The Journal of Experimental Biology 205, 3729–3737 (2002)
Printed in Great Britain © The Company of Biologists Limited
JEB4341

Stimulatory effects on Na^+ transport in renal epithelia induced by extracts of *Nigella arvensis* are caused by adenosine

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Accepted 21 August 2002

Summary

Effects of the extract of *Nigella arvensis* (*NA*) seeds on transepithelial Na^+ transport were studied in cultured A6 toad kidney cells by recording short-circuit current (I_{sc}), transepithelial conductance (G_T), transepithelial capacitance (C_T) and fluctuation in I_{sc}. Apical application of *NA* extract had merely a small stimulatory effect on Na^+ transport, whereas basolateral administration markedly increased I_{sc}, G_T and C_T. A maximal effect was obtained at 500 μl l^{-1} of lyophilized *NA* extract. The increase in C_T suggests that the activation of I_{sc} occurs through the insertion of transport sites in the apical membrane. In experiments performed in the absence of Na^+ transport [apical Na^+ was replaced by *N*-methyl-D-glucamine ($NMDG^+$)], basolateral *NA* extract did not affect I_{sc} and G_T, indicating that Cl^- conductance was not influenced. Noise analysis of I_{sc} using 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC) showed that *NA* extract reduced single-channel current (i_{Na}) and decreased channel open probability (P_o) but evoked a threefold increase in channel density (N_T), which confirms the insertion of Na^+ channels. The separation of the compounds in the crude extract of *NA* was performed by fast protein liquid chromatography (FPLC) on a Superdex 200 gel-filtration column and by reverse-phase high-pressure liquid chromatography (RP-HPLC) on an μRPC C2/C18 SC2.1/10 column connected to a SMART system. Analysis of the purified active fraction by mass spectrometry demonstrated the presence of adenosine as the single organic compound in the extract that had a stimulatory effect on Na^+ transport. In a separate series of experiments, we confirmed that 1 μmol l^{-1} adenosine had similar effects on the parameters of Na^+ transport as did the *NA* extract. The action of adenosine was further identified by experiments in which *NA* extract was added after adenosine. In these experiments, *NA* extract did not affect I_{sc}, G_T or C_T. These results clearly demonstrate an essential role of adenosine in the stimulatory action of *NA* extract.

Key words: *Nigella arvensis* extract, sodium transport, adenosine, renal epithelia, short-circuit current, transepithelial capacitance, transepithelial conductance.

Introduction

The plant nigella is a genre of three species: *Nigella sativa* L., *Nigella damascena* L. and *Nigella arvensis* L., commonly known as black seeds and belonging to the botanical family of Ranunculaceae. The three species have been in use in many Middle Eastern and Far Eastern countries as a natural remedy for over 2000 years. Nigella seeds are ascribed to have many medicinal properties in traditional medicine. In Arabic countries, these seeds are considered as a real panacea. So, they are commonly taken alone, in combination with honey or added to many food preparations. Consequently, many researchers have studied the antibacterial, antifungal and antihelmintic effects of nigella seeds (Agarwal et al., 1949; Akhtar and Riffat, 1991; Rathee et al., 1982). Previous work on nigella seed extracts has shown that it inhibits the growth of the bacteria *Escherichia coli*, *Bacillus subtilis* and *Streptococcus faecalis* (Saxena and Vyas, 1986). The antimicrobial activity of *N. sativa* against several other species of pathogenic bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*) and pathogenic yeast (*Candida albicans*) has also been established (Hanafy and Hatem, 1991).

For a long time, plant remedies, including nigella, have been used to treat diabetes. It has been proposed that the anti-diabetic action of the nigella extracts may, at least partly, be mediated through decreased hepatic gluconeogenesis (al-Awadi et al., 1991). Traditionally, these seeds are well known for their action on stone dissolution in the kidney and bladder. Therefore, we wished to investigate the effects of *N. arvensis* at the molecular level in renal A6 cells.

We have demonstrated the effects of extracts of *N. arvensis* (*NA*) seeds on transepithelial Na^+ transport in a distal tubule cell line, A6, isolated from the kidney of the toad *Xenopus laevis*, by recording short-circuit current (I_{sc}), transepithelial

conductance (G_T) and transepithelial capacitance (C_T) and by analyzing the fluctuations induced by a reversible blocker of the apical Na^+ channel [6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC)]. Analysis of the purified active fraction by mass spectrometry demonstrated the presence of adenosine as the single organic compound in the *NA* extract that had a stimulatory effect on Na^+ transport.

Materials and methods

Cell culture

A6 cells (obtained from Dr J. P. Johnson, University of Pittsburgh, PA, USA) were cultured as described previously (De Smet et al., 1995; Jans et al., 2000), grown on permeable culture support (Anopore, Nunc Intermed, Roskilde, Denmark) and used after 15–30 days of growth (passages 103–109). The epithelial monolayers were mounted with minimal edge damage in Ussing-type chambers for electrophysiological measurements. These chambers are suited for continuous perfusion of both compartments and rapid exchange of the solutions. All experiments were carried out under short-circuit conditions by continuously clamping transepithelial voltage to zero using a low-noise or high-speed voltage-clamp for noise analysis or capacitance measurements, respectively.

Capacitance measurements

In a previous report from our laboratory (Van Driessche et al., 1999), we described in detail the equipment used and extensively discussed the theoretical background of the measurement of transepithelial capacitance (C_T). Briefly, in our study, we used sine-wave analysis at 2 kHz, 2.7 kHz, 4.1 kHz, 5.4 kHz and 8.2 kHz. The data in the present paper illustrate records at 4.1 kHz. Phase shift and amplitude ratio between the voltage and current signal was calculated using regression analysis. With these data, we calculated the parameters of the equivalent circuit of the epithelium represented by a simple RC network that consists of a series resistance, transepithelial capacitance and its equivalent parallel resistance. The graphical interface (Labview, National Instruments, Austin, TX, USA) enabled real-time display of transepithelial conductance (G_T), short-circuit current (I_{sc}) and C_T.

Model calculations based on a lumped two-membrane model demonstrated that, in the high-frequency range, C_T equals the equivalent capacitance of the series arrangement of the apical (C_{ap}) and basolateral (C_{bl}) capacitance (Van Driessche et al., 1999): $1/C_T=1/C_{ap}+1/C_{bl}$. As C_{bl} is approximately 12 times larger than C_{ap} (Erlij et al., 1994), changes in C_T will mainly reflect alterations at the apical membrane, i.e. the result of endo- and exocytotic processes at that border.

