by sulfate. G_t behaved in a roughly similar way, although clearly slower in uprise and decay. Current noise analysis revealed at both, peak and steady-state of the current, a ''spontaneous'' Lorentzian noise component the plateau of which closely followed the I_{sc} magnitude and vanished after Cl^- withdrawal (Fig. 1*B*). Mean values for I_{sc}, G_t, S_o (Lorentzian plateau value) and f_c (corner frequency) at the respective positions 1 and 2 as indicated by numbers in Fig. 1*A* are listed in Table 1. Washing out the hormone in presence of basolateral chloride abolished the Cl^--related parameters in I_{sc}, G_t or

Table 1. Cl^- secretion stimulated by AVT, CPT-cAMP, forskolin and caffeine

		I_{sc} ($\mu A/cm^2$)	G_t (mS/cm^2)	$S_o \cdot 10^{21}$ (A^2 sec/cm^2)	f_c (Hz)	n
AVT	Peak	8.00 ± 0.81	0.16 ± 0.02	2.26 ± 0.56	49.50 ± 2.17	6
	Steady state	2.43 ± 0.36	0.11 ± 0.02	0.52 ± 0.06	52.33 ± 1.58	
CPT-cAMP	Peak	6.30 ± 0.88	0.15 ± 0.01	2.31 ± 0.53	51.50 ± 1.34	6
	Steady state	5.30 ± 0.78	0.14 ± 0.01	1.01 ± 0.13	60.50 ± 2.47	
Fsk	Peak	6.35 ± 0.50	0.10 ± 0.02	2.71 ± 0.33	41.55 ± 2.06	7
	Steady state	5.48 ± 0.59	0.12 ± 0.02	0.99 ± 0.10	51.18 ± 1.29	
Caffeine (1 mM)	Peak = Steady state	1.03 ± 0.15	0.09 ± 0.01	0.94 ± 0.43	46.15 ± 3.96	5
Caffeine (10 mM)	Peak	5.03 ± 0.77	0.11 ± 0.02	1.33 ± 0.51	63.12 ± 3.32	5
	Steady state	2.04 ± 0.36	0.11 ± 0.02	0.36 ± 0.08	70.85 ± 4.92	

Mean values ± SEM for peak and steady-state values of I_{sc}, G_t and the Lorentzian parameters S_o and f_c.

the current noise within a few minutes. With oxytocin as stimulant, we generally obtained a similar picture (*see* for instance Fig. 3 and Table 2). It was a general finding that the I_{sc} peak heights obtained with the hormones at the defined concentrations (AVT: 0.1 μM; oxytocin: 0.1 I.U./ml) were quite similar whereas the plateau value was lower in case of AVT as compared to oxytocin. Like with AVT, all Cl^--related signals induced by oxytocin disappeared upon withdrawal of basolateral Cl^-.

Raising the Second Messenger cAMP: Short-Circuit Current, Transepithelial Conductance and Current Noise

For a variety of tight epithelia it has been suggested that antidiuretic hormones act via a G-protein-coupled rise of cAMP after activation of adenylate cyclase. There is also evidence that this type of stimulation elicits apical exocytosis (Garty & Palmer, 1997) which mostly requires an increase in cytoplasmic Ca^{2+} (Ca_c) (Burgoyne & Morgan, 1993). So, our next attempt was to try to mimic the action of AVT and oxytocin by either directly activating the adenylate cyclase using forskolin, or by applying a membrane-permeant nonhydrolyzable cAMP analogue (CPT-cAMP), or else, by preventing the destruction of endogenous cAMP using phosphodiesterase inhibitors.

With CPT-cAMP (1 mM), the current peaked as with the hormones (but at lower level) and the peak-to-plateau ratio is estimated to 1.6 (Fig. 1*C*). Most remarkable, however, was that G_t did not peak but remained high as long as basolateral Cl^- was present. The averaged parameters for this type of experiment are given in Table 1. cAMP generated also a Lorentzian noise (Fig. 1*D*) whose magnitude was comparable to that obtained with AVT although the I_{sc} response with cAMP is much smaller. However, since dose-response relationships were not systematically established for the different stimulators, a strict quantitative comparison of e.g., AVT with cAMP may be premature.

We also tested the effect of forskolin, and the behavior of I_{sc}, G_t (Fig. 2*A*) and current noise (Table 1) after stimulation was comparable to that obtained with cAMP. Forskolin or cAMP seem to work synergically and each may be used alternatively. Further addition of the phosphodiesterase blocker 3-isobutyl-1-methylxanthine (IBMX) to the forskolin-containing solution did not augment the response (*not shown*).

