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A Calcium-activated and Nucleotide-sensitive Nonselective Cation Channel in M-1 Mouse Cortical Collecting Duct Cells

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Abstract. We recently reported that M-1 mouse cortical collecting duct cells show nonselective cation (NSC) channel activity (*Proc. Natl. Acad. Sci. USA* **89:**10262–10266, 1992). In this study, we further characterize the M-1 NSC channel using single-channel current recordings in excised inside-out patches. The M-1 NSC channel does not discriminate between Na^+, K^+, Rb^+, Cs^+, and Li^+. It has a linear *I-V* relation with a conductance of 22.7 ± 0.5 pS (n = 78) at room temperature. The P_{cation}/P_{anion} ratio is about 60 and there is no measurable conductance for NMDG, Ca^{2+}, Ba^{2+}, and Mn^{2+}. Cytoplasmic calcium activates the M-1 NSC channel at a threshold of 10^{-6} M and depolarization increases channel activity (NP_o). Cytoplasmic application of adenine nucleotides inhibits the M-1 NSC channel. At doses of 10^{-4} M and 10^{-3} M, ATP reduces NP_o by 23% and 69%, respectively.

Furthermore, since ADP (10^{-3} M) reduces NP_o by 93%, the inhibitory effect of adenine nucleotides is not dependent on the presence of a γ-phosphoryl group and therefore does not involve protein phosphorylation. The channel is not significantly affected by 8-Br-cGMP (10^{-4} M) or by cGMP-dependent protein kinase (10^{-7} M) in the presence of 8-Br-cGMP (10^{-5} M) and ATP (10^{-4} M). The NSC channel is not sensitive to amiloride (10^{-4} M cytoplasmic and/or extracellular) but flufenamic acid (10^{-4} M) produces a voltage-dependent block, reducing NP_o by 35% at depolarizing voltages and by 80% at hyperpolarizing voltages.

We conclude that the NSC channel of M-1 mouse cortical collecting duct cells belongs to an emerging family of calcium-activated and nucleotide-sensitive nonselective cation channels. It does not contribute to amiloride-sensitive sodium absorption and is unlikely to be a major route for calcium entry. The channel is normally quiescent but may be activated under special physiological conditions, e.g., during volume regulation.

Key words: Cortical collecting duct — Flufenamic acid — Amiloride — Adenine nucleotides — cGMP-dependent protein kinase — Patch clamp

Introduction

Patch clamp experiments have shown the presence of nonselective cation channels in many excitable and nonexcitable tissues including epithelia. They constitute a heterogeneous group of channels: (i) Ligand-gated channels such as the nicotinic acetylcholine receptor (Changeux, Devillers-Thiéry & Chemouilli, 1984); (ii) Cyclic nucleotide-gated channels such as the cGMP-gated photoreceptor channel (Kaupp, 1991); (iii) Stretch-activated channels (Guharay & Sachs, 1984; Christensen, 1987); (iv) Nonselective cation channels that underly receptor mediated calcium entry (Benham & Tsien, 1987; Fasolato et al. 1993); and (v) Calcium-activated nonselective cation channels (Patridge & Swandulla, 1988; Cook, Poronnik & Young, 1990). Their common property is high selectivity for cations over anions and poor discrimination between sodium and potassium. Whereas detailed information on the molecular structure of the nicotinic acetylcholine receptor and several cyclic nucleotide-gated channels is available, less is known about other nonselective cation channels. Because of the incomplete functional and molecular understanding of these diverse nonselective cation channels, a conclusive classification has not yet been possible (for review *see* Siemen, 1993). To further define their physiological role it is important to determine how various nonselective

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cation channels are functionally and genetically related to each other.