Noise analysis

As we wanted to investigate the effect of the *NA* extract on the kinetics of the Na^+ channel in A6 epithelia, fluctuation analysis of I_{sc} was applied using increasing concentrations of CDPC. The macroscopic current, which equals I_{sc}, fluctuates around its mean value. These oscillations are, in fact, the sum of small currents through many channels that switch randomly between an open and a closed state. Their transition states depend on voltage, temperature and blocker concentration. Interaction of the blockers (amiloride or CDPC) with the Na^+ channels induces interruptions of the current through the individual channels and consequently causes a third, blocked state. Analysis of such a process gives power-density spectra (PDS) with a single Lorentzian noise, which can be described by the following equation:

$$S(f) = \frac{S_o}{1 + (f/f_c)^2}\,, \qquad (1)$$

where $S(f)$ is the power density spectral function, S_o is its plateau value and f_c is the corner frequency. f_c depends on the *ON* (k_{ob}) and *OFF* (k_{bo}) rates of the interaction between the blocker and the channel and varies linearly with the blocker concentration (Van Driessche and Lindemann, 1979). The k_{ob} and k_{bo} rates were calculated from the following equation: $2\pi f_c=k_{ob}[CDPC]+k_{bo}$, where [CDPC] is the blocker concentration. S_o depends on the concentration of the blocker, the transition rate constants (k_{ob} and k_{bo}), the number of active channels (N_T) and the single-channel current (i_{Na}), which, all together, determine the Na^+ transport rate: $I_{sc}=N_T\,i_{Na}P_o$, where P_o is the open probability of the channel in the absence of the blocker. The PDS were calculated during a stepwise increase of CDPC concentrations, ranging between 10 μmol l^{-1} and 100 μmol l^{-1}. CDPC is a weaker blocker than amiloride, thus making it better suited for extrapolation to determine the *OFF* rate and channel densities (Helman and Baxendale, 1990).

We used a pulse protocol similar to that described by Blazer-Yost et al. (1998). The apical surface was alternately exposed to 10 μmol l^{-1} and 40 μmol l^{-1} CDPC for 5 min. Current noise at both blocker concentrations was amplified, digitized and Fourier transformed to yield PDS during each 5 min period. The amiloride-insensitive current *(*I_{ami}*)* was measured by blocking the channels at the apical side with 50 μmol l^{-1} amiloride. The blocker-sensitive macroscopic current (I^B_{Na}) was calculated as: $I^B_{Na}=I_{sc}-I_{ami}$.

The single-channel currents in the presence of 10 μmol l^{-1} CDPC (i^{10}_{Na}) were regarded as single-channel currents in the absence of blocker, as they do not differ significantly (Blazer-Yost et al., 1998) according to:

$$i_{Na} = i^{10}_{Na} = \frac{S^{10}_o(2\pi f^{10}_c)^2}{4I^{10}_{Na}k_{ob}[CDPC]}\,, \qquad (2)$$

where S^{10}_o, $f^{10}_c$and I^{10}_{Na} are the values of S_o, f_c and I_{Na} in the presence of 10 μmol l^{-1} CDPC.

Channel density at 10 μmol l^{-1} CDPC (N^{10}_o) is given by the following equation: $N^{10}_o=I^{10}_{Na}/i^{10}_{Na}$. In the absence of CDPC, channel density (N_o) is calculated as:

$$N_o = N^{10}_o\left(1 + P_o\,\frac{[CDPC]}{K_B}\right), \qquad (3)$$

where K_B is the equilibrium coefficient for the effect of the blocker on open channels (k_{bo}/k_{ob}) in μmol l^{-1}. Open-channel probability (P_o) was calculated with the values of K_B from the fractional inhibition of the blocker-sensitive Na^+ transport, $I_{Na}^{40/10}$, caused by increasing the CDPC concentration from 10 μmol l^{-1} to 40 μmol l^{-1}:

$$P_o = \left(\frac{1 - I_{Na}^{40/10}}{40 I_{Na}^{40/10} - 10} \right) K_B . \quad (4)$$

The total number of channels (N_T) was calculated as: $N_T = N_o/P_o$.

Methods of extraction and identification of the active principal molecule of NA

Preparation of the plant extract

The decoction was prepared by boiling 5 g of dried and pulverized *Nigella arvensis* seeds in 100 ml distilled water for 10 min. Using filter paper, the plant extract was then filtered (filtrate I). Subsequently, a sample (2 ml) of *NA* extract was filtered through a polyvinylidene difluoride (PVDF) syringe filter with a pore size of 0.45 μm (Alltech Europe, Laarne, Belgium) (filtrate II). In addition, the PVDF filter was washed with 1 ml 5% isopropanol in high-pressure liquid chromatography (HPLC) water (filtrate III). Filtrates II and III were evaporated in a Savant Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and then dissolved in 2.5 ml and 100 μl of distilled water, respectively. These fractions were used at a concentration of 250 μl l^{-1} to analyze activity on the Na^+ channel. Filtrate II showed the maximum activity on Na^+ transport.

Fast protein liquid chromatography (FPLC)

A sample (2.5 ml) of filtrate II was lyophilized in a Savant Speed-Vac concentrator. The sample was dissolved in 300 μl of FPLC column buffer (100 mmol l^{-1} ammonium bicarbonate in HPLC water, pH 7.5), and 250 μl was loaded onto a Superdex 200 gel-filtration column (Marsha Pharmacia Biotech AB, SE751-84, Upscale, Sweden). Flow rate was 0.5 ml min^{-1}, and fractions of 250 μl were collected. Elution of the various compounds was established by monitoring the absorbance at 280 nm. Each fraction was lyophilized and analyzed for its activity on the Na^+ channel. The highest stimulating activity on Na^+ transport was detected in fraction 108. It should be noted that the volatile column buffer allowed for a complete lyophilization without subsequent generation of high salt concentrations.

Reverse-phase HPLC (RP-HPLC)

Fraction 108 was further purified by performing RP-HPLC using a C2/C18 column (μRPC C2/C18 SC2.1/10 column, Amersham Pharmacia Biotech, Buckinghamshire, UK) connected to a SMART system (Amersham Pharmacia Biotech). Operating conditions were as follows: solvent A, 0.1% trifluoracetic acid (TFA) in HPLC water, solvent B, 95% acetonitrile in 0.1% TFA. Column conditions were as follows: 0% solvent B for 7 min followed by a linear gradient to 70% solvent B in 83 min at a flow rate of 80 μl min^{-1}.

The sample was injected onto the reverse-phase column, which had previously been equilibrated in solvent A. The column was washed with 420 μl of solvent A. Subsequently, a linear gradient from 0% to 70% acetonitrile in solvent A was performed. Elution was monitored at three wavelengths (215 nm, 254 nm and 280 nm), and peak fractions of fraction 108 were collected manually in 500 μl Eppendorf tubes based on the absorbance at 215 nm. Each fraction was lyophilized, dissolved in analysis buffer (Ringer solution; see composition below) and analyzed for its activity on Na^+ transport in the renal epithelial cells. Activity was demonstrated in a 215 nm peak eluting at 20% acetonitrile, which corresponded to fraction 12. To identify the molecular identity of the active principle, this fraction was further analyzed by mass spectrometry.