Also the naturally occurring diuretics, theophylline and caffeine, are known to inhibit the phosphodiesterase and are therefore thought to maximally raise the cytosolic cAMP content. Figure 2*B* and *C* illustrates caffeine as representative case. While it may be anticipated that a higher dose of stimulant augments the responses of conductance, current and Lorentzian magnitude, the finding of a clear and considerable upwards shift in f_c (Fig. 2*C*) was unexpected. One mM caffeine led to only a small I_{sc} peaking and an equally small increase in G_t (Fig. 2*B*) although a Lorentzian noise was clearly obtained (Fig. 2*C*). Caffeine washout quickly resulted in a decrease of I_{sc} and G_t to the levels before the addition of the stimulant, while the Lorentzian noise component disappeared. A 10-fold higher caffeine concentration in the same tissue not only produced larger responses in current and conductance but also a very marked and rapidly attained peak followed by a sharp decay, much reminding the I_{sc} time course obtained with AVT. In contrast, the final Lorentzian noise was somewhat (Fig. 2*C*) or markedly (Table 1), but always smaller at 10 mM than at 1 mM caffeine. Considering the inverse relationship between S_o and f_c (Eq. 1), this could be related to the increased corner frequency recorded at 10 mM caffeine. Similar observations (*not shown here*) were made with theophylline as stimulant.

Repetitive Stimulation with Hormone: Refractory Responses

The reversal of Cl^- secretion after removal of the stimulants, shown e.g., in Fig. 2*B*, suggests fast and complete

Table 2. Parameters changes at different periods of repetitive stimulation with oxytocin

Stimul. period		I_{sc} ($\mu A/cm^2$)	G_t (mS/cm^2)	$S_o \cdot 10^{21}$ (A^2 sec/cm^2)	f_c (Hz)	n
1	Peak	8.00 ± 0.81	0.16 ± 0.03	3.12 ± 0.46	46.70 ± 2.03	6
	Steady state	3.55 ± 0.45	0.13 ± 0.02	0.84 ± 0.09	53.53 ± 3.27	
2	Peak	4.32 ± 0.67	0.12 ± 0.02	1.68 ± 0.29	46.12 ± 2.13	
	Steady state	2.83 ± 0.41	0.11 ± 0.02	0.73 ± 0.06	51.10 ± 1.36	
3	Peak	2.63 ± 0.51	0.10 ± 0.01	1.51 ± 0.19	41.05 ± 1.74	
	Steady state	2.19 ± 0.33	0.10 ± 0.01	0.72 ± 0.08	48.87 ± 2.10	
4	Peak	1.70 ± 0.44	0.10 ± 0.01	1.20 ± 0.22	38.57 ± 2.10	
	Steady state	1.45 ± 0.34	0.11 ± 0.02	0.62 ± 0.06	45.66 ± 1.86	

Mean values ± SEM for peak and steady-state values of I_{sc}, G_t and the Lorentzian parameters S_o and f_c.

recovery from stimulant-induced parameter changes. However, repetitive hormonal stimulation (Fig. 3) interrupted by a washout period of 10 min, demonstrated a general refractory behavior. Interestingly, the peak-to-plateau ratios do not appear to change much throughout the series of stimulations. After four repetitive stimulations, even an increased washout time of about 1 hr leads to no recovery from this refractory behavior. For 6 tissues, the data from repetitive stimulation have been pooled in Table 2.

A dramatic and unexpected result was obtained (Fig. 4) when, after repetitive stimulation with oxytocin and fully developed refractory behavior, additional forskolin was given after the fourth challenge with the hormone: the almost abolished response to oxytocin could be completely overcome, and the current height obtained in presence of forskolin was similar to the one attained with the first hormonal challenge. G_t behaved identically; opposite to previous findings (Fig. 2), the current showed no clear peak with forskolin. Also, the Lorentzian plateau in the presence of forskolin was markedly augmented while f_c remained unchanged. This experiment is representative for a total of seven tissues treated the same way (*see* Table 3).

Monitoring the Electrical Capacitance During Cl^- Secretion Stimulation

Measurement of membrane capacitance is a convenient approach (Van Driessche, 1994) to correlate changes in membrane surface area with alterations in membrane conductance, e.g., evoked by endo-/exocytosis of ion channel- or ion pump-containing vesicles. Figure 5 depicts the simultaneous recording of I_{sc}, G_t and transepithelial capacitance (C_T). Starting from a value of 0.83 $\mu F/cm^2$ in standard nonsecreting conditions, addition of AVT (Fig. 5*A*) caused an immediate rise in C_T by about 8% with, however, a delay in reaching the maximum when compared to the maxima of I_{sc} and G_t. Washing out the stimulant led to a fast restoring of I_{sc} and G_t to their previous values before stimulation. Likewise, C_T started to decrease but at a slower pace. With forskolin as stimulant (Fig. 5*B*) we obtained roughly the same feature however, without the pronounced current and conductance peaks. Also here, the G_t increase (about 70%) and the C_T increase (about 8%) occurred simultaneously, and both showed no peaking. The forskolin washout has features comparable to the washout of AVT. Pooled data in Table 4 show that AVT and forskolin augmented C_T significantly ($P < 0.05$) by 8.8 and 9.4%, respectively. Thus, it is tempting to assume that an apical membrane area change, as documented in each experiment by the parallel increase of conductance and capacitance, is a major factor in the Cl^- current responses to stimulants of even a different nature.