In renal epithelia, nonselective cation channels have been found in the proximal tubule (Gögelein & Greger, 1986; Merot et al. 1988; Marom et al. 1989; Filipovic & Sackin, 1991, 1992), in cortical thick ascending limb cells (Teulon, Paulais & Bouthier, 1987; Paulais & Teulon, 1989; Merot et al. 1991), in rabbit distal bright convoluted tubule (Poncet, Merot & Poujeol, 1992) and in a number of collecting duct preparations (Light et al. 1988; Palmer & Frindt, 1988; Laskowski et al. 1990; Ling, Hinton & Eaton, 1991; Korbmacher, Segal & Boulpaep, 1992*a*; Ahmad et al., 1992; Chraibi et al., 1994; Ono et al., 1994). The physiological role of these channels is not fully understood and may vary in different nephron segments. They may be involved in sodium reabsorption, calcium entry, cell proliferation, and volume regulation, (for review *see* Korbmacher & Barnstable, 1993).

In the present study, we characterize the electrophysiological properties and regulation of the M-1 nonselective cation channel and attempt to functionally classify this channel within the group of nonselective cation channels. We investigated ion selectivity for various monovalent and divalent cations, regulation by cytosolic calcium, ATP, ADP, 8-Br-cGMP and cGMP-dependent protein kinase, and the effects of amiloride and flufenamic acid. The presence of conductance substates in some of the single-channel recordings suggests that the channel may have more than one functional state. Taken together, the properties of the M-1 nonselective cation channel indicate that this channel belongs to a group of calcium-activated nonselective cation channels that are inhibited by cytosolic adenine nucleotides and appear to be widely distributed in secretory epithelial cells (Thorn & Petersen, 1992; Champigny, Verrier & Lazdunski, 1991; Cook et al., 1990). Recent evidence from whole-cell experiments suggests that the nonselective-cation channel described in the present study is involved in volume regulation during cellular shrinkage (Volk, Korbmacher & Frömter, 1994; Korbmacher, Volk & Frömter, 1994).

Part of this work was presented in abstract form (Korbmacher et al., 1992*a* and 1993*a*).

Materials and Methods

Patch Clamp Technique

The conventional patch clamp technique (Hamill et al., 1981) was used to record single-channel currents in excised inside-out membrane patches of M-1 cells. Single channel currents were amplified with an EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) using an ATARI computer system for data acquisition (E9-Screen program) and analysis. Patch pipettes were pulled either from Corning 7052 glass capillaries (A-M Systems, Everett, WA) or from Clark glass capillaries (Clark Electromedical Instruments, Pangbourne, UK), using either a L/M-3PP-A Puller (List Electronics, Darmstadt, Germany) or a Flaming/Brown Micropipette puller Model P-87 from Sutter Instruments Company (San Rafael, CA). Pipettes were coated with Sylgard 184™ and heat-polished. Pipette resistance was 2–12 MΩ in NaCl/Ringer.

Experiments were performed at room temperature in an elongated chamber (25 mm long, 5mm wide, bath depth 2–4 mm) containing 250–500 μl of bath solution. The reference electrode was an Ag/AgCl pellet bathed in the same solution as that used in the pipette, and connected to the bath via an agar/pipette-solution bridge or an agar/3M-KCl bridge in the outflow path of the chamber. The trans-patch potential difference is defined as the negative holding potential ($-V_{pip}$), or cytoplasmic potential referred to pipette potential. Liquid junction potentials occurring at the bridge/bath junction were measured using a 3M KCl flowing boundary electrode and ranged from −4 to +9 mV. For data analysis the $-V_{pip}$ values were corrected accordingly. Upward current deflections correspond to cell membrane outward currents. Data were recorded at 10 kHz bandwidth and stored on videotape after pulse code modulation (PCM 501, Sony, Japan). For data analysis, currents were usually filtered at 200 Hz, and for kinetic analysis at 1 kHz, using an 8-pole Bessel filter, and were read into the computer via the ITC-16 interface of the EPC-9 patch clamp amplifier at a sample rate of 1 or 5 kHz, respectively. The TAC program (Instrutech, Elmont, NY) was used to estimate channel open and closed times. All other computer software for single-channel data analysis was written by J. Disser and A. Rabe in our laboratory. Channel activity was estimated from binned amplitude histograms as the product NP_o, where N is the number of channels and P_o is single-channel open probability. The time integral of current divided by the product of mean single-channel current (i) and total time of recording is equal to NP_o. The program for calculating NP_o from integration of the areas under the peaks of amplitude histograms uses the following equation:

$$NP_o = \Sigma(n_j \cdot \Delta I_j)/(i \cdot \Sigma n_j) \quad (1)$$

where j refers to the j^{th} current amplitude bin and j ranges from 1 to the total number of bins; n_j = number of events within bin j; $\Delta I_j = I_j - I_c$, where I_j is the current of bin j and I_c is the current at which all channels are closed.