Mass spectrometry

Mass spectrometry analysis was performed on a Perkin Elmer API 3000 LC/MS/MS system (PE Biosystems, Foster City, CA, USA) equipped with a nanospray (Protana Engineering, Odense M, Denmark) at a flow rate of 1 μl h^{-1}. Calibration was performed externally with a polypropylene glycol test solution (Perkin Elmer test-kit) and horse heart myoglobin (16.95 kDa). 2 μl of the prepared fraction was introduced into the nanospray. Scans were made between m/z 5 and m/z 2000. Data from 50 shots to 100 shots were averaged to obtain the final spectrum.

Solutions

In all experiments, the apical and basolateral Ringer solutions contained 102 mmol l^{-1} Na^+, 2.5 mmol l^{-1} K^+, 2.5 mmol l^{-1} HCO_3^-, 1 mmol l^{-1} Ca^{2+} and 104 mmol l^{-1} Cl^- (pH 8; osmolality, 200 mosmol kg^{-1} H_2O). For the experiments in which we investigated Cl^- secretion, the apical solution was NaCl-free and, instead, contained 69 mmol l^{-1} *N*-methyl-D-glucamine sulphate [$(NMDG)_2SO_4$]; osmolality was 180 mosmol kg^{-1} H_2O. At the end of this type of experiment, we removed Cl^- from the basolateral solution, which contained 102 mmol l^{-1} Na^+, 2.5 mmol l^{-1} K^+, 2.5 mmol l^{-1} HCO_3^-, 1 mmol l^{-1} Ca^{2+} and 52 mmol l^{-1} SO_4^{2-} (the osmolality was adjusted to 200 mosmol kg^{-1} H_2O with sucrose).

Amiloride (50 μmol l^{-1}; Sigma, St Louis, MO, USA) was used to determine the amiloride-sensitive component of the I_{sc}. CDPC (Aldrich Chemical, Milwaukee, WI, USA; stock solution in dimethyl sulfoxide) was used in concentrations of up to 100 μmol l^{-1}. *Nigella arvensis* was brought from Fès, Morocco. The lyophilized *NA* extract was used in most experiments at a concentration of 250 μl l^{-1}. We chose to use this concentration of *NA* extract because an absolute maximal stimulation was recorded with 500 μl l^{-1} (see Results) and because of the limited access to the extract. Adenosine (1 μmol l^{-1}, 9-β-D-ribofuranosyladenine) was also purchased from Sigma.

Statistics

For pooled data, means ± S.E.M. were calculated. Statistical significance was evaluated using a Student's *t*-test. $P<0.05$ was accepted as significant.

Results

Stimulation of Na^+ transport by the NA *extract*

In the presence of NaCl-Ringer on both sides of the A6 epithelia, the addition of *NA* extract to the apical side evoked a small transient increase in I_{sc} and G_T (dashed lines, Fig. 1). The augmentation of I_{sc} and G_T following apical treatment was not significant and therefore is not further discussed in this paper. By contrast, addition of *NA* extract to the basolateral compartment gave rise to a pronounced increase in I_{sc}, G_T and C_T (solid lines, Fig. 1). Within 20 min, this increase reached its maximum effect of twice the initial value of I_{sc} and was followed by a steady state. The response was abolished when amiloride (50 μmol l^{-1}) was added to the apical bath. Mean values of I_{sc}, G_T and C_T are listed in Table 1.

Effect of the NA *extract on Cl^- secretion*

Although amiloride is able to almost completely block I_{sc} (Fig. 1; Table 1), we verified the effect of *NA* extract on Cl^- secretion through the epithelium. We therefore abolished Na^+ transport by incubating the epithelium with Na^+-free apical solutions (Fig. 2A). The addition of basolateral *NA* extract in the absence of apical Na^+ (replaced by $NMDG^+$) did not lead to an increase in I_{sc} and G_T. Subsequently, when apical NMDGCl-Ringer was replaced by NaCl-Ringer, I_{sc} increased

Table 1. *Effects of the* Nigella arvensis *(*NA*) extract and adenosine on short-circuit current (I_{sc}), transepithelial conductance (G_T) and transepithelial capacitance (C_T)*

		I_{sc} (μA cm^{-2})	G_T (mS cm^{-2})	C_T (μF cm^{-2})
PT 1	Control	9.93±1.74	0.35±0.11	0.79±0.01
	+*NA* Bl	22.89±2.26	0.49±0.13	0.85±0.01
	+Ami	0.62±0.19	0.26±0.13	0.86±0.01
	Control	7.98±1.45	0.17±0.05	0.83±0.02
	+*NA* Ap	8.79±1.78	0.20±0.04	0.81±0.01
PT 2	Control	10.63±1.61	0.33±0.06	0.90±0.01
	+Adenosine	18.18±1.86	0.42±0.04	0.98±0.01
	+*NA* Bl	17.51±1.53	0.42±0.04	0.98±0.01

Means ± S.E.M. (N=6) were calculated from experiments following the protocol depicted in Fig. 1 (PT 1) or in Fig. 7 (PT 2).

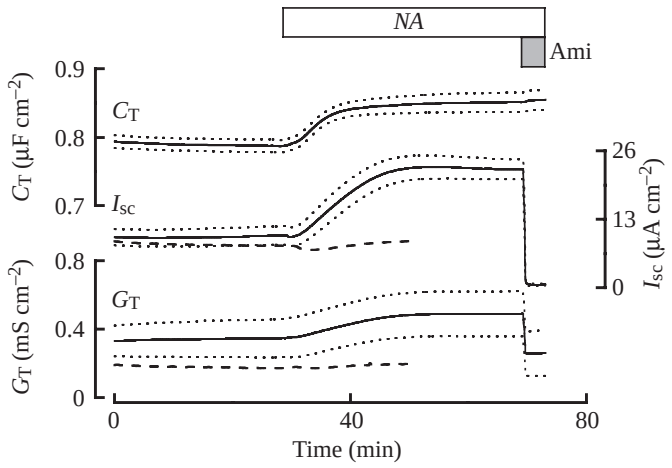

Fig. 1. Effects of the *Nigella arvensis* (*NA*) extract on short-circuit current (I_{sc}), transepithelial conductance (G_T) and transepithelial capacitance (C_T) in cultured monolayers of A6 epithelia under Na^+-transporting conditions. The A6 cells were incubated in 102 mmol l^{-1} NaCl-Ringer solutions on both surfaces. 250 μl l^{-1} *NA* extract was applied either to the apical side (dashed line) or basolateral side (solid line) of the monolayer. At the end of the experiment, Na^+ current was inhibited by apical 50 μmol l^{-1} amiloride (Ami). The traces are mean values from six tissues; dotted lines represent means ± S.E.M.