Cl^- Channel Blockers

5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; *see* Ling et al., 1997; Niisato & Marunaka, 1997) and mefenamate (De Smet & Van Driessche, 1992) dose dependently abolish the stimulant-induced Cl^- secretion by A6 cells. Here, we report experiments using another Cl^- channel blocker, flufenamate (FFNM) (White & Aylwin, 1990). Figure 6*A* shows one of 7 experiments (summarized in Table 5) where, after stimulation with oxytocin and reaching the I_{sc} plateau value, apical addition of flufenamate and successive increments of its concentration reduced I_{sc} and G_t in a dose-dependent and quickly reversible manner. 50% current reduction was achieved with 75 μM, full current suppression with 150 μM FFNM. Omission of the blocker caused a quick return of I_{sc}, G_t and the Lorentzian plateau to their former values in the absence of the drug. Since FFNM is a membrane-permeant substance, an intracellular location of its site of action may be considered. Current-noise analysis revealed that the spectrum was still satisfactorily fittable with a single Lorentzian (plus linear background). In the presence of the Cl^- channel blocker, both S_o as well as f_c were higher; with rising drug concentration, the former increased transiently (Table 5), the latter continuously and linearly (Table 5; Fig. 6*B*). According to Eq. 2, this

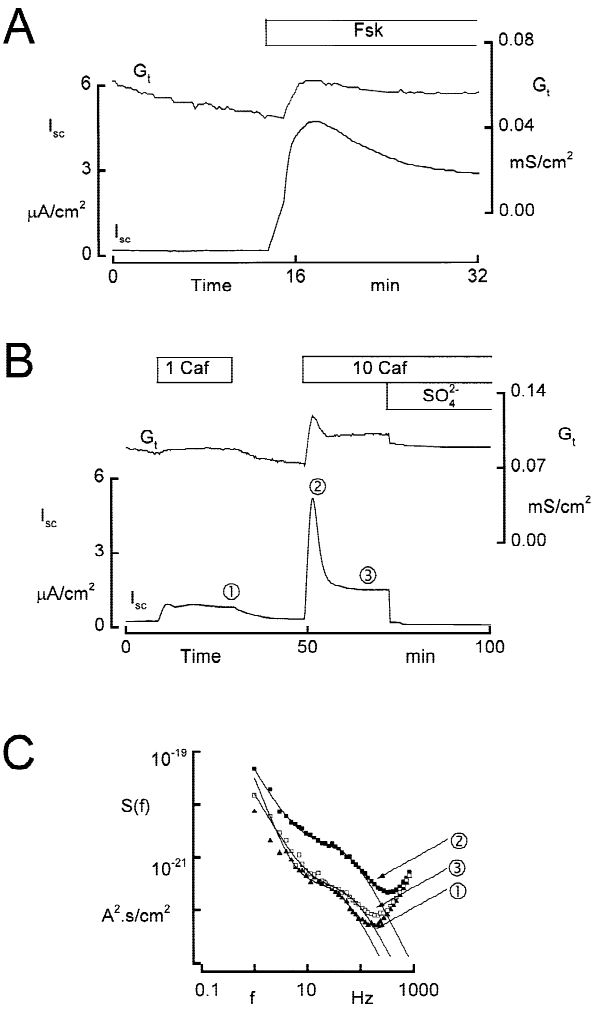

Fig. 2. Activation of Cl^- secretion by forskolin or by caffeine. (*A*) Time course of I_{sc} and G_t after basolateral addition of forskolin (Fsk) (5 μM). Solution conditions as in Fig. 1. (*B*) Time course of I_{sc} and G_t after basolateral addition of 1 or 10 mM caffeine (Caf). Solution conditions as in Fig. 1. (*C*) Power spectra of the current noise at times indicated in *B*. Lorentzian components: ① $S_o = 0.32 \cdot 10^{-21}$ A^2 sec/cm², $f_c = 46.7$ Hz. ② $S_o = 1.70 \cdot 10^{-21}$ A^2 sec/cm², $f_c = 68.0$ Hz. ③ $S_o = 0.26 \cdot 10^{-21}$ A^2 sec/cm², $f_c = 81.6$ Hz.

could be interpreted as the fingerprint of blocker-induced channel noise (Van Driessche, 1994). For a pool of 3–7 tissues that were studied with flufenamate, the mean f_c of the spontaneous Lorentzian noise at zero [FFNM], was clearly — although not much — lower (10 to 20%) than the extrapolated straight line ordinate intercept. For a first-order channel block, the ratio of ordinate intersection and slope in the f_c-[FFNM] graph should equal K_F, the drug concentration for halfmaximal current inhibition (*cf.* Materials and Methods). From the fluctuation kinetics of 7 tissues, we obtain a K_F of 269.0 ± 36.4 μM which is, however, 4 times higher than the value found from the current inhibition, 67.6 ± 7.8 μM, thus in clear contrast to theory.

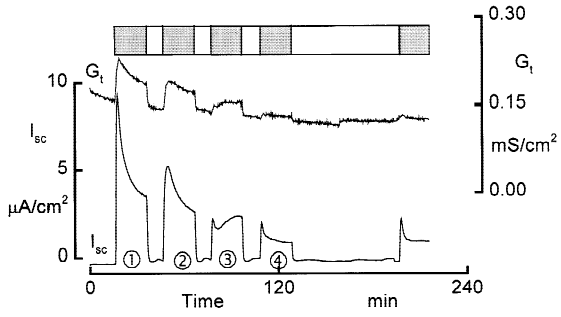

Fig. 3. Repetitive addition and removal of oxytocin to the basolateral bath. Time course of I_{sc} and G_t. Periods of repetitive addition of the hormone (0.1 I.U./ml) are indicated by numbers and shaded bars.