Cell Culture

The M-1 mouse cortical collecting duct cell line used in the present study was obtained from Dr. Fejes-Tóth (Stoos et al., 1991). Cells were used from passage 6 through 25 and were handled as described previously (Ahmad et al., 1992; Korbmacher et al., 1993*c*). Briefly, they were passaged with a split ratio of about 1:5 using 0.05% Trypsin/0.53 mM EDTA in calcium- and magnesium-free HBSS (Gibco Laboratories, Grand Island, NY). Cells were maintained in a 5% CO_2 atmosphere at 37°C and the culture medium was exchanged twice a week. PC1 culture medium (Ventrex, Portland, ME) was used and supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. PC1 medium is a low-protein, serum-free medium formulated in a specially modified DME:F12 base. We have previously shown that calf serum is not necessary to promote growth in M-1 cells (Korbmacher et al., 1993*c*). Nevertheless, for most of the patch clamp experiments cells were seeded in PC1 medium, which also contained 5% fetal calf serum as described by Stoos et al. (1991) in their original report on M-1 cells. For patch clamp experiments cells were seeded onto small pieces of glass cover slips (Baxter Scientific Products, McGaw Park, IL) and were used 1–3 days after seeding. Single or subconfluent M-1 cells were used because high resistance seals and successful i/o patches containing nonselective cation channels are more

easily obtained in 1–3 day old cells as compared to 5–14 day old confluent cells.

Solutions and Chemicals

The composition of the solutions is given in mM. The standard bath solution was NaCl/Ringer: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (adjusted to pH 7.5 with NaOH). KCl/Ringer: 140 KCl, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$ 10 HEPES (adjusted to pH 7.5 with KOH). Sucrose/Ringer: 10 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 260 sucrose, 10 HEPES (adjusted to pH 7.5 with NaOH). A calcium-free (0 mM Ca^{2+}) EGTA/NaCl/Ringer's solution was obtained by including 1 mM EGTA instead of 1 mM $CaCl_2$. Solutions with free calcium concentrations of 10^{-5}, 10^{-6}, 10^{-7} or 10^{-8} M were obtained by using EGTA/NaCl/Ringer's solution and adding 1.003, 0.934, 0.582 or 0.122 mM $CaCl_2$, respectively. Pipette solutions were either KCl/Ringer, NaCl/Ringer, or a Na_2SO_4/K_2SO_4/Ringer, which contained: 12.5 NaCl, 30 Na_2SO_4, 12.5 KCl, 30 K_2SO_4, 60 sucrose, 10 HEPES adjusted to pH 7.5 with NaOH/KOH. For testing monovalent cationic selectivity the experiments were started with 150/NaCl/Ringer both in pipette and bath (150 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES adjusted to pH 7.5 with Tris) and then the Na in the bath was replaced by an equal amount of K^+, Cs^+, Rb^+ or $Li.^+$ To test for divalent cation permeability the pipette solution was NaCl/KCl/Ringer containing: 75 NaCl, 75 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (adjusted to pH 7.5 with Tris). Initially the bath solution was 150/NaCl/Ringer, and the NaCl was subsequently replaced by 75 mM of $CaCl_2$, $BaCl_2$, or $MnCl_2$. To maintain osmolality, 75 mM mannitol was added to the divalent cation solutions. N-methyl-D-glucamine (NMDG) was used instead of sodium in 150/NMDG-Cl/Ringer's solution which contained: 150 NMDG-Cl/, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (adjusted to pH 7.5 with Tris). All bath solutions contained 5 mM glucose.