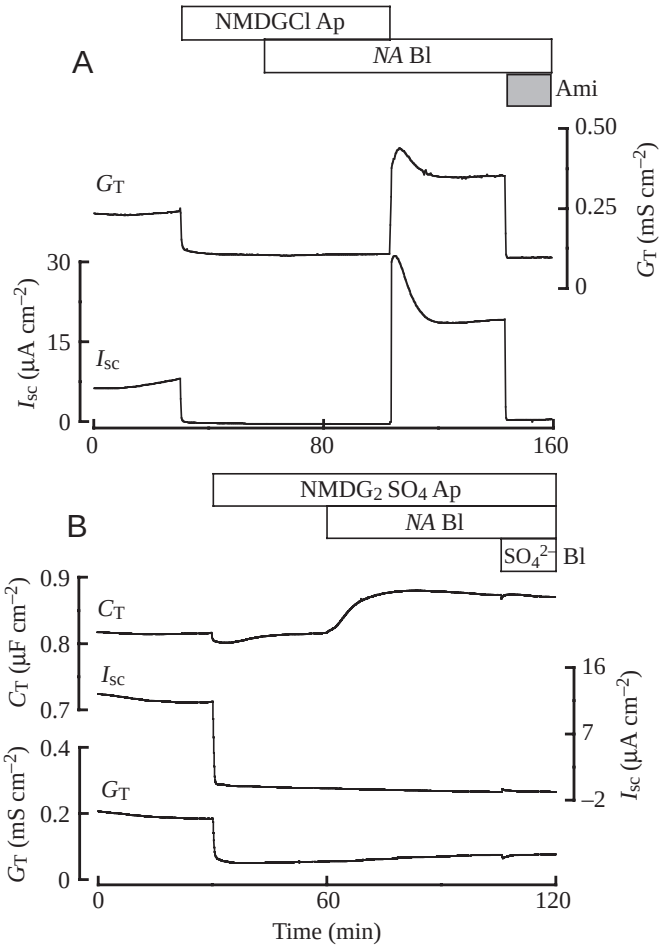

Fig. 2. Effects of the *Nigella arvensis* (*NA*) extract on Cl^- secretion. (A) Epithelia were incubated in 102 mmol l^{-1} NaCl-Ringer solutions on both apical (Ap) and basolateral (Bl) sides; *N*-methyl-D-glucamine chloride (NMDGCl)-Ringer was then added apically 30 min before the *NA* extract (250 μl l^{-1}) was administrated basolaterally. 50 μmol l^{-1} amiloride (Ami) was added at the end of the experiment. (B) Same experimental conditions as in A, but $NMDG_2SO_4$-Ringer was applied apically 30 min before the *NA* extract was added. At the end of the experiment, basolateral Cl^- was replaced by SO_4^{2-}.

rapidly, reached a peak and declined to a plateau. The peak in I_{sc} and decline to a steady plateau value are caused by Na^+ self-inhibition, which has been described for several Na^+-transporting epithelia. However, it should be noted that the plateau values of I_{sc} and G_T are markedly higher than the control values recorded at the beginning of the experiment. Moreover, amiloride was able to almost completely block I_{sc} and to strongly depress G_T. These observations confirm the effect of *NA* extract on Na^+ transport.

To further verify a possible effect of *NA* extract on Cl^- secretion, we performed experiments with a Cl^- gradient directed from the basolateral to the apical side (Fig. 2B). Apical NaCl-Ringer was replaced by $(NMDG)_2SO_4$-Ringer. No effect on I_{sc} and G_T was observed after addition of *NA* extract to the basolateral side. However, the increase in C_T was comparable with the effect observed in Fig. 1, demonstrating that the *NA* extract exerted its effect under these conditions. At the end of the experiment, basolateral NaCl-Ringer was replaced by Na_2SO_4-Ringer to check the Cl^- current, but practically no effect was observed. These data show that, with both protocols (Fig. 2A,B), the *NA* extract does not activate a Cl^- pathway.

Noise analysis parameters: blocker rate coefficients and determination of i_{Na}, N_T *and* P_o *values in control conditions and in the presence of* NA *extract*

Fig. 3 depicts the different steps that lead to the determination of the *ON* and *OFF* rates (k_{ob} and k_{bo} kinetics) of the interaction of CDPC with the Na^+ channel. Fig. 3A shows the inhibition of I_{sc} caused by apical application of increasing concentrations of CDPC, ranging from 10 μmol l^{-1} to 100 μmol l^{-1}, before and after basolateral stimulation with *NA* extract. The observed relative instability of I_{sc} after the application of different CDPC concentrations has been reported and discussed in the literature (Baxendale-Cox et al., 1997). It was attributed to the feedback regulation of Na^+ transport and therefore becomes more pronounced at higher transport rates, as observed after stimulation of I_{sc} by the *NA* extract. Similarly, after washout of CDPC from the apical solution, just before *NA* extract application, relatively high transient overshoots in I_{sc} were observed. Typically, amiloride inhibited Na^+ transport rapidly and completely when applied at the end of the experiment following the highest dose of CDPC.

Fig. 3B illustrates that $2\pi f_c$ data correlate linearly with the CDPC concentrations. Therefore, k_{ob} and k_{bo} can be determined by linear regression analysis using the following equation: $2\pi f_c = k_{ob}[CDPC] + k_{bo}$ (see Materials and methods). The *ON* and *OFF* rates for CDPC during the control period were consistent with the rates previously reported (Jans et al., 2000). Treatment with *NA* extract did not change k_{ob} and slightly increased k_{bo}. Mean values of the kinetic parameters (k_{ob}, k_{bo} and K_B) are presented in Table 2.

As Na^+ channels in the apical membrane of A6 cells are rate limiting for transepithelial Na^+ transport (Granitzer et al., 1991), the increase in I_{sc} observed during application of *NA*