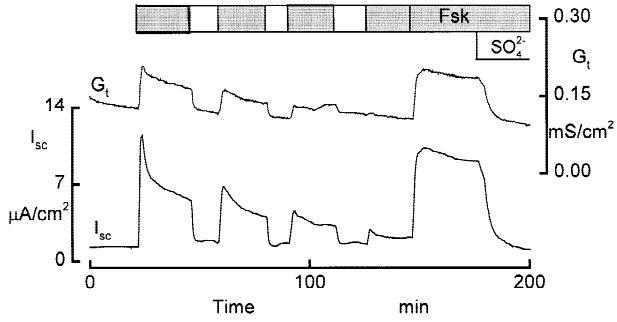

Fig. 4. Repetitive addition and removal of oxytocin and subsequent forskolin. Time course of I_{sc} and G_t during serial application of 0.1 I.U./ml oxytocin (shaded bar) followed by addition of 5 μM forskolin to the hormone-containing bath. Other conditions like in Fig. 1.

Most remarkably, it should be stressed that linear extrapolation of the f_c-[FFNM] relationship to zero drug concentration (i.e., the situation where only the spontaneous channel noise will be visible) gave, for two out of seven tissues, exactly the same f_c which had been obtained for the ''spontaneous'' Lorentzian noise before the use of the blocker. This, of course, could be fortuitous. It might be, on the other hand, that the linear f_c increase with increasing FFNM concentration may in this case not be indicative of a first-order channel block but rather represents a shift of the ''spontaneous'' channel conductance fluctuations to higher frequencies.

Discussion

Cl^- Channel Properties

As can be seen in the present figures, the transepithelial conductance is usually small and the short-circuit current is close to or equals zero in the absence of stimulators like hormones, indicating a negligible passive ion permeation through the tight junctions under conditions of asymmetrical solutions. Most recently, Ling et al. (1997) showed the apical route for Cl^- secretion by

Table 3. Parameter changes from last repetitive stimulation with oxytocin and subsequent addition of forskolin

	I_{sc} ($\mu A/cm^2$)	G_t (mS/cm^2)	$S_o \cdot 10^{21}$ (A^2 sec/cm^2)	f_c (Hz)	n
During last oxytocin stimulation	2.55 ± 0.30	0.11 ± 0.02	0.62 ± 0.09	47.89 ± 4.04	7
Addition of forskolin	7.42 ± 0.49	0.16 ± 0.02	0.95 ± 0.11	52.78 ± 2.65	

Mean values ± SEM for steady-state values of I_{sc}, G_t and the Lorentzian parameters S_o and f_c.

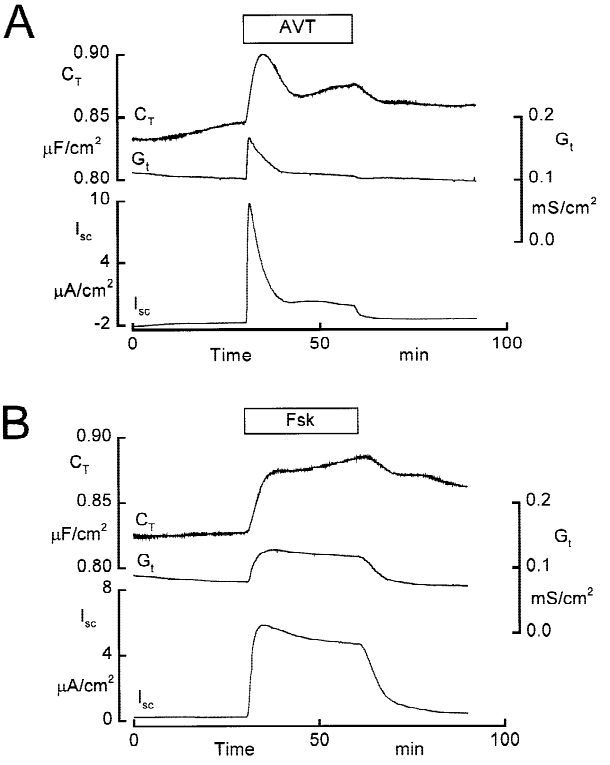

Fig. 5. Time course of C_T, I_{sc} and G_t during stimulation with AVT or forskolin. (*A*) Effect of stimulation by AVT (0.1 mM). Other conditions like in Fig. 1. (*B*) Effect of stimulation by forskolin (5 μM).

A6 cells to be CFTR and to be stimulatable e.g., by forskolin. Our experiments were performed with apical $(NMDG)_2SO_4$ and basolateral NaCl Ringer in order to avoid current- and noise interference from Na^+ transport, and to maximize serosa to mucosa Cl^- flux. Under such conditions, the noise spectrum seems to be fitted satisfactorily by the sum of a linear background plus one Lorentzian noise component which is consistent with the assumption of a single Cl^- channel type. For different stimuli, the ''fingerprint'' f_c values were quite comparable. However, we have to notice a tendency (*see* figures and tables) for a somehow inverse relationship between f_c and current magnitude, as obvious from peak-plateau time courses, but especially in the experiments with rising caffeine doses.