ATP, ADP, and 8-Br-cGMP were purchased from Sigma, Deisenhofen, Germany. Stock solutions (10^{-2} or 10^{-3} M) in NaCl/Ringer were freshly prepared on the day of the experiment and were kept on ice. Immediately prior to use, an aliquot of the stock solution was added to NaCl/Ringer's bath solution to give the desired final concentration.

The cGMP-dependent protein kinase (cGMP-PK) was kindly provided by Dr. S. Lohmann and Prof. Dr. U. Walter (Medizinische Universitätsklinik Würzburg, Germany). It was isolated from bovine lung according to the protocol described by Walter et al. (1980). The kinase was stored at –20°C in a 50% glycerol buffer containing 10 mM potassium phosphate buffer (pH 6.8), 1 mM EDTA, 15 mM β-mercaptoethanol, 20 U/ml Trasylol. The glycerol stock solution from three different enzyme batches contained 7.9, 10.7 and 16.6 μM cGMP-PK, respectively. These enzyme preparations had kinase activities greater than 1 μmol/min/mg cGMP-PK in in vitro kinase assays using Kemptide as a substrate and 5 μM cGMP stimulation. Immediately before use cGMP-PK from the glycerol stock was added to NaCl/Ringer's bath solution to give a final concentration of 10^{-7} M. The bath solutions prior to the application of cGMP-PK contained an equal amount of the 50% glycerol buffer.

Results

Channel Activity in Symmetrical NaCl and Voltage Dependence of Open Probability

Figure 1*A* shows a typical recording of nonselective cation channel activity in an excised inside-out membrane patch with 150 mM NaCl/Ringer in the pipette and in the bath. Current transitions indicate that the patch contains at least three individual channels. The number of channel levels observed per patch ranged from one up to more than ten. Typically, two to four levels were seen. Channel activity was observed in about every fourth excised patch, while the rest of the patches remained 'silent'. No channel activity was observed in the cell-attached configuration prior to excision. As shown in Fig. 1*B* the single-channel *I-V* relationship is ohmic under symmetrical NaCl conditions with a slope conductance of 22.1 ± 0.6 pS (n = 5). Note that the channels shown in Fig. 1*A* are more active at depolarizing holding potentials. This is illustrated in Fig. 1*C* in which the open probability is plotted versus $-V_{pip}$.

Channel Kinetics

Analysis of channel kinetics was limited to the few experiments in which only one channel level was observed in a patch. From those current traces, single-channel dwell-time histograms were generated using a logarithmic time axis and a square-root scaling for the number of events on the ordinate as described by Sigworth and Sine (1987). The positions of the peaks in such histograms correspond directly to the time constants. Dwell-time data from four experiments are summarized in the histograms shown in Fig. 1*D*. The open-time histogram reveals a maximum of events with long durations (up to several hundred msec), while the closed-time histogram peaks at a duration of less than 1 msec. Using a maximum likelihood fitting routine, three open-time constants and three closed-time constants were estimated from these histograms. The open-time constants (with percentage contribution) were: 1.7 msec (15.4%), 83.5 msec (43.1%), and 576 msec (41.5%). The closed-time constants were: 0.3 msec (74.5%), 7.3 msec (14.4%) and 297 msec (11.1%). Given the relatively small number of events analyzed and the rather poor fit obtained, the presence of only two time constants as well as the presence of additional time constants cannot be excluded. Furthermore, only the short closed-time constant of less than 1 msec was consistently found in each of the four experiments, while the other time constants and their relative contribution varied somewhat from patch to patch.

Selectivity for Monovalent Cations

Figure 2 shows an average *I-V* relationship from single-channel current measurements obtained in experiments in which the pipettes were filled with Na_2SO_4/K_2SO_4/Ringer and the bath contained control NaCl/Ringer's solution. Under these conditions E_K is +72 mV, E_{Na} is –18 mV, and E_{Cl} is +48 mV. The reversal potential of 0.7 ± 0.6 mV (n = 37) demonstrates that the channel is a nonselective cation channel which does not discriminate be-