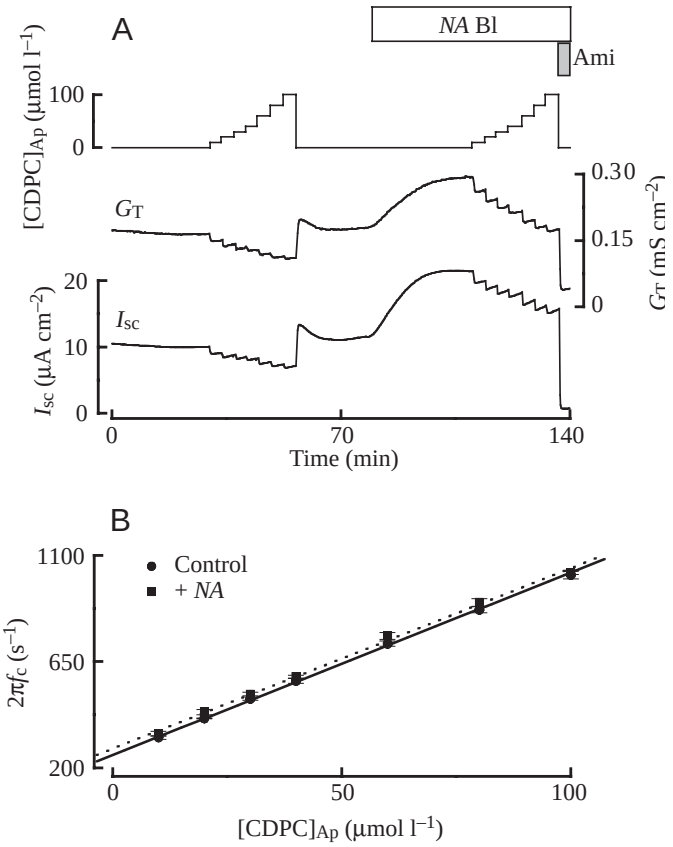

Fig. 3. Effects of the *Nigella arvensis* (*NA*) extract on kinetic parameters k_{ob}, k_{bo} and K_B of the interaction of 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC) with the Na^+ channel. (A) Effect of stepwise application of apical CDPC concentrations (ranging from 10 μmol l^{-1} to 100 μmol l^{-1}) on short-circuit current (I_{sc}) and transepithelial conductance (G_T) of A6 cells before and after basolateral (Bl) stimulation with 250 μl l^{-1} *NA* extract (N=6). 50 μmol l^{-1} amiloride (Ami) was added after the highest dose of CDPC at the end of the experiment. Solutions were as in Fig. 1. (B) $2\pi f_c$ data at different doses of apical CDPC ($[CDPC]_{Ap}$). The slope and intercept of the linear regressions for the control ($2\pi f_c$=7.66$[CDPC]_{Ap}$+259.29) and in the presence of *NA* extract ($2\pi f_c$=7.64 $[CDPC]_{Ap}$+281.51) indicate the *ON* and *OFF* rates, respectively, of the interaction between CDPC and the Na^+ channel.

Table 2. *Effects of basolateral* Nigella arvensis *(*NA*) extract on parameters of CDPC blocker rate coefficients and* K_B

	k_{ob} (μmol^{-1} l s^{-1})	k_{bo} (s^{-1})	K_B (μmol l^{-1})
Control	7.66±0.16	259.23±5.79	33.92±1.22
NA extract	7.64±0.20	281.39±10.95	37.01±2.08

CDPC, 6-chloro-3,5-diaminopyrazine-2-carboxamide.

Independent *t*-test on control and *NA* extract (P<0.05); the values are not significantly different. Values are means ± S.E.M. (N=6).

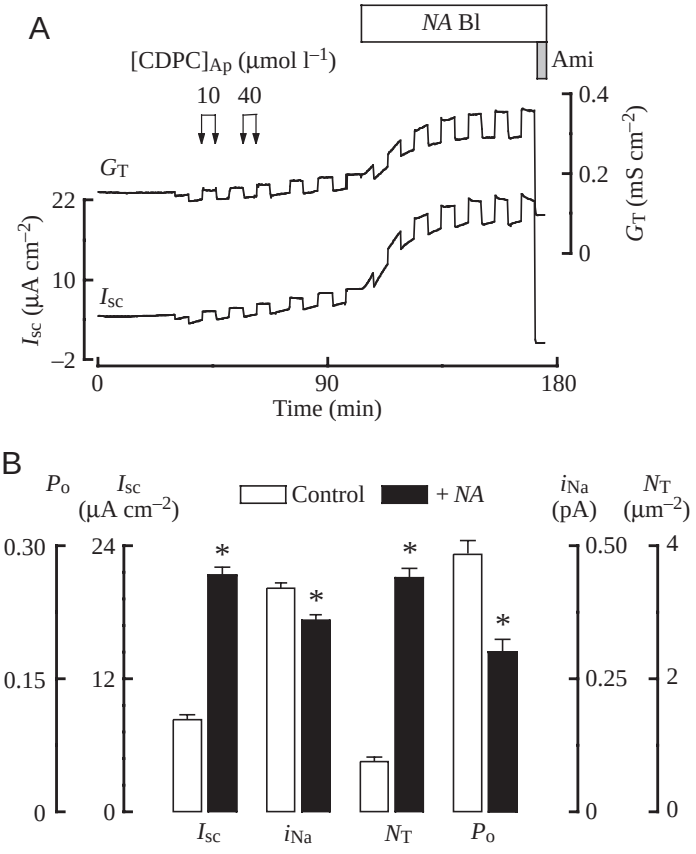

Fig. 4. Effects of the *Nigella arvensis* (*NA*) extract on short-circuit current (I_{sc}), single-channel current (i_{Na}), transepithelial conductance (G_T), total number of channels (N_T) and open-channel probability (P_o). (A) Pulse protocol of 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC)-induced noise before and after addition of the *NA* extract basolaterally. CDPC-induced noise was recorded by switching the apical CDPC concentration ($[CDPC]_{Ap}$) alternately from 10 μmol l^{-1} to 40 μmol l^{-1} every 5 min. 50 μmol l^{-1} amiloride (Ami) was administered apically at the end of the experiment. Solution conditions were as in Fig. 1. (B) I_{sc}, i_{Na}, N_T and P_o during control (open bars) and after exposure to *NA* extract (filled bars). The values were calculated using a three-state model. * The values of the control and the *NA* extract are significantly different (P<0.05) for all parameters.

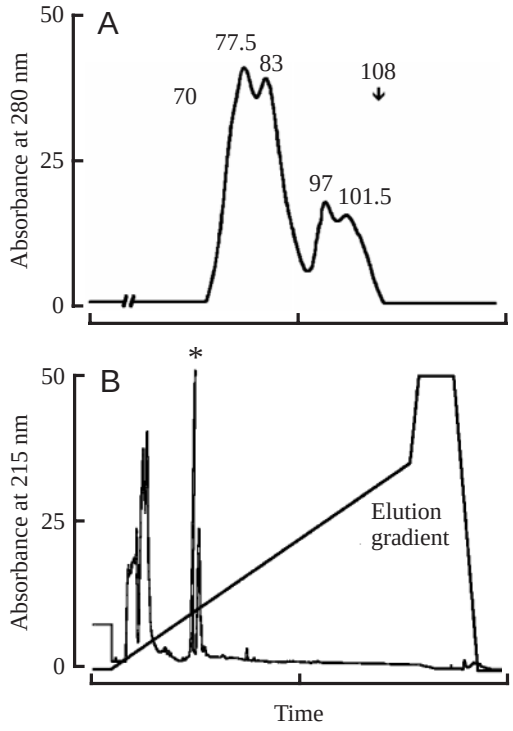

Fig. 5. Identification of the active compound in the *Nigella arvensis* (*NA*) extract. (A) Fast protein liquid chromatography (FPLC) profile of the *NA* extract. The elution position of fraction 108, showing activity, is indicated by an arrow. (B) Further separation of fraction 108 by reverse-phase high-pressure liquid chromatography (RP-HPLC). The activity was demonstrated in a 215 nm peak eluting at 20% acetonitrile (fraction 12, indicated by an asterisk). The linear elution gradient is also shown.

extract could result from a rise in i_{Na} and/or N_T and/or P_o. To resolve this question, we performed noise analysis experiments. Fig. 4A illustrates typical I_{sc} responses to basolateral *NA* extract in such an experiment where apical [CDPC] was switched alternately between 10 μmol l^{-1} and 40 μmol l^{-1} every 5 min. It should be noted that basolateral *NA* was added in the presence of 10 μmol l^{-1} apical CDPC and that the first 40 μmol l^{-1} CDPC pulse was executed approximately 10 min after addition of the *NA* extract. I_{sc} increased from 8.62 μA cm^{-2} to 22.12 μA cm^{-2}, and G_T increased from 0.20 mS cm^{-2} to 0.36 mS cm^{-2}. In addition, indicated on these tracings is the consistent finding that the I_{sc} in these studies is amiloride sensitive, as shown by the depression of I_{sc} and G_T by 50 μmol l^{-1} amiloride at the end of the experiment.