Of the membranes in question, Lorentzian noise will be prominent from that membrane which has the highest resistance (Van Driessche & Gögelein, 1980; Van Driessche, 1994). In A6 cells (Grantizer et al., 1991) as in most epithelia, this is the apical membrane. Here, regulatory rate-limiting transport mechanisms should be expected. Just like high resistances in series with the noise source, also low resistances in parallel will tend to attenuate the fluctuating Lorentzian signal (Van Driessche & Gögelein, 1980). In preliminary experiments (*unpublished*) we introduced such parallel low-resistance apical pathways by creating poorly selective ion pores through apical incorporation of the antibiotic amphotericin B. Indeed, as predicted for an apical noise source, its magnitude was attenuated to such an extent that it merged with the background noise.

Generally, the Lorentzian plateaus seem to follow the time course of he current but it must be kept in mind that the former parameter is not only reflecting the net apical current but rather depends on the square of the single-channel current as well as on the channel fluctuation kinetics (*cf.* Eq. 1). This allows only a semiquantitative comparison of conductance, current and current-noise parameters. Unfortunately, no detailed information is presently available about the fluctuation kinetics as well as about the time- and stimulant-specific changes in the driving forces, so further analyses of the measured parameters (e.g., in terms of single-channel current- or conductance, or channel density) are currently impossible.

The fluctuations as observed in apical patches containing CFTR (Schultz et al., 1996; Ling et al., 1997) are quite slow, around a few Hz. However, fast flickering of

Table 4. Transepithelial capacitance (C_T) before or after stimulation of Cl^- secretion

	C_T ($\mu F/cm^2$) before stimulation	C_T ($\mu F/cm^2$) after stimulation	n
AVT	0.80 ± 0.02	0.87 ± 0.03	3
Forskolin	0.85 ± 0.04	0.93 ± 0.04	6

Mean values ± SEM of C_T before and after stimulation of Cl^- secretion with AVT and forskolin. Paired Student's *t*-test was used to determine the significance of difference between the control and experimental periods: data are significantly different with $P < 0.05$.

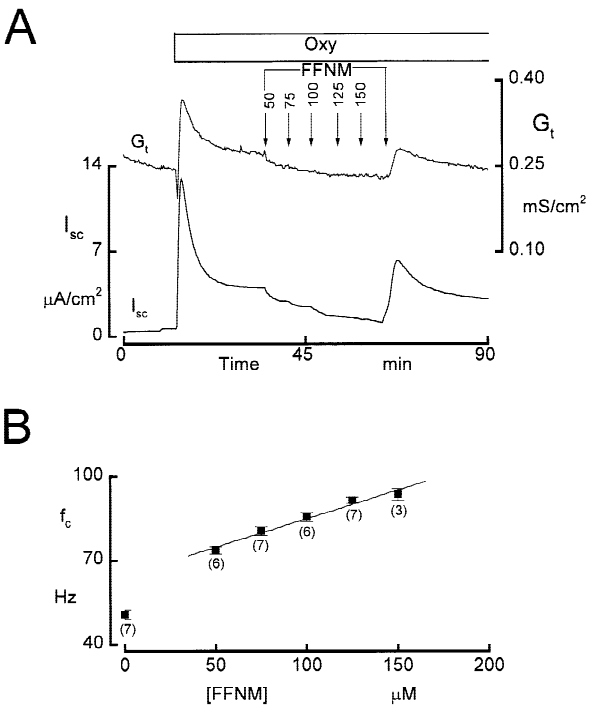

Fig. 6. Inhibitory effect of apical flufenamate (FFNM) on electrical parameters after stimulation by oxytocin. (*A*) Time course of I_{sc} and G_t for Cl^- secretion stimulation by oxytocin (Oxy; 0.1 I.U./ml). Flufenamate (FFNM) concentrations are given in μM. Other conditions as in Fig. 1. (*B*) Pooled diagram of linear relationship between corner frequency in the presence of different concentrations of flufenamate, and comparison to the ''spontaneous'' f_c before addition of the drug. In parentheses number of tissues. Mean values of f_c ± SEM.

the non-blocked channel has been described most recently (Larsen et al., 1996) with a fluctuation frequency in a range of that described in the present paper. Our method cannot clearly distinguish a low-frequency Lorentzian from the large linear background noise so that we will probably miss the expected, slowly fluctuating Lorentzian component in the power spectrum of our Cl^- current noise. The f_c range as observed in our experiments deserves special attention: (i) as mentioned above, a ''systematic'' inverse relationship between I_{sc} and f_c seems to exist; (ii) f_c increased with the caffeine as well as with the theophylline concentration; (iii) a clearly discriminating point is hard to make whether the shift of f_c in the presence of flufenamate represents ''true'' blocker kinetics of merely a drug-dependent acceleration of the spontaneous fluctuations. One possible explanation for these difficult to interpret findings could be that the ''spontaneous'' f_c is voltage-dependent. For instance, membrane potential changes after apical Cl^- channel block may be expected, or voltage shifts may underlie the peak-to-plateau current transients.