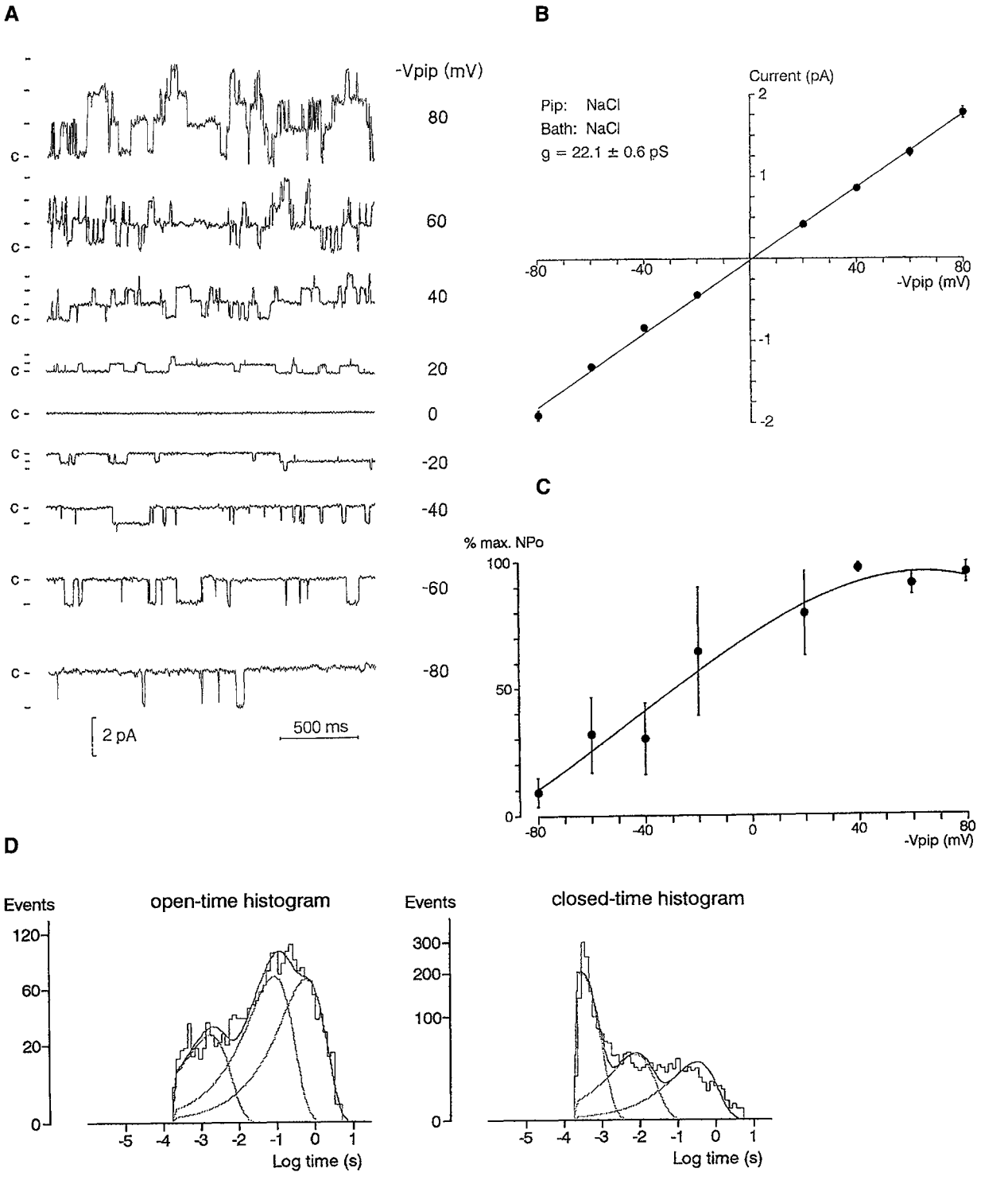

Fig. 1. Nonselective cation channels in excised inside-out patches from M-1 cells. (*A*) Current traces showing nonselective cation channel activity at various trans-patch potentials ($-V_{pip}$) with 150/NaCl/Ringer's solution in pipette and bath. (*B*) Average single-channel *I-V* plot from five experiments similar to that shown in (*A*). Each data point represents an average of at least four measurements. SEM-values are indicated by vertical bars, unless they were smaller than the symbols used. Linear regression yields a single-channel conductance of 22.1 ± 0.6 pS and a reversal potential of E_{Rev} = 0.6 ± 0.7 mV (n = 5). (*C*) Voltage dependence of channel activity (NP_o). In three experiments similar to that shown in (*A*), NP_o was determined at various trans-patch potentials using amplitude histograms that were generated from current records with an average length of 20.5 ± 2.9 sec (range 6–65 sec). NP_o values were normalized to the highest NP_o value observed in each experiment (% max. NP_o). Mean values are plotted *vs.* $-V_{pip}$; vertical bars indicate SEM-values. (*D*) Open-time and closed-time histograms. Cumulative dwell-time data were taken from four experiments in which only one channel level was observed in each patch. Histograms were generated according to the method described by Sigworth and Sine (1987). The pipette contained Na_2SO_4/K_2SO_4/Ringer's solution; the bath solution was NaCl/Ringer. The trans-patch potential ($-V_{pip}$) was 69 mV in three out of the four experiments, and 49 mV in the fourth experiment.

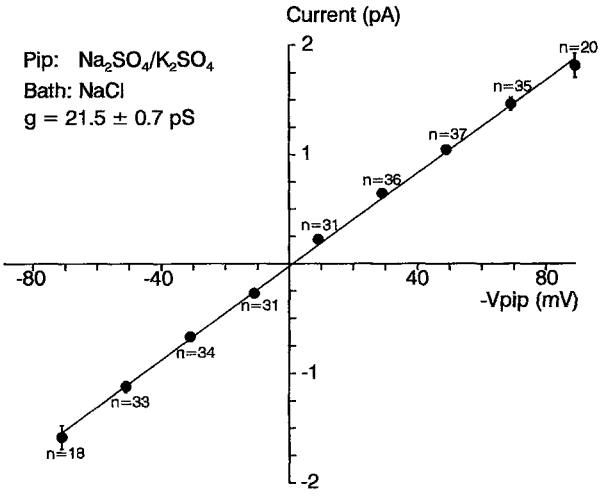