We used noise analysis to determine the contributions of i_{Na}, P_o and N_T to the I_{sc}. Current noise PDS were alternately measured during exposure to 10 μmol l^{-1} and 40 μmol l^{-1} CDPC, thus providing the S_o and f_c values of the blocker-induced Lorentzians. Fig. 4B summarizes the influence of *NA* extract on I_{sc}, i_{Na}, N_T and P_o. *NA* extract increased I_{sc} from 8.3±0.44 μA cm^{-2} to 21.36±0.71 μA cm^{-2}. The most important factor involved in the activation of I_{sc} after application of *NA* extract was N_T, which increased from 0.75±0.06 μm^{-2} to 3.54±0.14 μm^{-2}. The relatively small decrease in i_{Na} from 0.42±0.01 pA to 0.36±0.01 pA during exposure to *NA* extract probably represents the immediate response to depolarization of the apical membrane by activation of the Na^+ permeability. P_o in the presence of basolateral *NA* decreased from 0.29±0.02 to 0.18±0.01. Results are presented as means ± S.E.M. (N=6).

Identification of the principal active component of the NA *extract*

Fig. 5A shows the elution position (fraction 108) of the activity during the separation of the *NA* extract by FPLC on a Superdex 200 column. This corresponds to compounds with a molecular mass of <2000 Da. Fraction 108, in turn, was separated using RP-HPLC (Fig. 5B). Fraction 12 of the

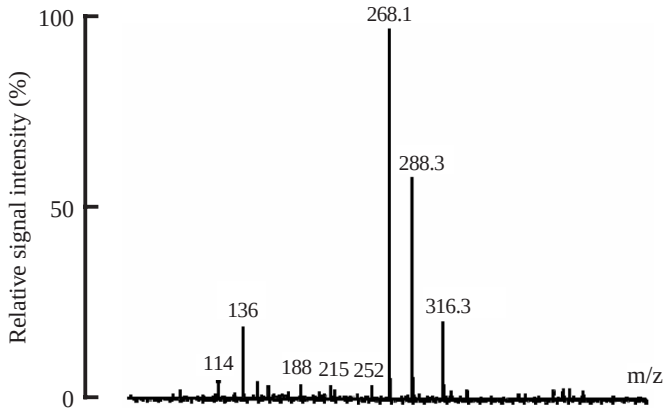

Fig. 6. Mass spectrum of fraction 12. The presence of three distinct molecules with masses of 316, 288 and 268 are visible. The compounds with masses of 316 and 288 could be identified as contaminants generated during the purification procedure. The 268-mass compound was identified as adenosine (M_r 267.24).

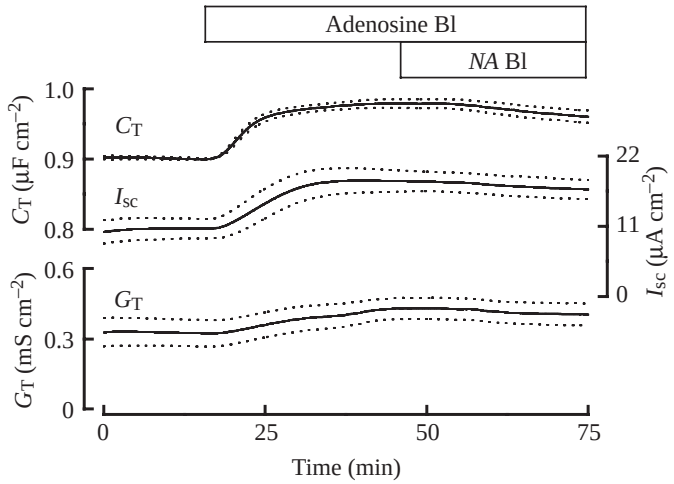

Fig. 7. Time courses of short-circuit current (I_{sc}), transepithelial conductance (G_T) and transepithelial capacitance (C_T) during basolateral (Bl) serial stimulation with adenosine and the *Nigella arvensis* (*NA*) extract. First, 1 μmol l^{-1} adenosine was added at the basolateral side, followed by addition of 250 μl l^{-1} *NA* extract under transporting conditions. The same solution conditions were used as in Fig. 1. The traces are mean values from six tissues; dotted lines represent means ± S.E.M.

separation of fraction 108, corresponding to a 215 nm peak eluting at 20% acetonitrile, showed maximum stimulatory effect on I_{sc} (data not shown). Therefore, it was taken for further identification. Functional tests with other fractions of FPLC and RP-HPLC did not demonstrate a stimulatory activity.

Mass spectrometry analysis (Fig. 6) allowed the identification of a 268-mass compound as adenosine (M_r 267.24), from which adenine (M_r 135.15) is further derived. The compounds with other masses could be identified as contaminants generated during the purification procedure.

The possible role of adenosine was functionally tested by verifying its effect on Na^+ transport and by subsequently investigating the effect of *NA* extract on Na^+ transport (Fig. 7). The addition of *NA* extract 30 min after an adenosine response (Fig. 7) did not show an additive effect on apical membrane Na^+ influx. We obtained similar effects on Na^+ transport with adenosine. Moreover, adenosine blunted the effect of *NA* extract. The *NA* extract concentrations were chosen to elicit maximal responses. These data suggest that the stimulation by both agents occurs through a common signaling pathway and confirm that adenosine is the single organic compound in the *NA* extract that increased Na^+ transport. Fig. 7 represents the mean of six experiments.