Despite the primary assumption of the stimulants caffeine and theophylline to be phosphodiesterase inhibitors, additional messenger pathways cannot be excluded (Fredholm, 1995), so the finding that a tenfold change in their concentration shifts the spontaneous Lorentzian to higher frequencies by doubling the f_c may indicate that additional intracellular factors (other than cAMP) influence the channel fluctuation. f_c changed also linearly with increasing concentrations of the Cl^- channel blocker flufenamate but, despite this normally typical sign for a first-order rate channel blockade (Van Driessche, 1994), the blocker inhibition constants derived from current- and from noise kinetics, respectively, differ by a factor of four. Moreover, and contrary to other findings obtained with high-rate blocker noise on CFTR (Schultz et al., 1996), f_c at the lowest blocker concentrations is in our case quite close to that recorded in the absence of flufenamate, and the case cannot convincingly be made that f_cs in the absence and in the presence of the blocker are of distinct nature. In this context, it is important to recall that protonated FFNM and other diphenylamine carboxylates are quite membrane-permeant, so a cytosol acidification could occur after membrane passage (Brown & Dudley, 1996). Since this is known to impair epithelial K^+-permeable channels (Harvey, 1995) a so-obtained depolarization of the cell could reduce the Cl^- current and noise, which would then be misinterpreted as a direct chloride channel block by FFNM.

Many apical Cl^- channels including the one discussed here have been shown to be subject to regulation by cAMP which, after more differential analysis, classified them as CFTR-type channels (Cliff & Frizzell, 1990; Frizzell & Morris, 1994). In A6 cells, CFTR has been located (Ling et al., 1997) in intracellular vesicles as well as in the apical membrane. Treatment with antisense oligonucleotide reduced the allover current response to forskolin, the immunologically detectable apical CFTR expression as well as the Cl^- channel activity at the single channel level (Ling et al., 1997). P_o became virtually zero. This would be consistent with a role of vesicle shuttling in the response to the stimulant. Cell-attached patch clamp experiments have shown that the 8pS (CFTR) apical A6 Cl^- channel (Ling et al., 1997; Marunaka & Eaton, 1990) is blocked by NPPB. It was further demonstrated (Shintani & Marunaka, 1996) that cAMP left the open probability of the 8pS channel unaffected at 0.4. On the other hand, the channel number per patch was augmented 6-fold. Only the channel number decreased with brefeldin which points to a role of cAMP in Cl^- channel exocytosis.

Within a given experimental and analytical frame, capacitance measurements will reflect cell membrane surfaces (Van Driessche, 1994). Their series arrangement as in epithelia dictates that the smallest area, i.e., the apical membrane, determines the entire capacitance signal. cAMP as well as cell Ca^{2+} (Burgoyne & Morgan, 1993), following stimulation by secretagogues, will con-

Table 5. Parameter changes with 75 and 125 μM FFNM after stimulation with oxytocin

FFNM (μM)	I_{sc} ($\mu A/cm^2$)	G_t (mS/cm^2)	$S_o \cdot 10^{21}$ ($A^2 sec/cm^2$)	f_c (Hz)	n
0	2.84 ± 0.29	0.18 ± 0.03	3.67 ± 0.30	50.57 ± 1.22	7
75	1.76 ± 0.20	0.16 ± 0.03	4.82 ± 0.75	81.08 ± 1.85	
125	1.49 ± 0.30	0.16 ± 0.03	4.07 ± 0.68	91.63 ± 1.40	

Mean values ± SEM of I_{sc}, G_t and the Lorentzian parameters S_o and f_c for different concentrations of FFNM after stimulation with oxytocin.

tribute to rate-limiting mechanisms that evoke exocytosis and therefore an increase in C_T like it has been shown for similar processes of Cl^- secretion where CFTR is involved (Greger et al., 1996; Takahashi et al., 1996). In our experiments using forskolin the increases of total conductance and capacitance occurred with about the same time course which was, however, somewhat slower than the development of the peak current, a phenomenon ascribed further below to the possible dominance of the driving force in generating the peak. But no such correlation could recently be found in CHO cells overexpressing CFTR (Hug et al., 1997). In the case of AVT, a synchrony of G_t and C_T appears to exist only in the very initial phase of stimulation. The peak phase in both, G_t and current, precedes the peak in C_T which increases monotonically just as observed with forskolin. Therefore, the initial increase in G_t and I_{sc} may, at least partly, reflect the activation of previously silent apical Cl^- channels, whereas C_T rather monitors subsequent exocytosis and activation of a further CFTR population.

Which Second Messenger Route?

Equally complicated may be the answer which second messenger has a dominant function at which point in time. The case seems clear for forskolin and cAMP stimulation; however, a number of reports (Perez-Reyes et al., 1994; Yuan & Bers, 1995) have appeared lately that indicate that cAMP, also in A6 cells (Niisato & Marunaka, 1997), might generate a Ca^{2+}-influx. Equally, the Na^+ uptake-stimulating antidiuretic hormone (Hayslett et al., 1995) was reported to raise cell Ca^{2+} as well as cAMP. At the same time interference with the cAMP pathways had no effect on Na^+ uptake whereas impairment of the Ca_c reaction chain had.