Discussion

The present study demonstrates that the *NA* extract activates transepithelial Na^+ transport in A6 cells. The receptors and/or mechanisms are located at the basolateral side of the cells, as apical administration of *NA* extract had only a small stimulatory effect on I_{sc} and G_T. The activation of transepithelial Na^+ transport in A6 cells by basolateral *NA* extract evokes a parallel increase in C_T, I_{sc} and G_T (Fig. 1). As demonstrated previously (Van Driessche et al., 1999), the value of C_T is dominated by the value of C_{ap}, which, in turn, is proportional to the apical membrane area. Hence, our findings indicate that *NA* extract causes an increase of the apical membrane area of the epithelium. This increase in membrane area could be due to membrane trafficking and Na^+-channel insertion in the apical membrane. Based on this observation, we performed noise analysis to further explore this hypothesis. As shown by the analysis of the CDPC-induced noise, the *NA* extract increases the number of open amiloride-sensitive Na^+ channels in the apical membrane of A6 cells. This finding, together with the increase in apical membrane area, suggests a stimulation of epithelial transport by promoting the exocytotic transfer of membranes from the cytoplasmic compartment into the membrane surface. However, the possibility that pre-existing Na^+ channels are activated by *NA* extract, independently of the transfer of new membranes, cannot be ruled out. The number of open Na^+ channels increased threefold, while C_T increased by only 8%. These numbers could be interpreted as indicating that the additional membranes contain a considerably higher density of Na^+ channels than the resting apical membrane. Similar results were obtained with insulin (Erlij et al., 1994) and aldosterone (Blazer-Yost et al., 1998). Previous data (Coupaye-Gerard et al., 1994) showed that aldosterone-induced Na^+ transport in A6 cells was sensitive to brefeldin A (BFA), an inhibitor of exocytotic events. In addition, the decrease of I_{sc} after BFA was primarily the result of a decrease in open-channel density in the apical membrane of A6 cells (Fisher et al., 1996). Full elucidation of these phenomena will, however, require further investigation.

It is remarkable that the activation of Na^+ transport by *NA* extract is exclusively due to an increase of apical Na^+ channels,

whereas the i_{Na} and P_o decreased. The decrease in i_{Na} probably results from cell depolarization, as previously reported (Van Driessche and Zeiske, 1985). In the present study, the depolarization was caused by an increase in apical membrane Na^+ conductance (Fig. 4A). Possible mechanisms that could explain the decrease of P_o by 38% are, as yet, unknown. Despite appreciable *NA*-extract-related decreases of P_o, the net stimulation of transport occurs as a result of a considerable increase in N_o. Studies with forskolin, which is known to increase cytosolic cyclic adenosine monophosphate (cAMP), have shown similar effects (Els et al., 1991), suggesting a possible role for cAMP in the regulation of P_o. It is also possible that cytosolic Ca^{2+} causes this decrease in P_o. In recent experiments with ionomycin, elevation of cytosolic Ca^{2+} induced by this ionophore led to a decrease of P_o in A6 epithelia (Helman et al., 1998).

To elucidate the mechanisms of action of the *NA* extract on transepithelial Na^+ transport in A6 cells, we attempted to identify the principle active compound(s) by using biochemical approaches: FPLC, RP-HPLC and mass spectrometry. The molecule was identified as adenosine (Fig. 6). The function tests with adenosine confirmed that it stimulates active Na^+ transport in A6 epithelia and that its action blunted the effect of the *NA* extract (Fig. 7).

Adenosine regulates both Na^+ uptake and Cl^- secretion in A6 cells, as in other epithelia. It has been reported that the regulation of Cl^- secretion is mediated by A_1 adenosine receptors located at the apical cell surface and transduced by Ca^{2+} release from intracellular stores (Banderali et al., 1999; Schwiebert et al., 1992). Similar results were shown by a metabolically stable analogue of adenosine N^6-cyclopentyladenosine (CPA), which binds to both the A_1 and A_2 adenosine receptors in A6 cells (Casavola et al., 1996) but has a higher affinity for the A_1 receptors. When CPA was added to the apical side, it induced an increase in Cl^- conductance by acting on the A_1 receptors. Recently, Di Sole et al. (1999) reported results indicating that stimulation of A_3 receptors induced an elevation of cytosolic Ca^{2+} in A6/C_1 cells. These findings were confirmed by Reshkin et al. (2000), who demonstrated the presence of A_3 receptors in the apical membrane. When added to the basolateral side, adenosine increases Na^+ transport by interacting with basolaterally located A_2 receptors. The activation of Na^+ conductance occurs through an adenylate cyclase-dependent mechanism (Lang et al., 1985). Moreover, Dobbins et al. (1984) have demonstrated that adenosine and some of its analogues increase cAMP levels, which results in secondary Cl^- secretion, indicating the presence of an A_2 adenosine receptor on rabbit ileum mucosal cells that activates adenylate cyclase. On the other hand, adenosine stimulation of electrogenic Na^+ transport in renal cells has been demonstrated to occur, at least in part, through Ca^{2+}-dependent signal transduction events and not through regulation of adenylate cyclase (Hayslett et al., 1995). The observation that increased levels of intracellular Ca^{2+} correlated with a two- to threefold increase in inositol (1,4,5)-trisphosphate suggests that Ca^{2+} was released from intracellular stores. Furthermore, Kurtz (1988) has demonstrated that, in isolated juxtaglomerular cells, activation of A_1 receptors is associated with an elevation of cyclic guanosine-3′,5′-monophosphate (cGMP) but not with changes in either cytosolic Ca^{2+} or cAMP, suggesting the involvement of yet another second messenger system for adenosine. An effect of adenosine on intracellular Ca^{2+} was also found in experiments where A_3 receptors were stimulated by 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-methyluronamide, which activates Cl^- secretion by Ca^{2+} and cAMP-regulated channels.

In the present study, we did not find Cl^- secretion induced by adenosine or *NA* extract in A6 cells (Fig. 2). On the other hand, we have evidence that cAMP and/or Ca^{2+} activate Cl^- channels in the same clone of A6 cells (Atia et al., 1999; Zeiske et al., 1998). It is puzzling that, in this study, we found a marked activation of Na^+ absorption without affecting Cl^- secretion. The magnitude of the effect on Na^+ transport suggests that, if the stimulation occurs through cAMP, its rise in cytosolic concentration should be significant and probably sufficient to activate Cl^- secretion. However, in experiments where the additive effect of forskolin and adenosine was tested, we found that forskolin could still markedly activate Na^+ transport in tissues pretreated with adenosine, whereas the effect of adenosine after forskolin addition was rather small (F. Atia, unpublished observation). So, it remains possible that the cAMP levels reached by adenosine or *NA* extract treatment are still too small to activate Cl^- conductance but are sufficiently large enough to activate Na^+ transport. This issue requires further investigation and leaves the possibility open of the involvement of a cAMP-independent pathway.

The present study is of importance because of the widespread use of spices and other plant products by humans. Plants have always been used in traditional medicine. Their effects may provide a source of inspiration for the development of new drugs based on careful scientific studies that are required to avoid adverse effects that may occur. In fact, there are numerous plants, including nigella, awaiting further investigation of their therapeutic potential.

This project was supported by research grants from the ‘Fonds voor wetenschappelijk onderzoek – Vlaanderen’ (G.0179.99), the Interuniversity Poles of Attraction Program – Belgian State, Prime Minister’s Office – Federal Office for Scientific, Technical, and Cultural Affairs IUAP P4/23. F. Atia was supported by a grant from the Belgian Ministry of Foreign Affairs (ABOS).