Methylated xanthines (caffeine, theophylline, IBMX) have given evidence (Fredholm, 1995) to stimulate purinergic receptors which would possibly qualify them as agents that rise cell Ca^{2+}, like antidiuretic hormone or ATP (Mori et al., 1996), in addition to their effect on the cAMP system. As putative link between Ca^{2+} and cAMP it has been proposed (Shintani & Marunaka, 1996; Niisato & Marunaka, 1997) that cAMP opens Ca^{2+} channels via protein kinase A (PKA) phosphorylation which could provide Ca^{2+} even in the absence of the vesicular Ca^{2+}-mobilizing IP_3 pathway: consistently, current peaking was much reduced by PKA inhibitors, and treatment with the Ca^{2+} ionophore ionomycin resulted in a sharp current peak that decayed to zero within a short time. However, this view completely ignores an involvement of exocytosis. In fact, a recent study in the rectal gland (Warth et al., 1998) provided evidence that cAMP enhances Ca^{2+} influx but not Ca^{2+} release.

The Stimulus-Induced Current Transient

In the case of stimulation by hormones but also by caffeine, I_{sc} rises and also decays faster as the corresponding time course of the conductance. This may suggest a major and rapid contribution of the driving force to the shape of the current response. However, treatment with forskolin or cAMP rather gives the impression that the development in time of conductance and current is more congruent. It may be speculated that hormones and caffeine — contrary to cAMP and forskolin — only transiently increase apical Cl^- conductance. Following the speculation of others (Niisato & Marunaka, 1997) Ca^{2+} is involved in the generation of the I_{sc} peak after Cl^- secretion stimulation. The subsequent decrease in driving force (I_{sc} decay) seems to be common to most stimulators and may be due to a change of the cell potential at short-circuit that could predominantly reflect — as in other Cl^--secreting systems (Greger et al., 1997) — the activity of a K^+ conductance.

Our results suggest initial effects (stimulating and then inhibiting) on the apical Cl^- conductance and possibly on the establishment of an electrical driving force (K^+-permeable channels) whereas the dominant long-term effect of cAMP seems to be the creation of a constant apical conductance and provides stable conditions for efficient Cl^- secretion. Most recently, we found strong evidence (Zeiske, Atia & Van Driessche, 1998) [and manuscript in preparation] that vesicular Ca^{2+}, released by extracellular ATP, generates sharply peaking CFTR channel current and -noise in A6 cells, which will put this inorganic ion into focus as second messenger in addition to cAMP.

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References

Brown, C.D., Dudley, A.J. 1996. Chloride channel blockers decrease intracellular pH in cultured renal epithelial LLC-PK1 cells. *Br. J. Pharmacol.* **118:**443–444

Burgoyne, R.D., Morgan, A. 1993. Regulated Exocytosis. *Biochem. J.* **293:**305–316

Chalfant, M.L., Coupaye-Gerard, B., Kleyman, T.R. 1993. Distinct regulation of Na^+ reabsorption and Cl^- secretion by arginine vasopressin in the amphibian cell line A6. *Am. J. Physiol.* **264:**C1480–1488

Cliff, W.H., Frizzell, R.A. 1990. Separate Cl^- conductances activated by cAMP and Ca^{2+} in Cl^--secreting epithelial cells. *Proc. Natl. Acad. Sci. USA* **87:**4956–4960

De Smet, P., Simaels, J., De Clercq, P.E., Van Driessche, W. 1995. Regulatory volume decrease in cultured kidney cells (A6): Role of amino acids. *J. Gen. Physiol.* **106:**525–542

De Smet, P., Van Driessche, W. 1992. Inhibition of Cl^- currents in A6 epithelia by mefenamic acid. *Arch. Int. Pharmacodyn. Ther.* **315:**123–124

De Wolf, I., Van Driessche, W., Nagel, W. 1989. Forskolin activated gated Cl^- channels in frog skin. *Am. J. Physiol.* **256:**C1239–C1249

Erlij, D., DeSmet, P., Van Driessche, W. 1994. Effect of insulin on area and Na^+ channel density of apical membrane of cultured toad kidney cells. *J. Physiol.* **481:**533–542

Fredholm, B.B. 1995. Astra Award Lecture. Adenosine, adenosine receptors and the actions of caffeine. *Pharmacol. Toxicol.* **76:**93–101

Frizzell, R.A., Morris, A.P. 1994. Chloride conductances of salt-secreting epithelial cells. *Curr. Top. Membr. Transp.* **42:**173–215

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

Granitzer, M., Leal, T., Nagel, W., Crabbé, J. 1991. Apical and basolateral conductance in cultured A6 cells. *Pfluegers Arch.* **417:**463–468

Greger, R. 1996. The membrane transporters regulating epithelial NaCl secretion. *Pfluegers Arch.* **432:**579–588

Greger, R., Mall, M., Bleich, M., Ecke, D., Warth, R., Riedemann, N., Kunzelmann, K. 1997. Regulation of epithelial ion channels by the cystic fibrosis transmembrane conductance regulator. *J. Mol. Med.* **74:**527–534

Greger, R., Bleich, M., Van Driessche, W., Ecke, D., Warth, R. 1996. The role of K^+ channels in colonic Cl^- secretion. *Comp. Biochem. Physiol.* **118A:**271–275

Harvey, B.J. 1995. Cross-talk between sodium and potassium channels in tight epithelia. *Kidney Int.* **48:**1191–1199

Hayslett, J.P., Macala, L.J., Smallwood, J.I., Kalghatgi, L., Gassala-herraiz, J., Isales, C. 1995. Vasopressin-stimulated electrogenic sodium transport in A6 cells is linked to a Ca^{2+}-mobilizing signal mechanism. *J. Biol. Chem.* **270:**16082–16088

Hug, M.J., Thiele, I.E., Greger, R. 1997. The role of exocytosis in the activation of the chloride conductance in Chinese hamster ovary cells (CHO) stably expressing CFTR. *Pfluegers Arch.* **434:**779–784

Jentsch, T.J., Gunther, W. 1997. Chloride channels: an emerging molecular picture. *Bioessays* **19:**117–126

Katz, U., Van Driessche, W. 1988. Effect of theophylline on the apical sodium and chloride permeabilities of amphibian skin. *J. Physiol.* **397:**223–236

Larsen, E.H., Price, E.M., Gabriel, S.E., Stutts, M.J., Boucher, R.C. 1996. Clusters of Cl^- channels in CFTR-expressing Sf9 cells switch spontaneously between slow and fast gating modes. *Pfluegers Arch.* **432:**528–537

Ling, B.N., Zuckerman, J.B., Lin, C., Harte, B.J., McNulty, K.A., Smith, P.R., Gomez, L.M., Worrell, R.T., Eaton, D.C., Kleyman, T.R. 1997. Expression of the cystic fibrosis phenotype in a renal amphibian epithelial cell line. *J. Biol. Chem.* **272:**594–600

Marunaka, Y., Eaton, D.C. 1990. Chloride channels in the apical membrane of a distal nephron A6 cell line. *Am. J. Physiol.* **258:**C352–C368

Mori, M., Nishizaki, T., Kawahara, K., Okada, Y. 1996. ATP-activated cation conductance in a *Xenopus* renal epithelial cell line. *J. Physiol.* **491:**281–290

Niisato, N., Marunaka, Y. 1997. Regulation of Cl^- transport by IBMX in renal A6 epithelium. *Pfluegers Arch.* **434:**227–233

Perez-Reyes, E., Yuan, W., Wei, X., Bers, D.M. 1994. Regulation of the cloned L-type cardiac calcium channel by cyclic-AMP-dependent protein kinase. *FEBS Lett.* **342:**119–123

Schultz, B.D., DeRoos, A.D., Venglarik, C.J., Singh, A.K., Frizzell, R.A., Bridges, R.J. 1996. Glibenclamide blockade of CFTR chloride channels. *Am. J. Physiol.* **271:**L192–L200

Shintani, Y., Marunaka, Y. 1996. Regulation of chloride channel trafficking by cyclic AMP via protein kinase A-independent pathway in A6 renal epithelial cells. *Biochem. Biophys. Res. Commun.* **223:**234–239

Simmons, N.L. 1993. Renal Epithelial Cl^- Secretion. *Exp. Physiol.* **78:**117–137

Smets, I., Zeiske, W., Steels, P., Van Driessche, W. 1998. Na^+ dependence of single-channel current and channel density generate saturation of Na^+ uptake in A6 cells. *Pfluegers Arch.* **435:**604–609

Takahashi, A., Watkins, S.C., Howard, M., Frizzell, R.A. 1996. CFTR-dependent membrane insertion is linked to stimulation of the CFTR chloride conductance. *Am. J. Physiol.* **271:**C1887–C1894

Van Driessche, W. 1994. Noise and Impedance Analysis. *In:* Methods in Membrane and Transporter Research. J.A. Schafer, G. Giebisch, P. Kristensen, H.H. Ussing, editors. pp. 27–80. R.G.L. Company, Austin, TX

Van Driessche, W., Gögelein, H. 1980. Attenuation of current and voltage noise signals recorded from epithelia. *J. Theor. Biol.* **86:**629–648

Warth, R., Thiele, I., Bleich, M., Greger, R. 1998. The role of cytosolic Ca^{2+} in the secretion of NaCl in isolated in vitro perfused rectal gland tubules of *Squalus acanthias. Pfluegers Arch.* **436:**133–140

White, M.M., Aylwin, M. 1990. Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. *Mol. Pharmacol.* **37:**720–724

Yanase, M., Handler, J.S. 1986. Adenosine 3′,5′-cyclic monophosphate stimulates chloride secretion in A epithelia. *Am. J. Physiol.* **251:**C810–C814

Yuan, W.L., Bers, D.M. 1995. Protein kinase inhibitor H-89 reverses forskolin stimulation of cardiac L-type calcium current. *Amer. J. Physiol.-Cell. Physiol.* **37:**C651–C659

Zeiske, W., Atia, F., Van Driessche, W. 1998. Stimulation of secretory apical chloride channels in A6 epithelia: CFTR shows Lorentzian noise. *Pfluegers Arch.* **435** (suppl):R116