References

Agarwal, R., Kharya, M. O. and Shrivasthava, R. (1949). Antimicrobial and antihelmintic activities of essential oil of *Nigella sativa*. *J. Exp. Biol.* **6**, 232-247.

Akhtar, M. S. and Riffat, S. (1991). Field trial of *Saussurea lappa* roots against nematodes and *Nigella sativa* seeds against cestodes in children. *J. Pak. Med. Assoc.* **41**, 185-187.

al-Awadi, F., Fatania, H. and Shamte, U. (1991). The effect of a plants mixture extract on liver gluconeogenesis in streptozotocin induced diabetic rats. *Diabetes Res.* **18**, 163-168.

Atia, F., Zeiske, W. and Van Driessche, W. (1999). Secretory apical Cl^- channels in A6 cells: possible control by cell Ca^{2+} and cAMP. *Pflügers Arch.* **438**, 344-353.

Banderali, U., Brochiero, E., Lindenthal, S., Raschi, C., Bogliolo, S. and Ehrenfeld, J. (1999). Control of apical membrane chloride permeability in the renal A6 cell line by nucleotides. *J. Physiol.* **519**, 737-751.

Baxendale-Cox, L. M., Duncan, R. L., Liu, X., Baldwin, K., Els, W. J. and Helman, S. I. (1997). Steroid hormone-dependent expression of blocker-sensitive ENaCs in apical membranes of A6 epithelia. *Am. J. Physiol.* **273**, C1650-C1656.

Blazer-Yost, B. L., Liu, X. and Helman, S. I. (1998). Hormonal regulation of ENaCs: insulin and aldosterone. *Am. J. Physiol.* **274**, C1373-C1379.

Casavola, V., Guerra, L., Reshkin, S. J., Jacobson, K. A., Verrey, F. and Murer, H. (1996). Effect of adenosine on Na^+ and Cl^- currents in A6 monolayers. Receptor localization and messenger involvement. *J. Membr. Biol.* **151**, 237-245.

Coupaye-Gerard, B., Kim, H. J., Singh, A. and Blazer-Yost, B. L. (1994). Differential effects of brefeldin A on hormonally regulated Na^+ transport in a model renal epithelial cell line. *Biochim. Biophys. Acta* **1190**, 449-456.

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

Di Sole, F., Casavola, V., Mastroberardino, L., Verrey, F., Moe, O. W., Burckhardt, G., Murer, H. and Helmle-Kolb, C. (1999). Adenosine inhibits the transfected Na^+–H^+ exchanger NHE3 in *Xenopus laevis* renal epithelial cells (A6/C1). *J. Physiol.* **515**, 829-842.

Dobbins, J. W., Laurenson, J. P. and Forrest, J. N., Jr (1984). Adenosine and adenosine analogues stimulate adenosine cyclic 3′,5′-monophosphate-dependent chloride secretion in the mammalian ileum. *J. Clin. Invest.* **74**, 929-935.

Els, W. J., Helman, S. I. and Mencio, T. (1991). Activation of epithelial Na channels by hormonal and autoregulatory mechanisms of action. *J. Gen. Physiol.* **98**, 1197-1220.

Erlij, D., De Smet, P. and 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.

Fisher, R. S., Grillo, F. G. and Sariban-Sohraby, S. (1996). Brefeldin A inhibition of apical Na^+ channels in epithelia. *Am. J. Physiol.* **270**, C138-C147.

Granitzer, M., Leal, T., Nagel, W. and Crabbé, J. (1991). Apical and basolateral conductance in cultured A6 cells. *Pflügers Arch.* **417**, 463-468.

Hanafy, M. S. M. and Hatem, M. E. (1991). Studies on the antimicrobial activity of *Nigella sativa* seed (black cumin). *J. Ethnopharmacol.* **34**, 275-278.

Hayslett, J. P., Macala, L. J., Smallwood, J. I., Kalghatgi, L., Gassalaherraiz, J. and 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.

Helman, S. I. and Baxendale, L. M. (1990). Blocker-related changes of channel density. Analysis of a three-state model for apical Na channels of frog skin. *J. Gen. Physiol.* **95**, 647-678.

Helman, S. I., Liu, X., Baldwin, K., Blazer-Yost, B. L. and Els, W. J. (1998). Time-dependent stimulation by aldosterone of blocker-sensitive ENaCs in A6 epithelia. *Am. J. Physiol.* **274**, C947-C957.

Jans, D., Simaels, J., Cucu, D., Zeiske, W. and Van Driessche, W. (2000). Effects of extracellular Mg^{2+} on transepithelial capacitance and Na^+ transport in A6 cells under different osmotic conditions. *Pflügers Arch.* **439**, 504-512.

Kurtz, A., Della Bruna, R., Pfeilschifter, J. and Bauer, C. (1988). Role of cGMP as second messenger of adenosine in the inhibition of renin release. *Kidney Int.* **33**, 798-803.

Lang, M. A., Preston, A. S., Handler, J. S. and Forrest, J. N., Jr (1985). Adenosine stimulates sodium transport in kidney A6 epithelia in culture. *Am. J. Physiol.* **249**, C330-C336.

Rathee, P. S., Mishra, S. H. and Kaughal, R. (1982). Antimicrobial activity of essential oil, fixed oil and insaponifiable matter of *Nigella sativa*. *J. Pharm. Sci.* **44**, 8-10.

Reshkin, S. J., Guerra, L., Bagorda, A., Debellis, L., Cardone, R., Li, A. H., Jacobson, K. A. and Casavola, V. (2000). Activation of A3 adenosine receptor induces calcium entry and chloride secretion in A6 cells. *J. Membr. Biol.* **178**, 103-113.

Saxena, A. P. and Vyas, K. M. (1986). Antimicrobial activity of seeds of some ethnomedicinal plants. *J. Eon. Taxon. Bot.* **8**, 291-299.

Schwiebert, E. M., Karlson, K. H., Friedman, P. A., Dietl, P., Spielman, W. S. and Stanton, B. A. (1992). Adenosine regulates a chloride channel via protein kinase C and a G protein in a rabbit cortical collecting duct cell line. *J. Clin. Invest.* **89**, 834-841.

Van Driessche, W., De Vos, R., Jans, D., Simaels, J., De Smet, P. and Raskin, G. (1999). Transepithelial capacitance decrease reveals closure of lateral interspace in A6 epithelia. *Pflügers Arch.* **437**, 680-690.

Van Driessche, W. and Lindemann, B. (1979). Concentration dependence of currents through single sodium-selective pores in frog skin. *Nature* **282**, 519-520.

Van Driessche, W. and Zeiske, W. (1985). Ca^{2+}-sensitive, spontaneously fluctuating, cation channels in the apical membrane of adult frog skin epithelium. *Pflügers Arch.* **405**, 250-259.

Zeiske, W., Atia, F. and Van Driessche, W. (1998). Apical Cl^- channels in A6 cells. *J. Membr. Biol.* **166**, 169-178.