Fig. 2. Average single-channel *I-V* relationship. Single-channel currents were recorded from excised inside-out patches. Pipettes were filled with Na_2SO_4/K_2SO_4/Ringer, and the bath contained NaCl/Ringer's solution. Current amplitudes were measured at different trans-patch potentials $-V_{pip}$ in a total of 37 patches. The filled circles indicate the mean current values obtained in different experiments (n = number of observations at a given trans-patch potential. The vertical bars indicate SEM values unless smaller than the symbol). The average conductance was 21.5 ± 0.7 pS and the average reversal potential was 0.7 ± 0.6 mV (n = 37).

tween sodium and potassium. The average slope conductance in these experiments was 21.5 ± 0.7 pS (n = 37). Summarizing the *I-V* data from all experiments in which the sum of the sodium and potassium concentration was 150 mM ($[Na]_i + [K]_i = [Na]_o + [K]_o = 150$ mM) on each side of the patch membrane yields a single-channel conductance of 22.7 ± 0.5 pS (n = 78).

To investigate the selectivity of the channel for different monovalent cations further, we performed experiments under bi-ionic conditions starting out with symmetrical 150/NaCl/Ringer's solution in bath and pipette. Subsequently, the bath solution was changed to solutions in which the NaCl was replaced by 150 mM KCl, RbCl, CsCl or LiCl. Single-channel *I-V* relationships were obtained before and after changing the bath from sodium to another monovalent cation. Results from four individual experiments are shown in Fig. 3. From these *I-V* plots it is apparent that neither the single-channel conductances nor the reversal potentials are significantly affected when sodium is replaced by any of the four other monovalent cations. This indicates that the M-1 nonselective cation channel does not discriminate between the monovalent cations Na^+, K^+, Rb^+, Cs^+ and Li^+.

Permeability for NMDG and Divalent Cations

When bath sodium was completely replaced by 150 mM NMDG, a large organic monovalent cation, no outward current deflections could be observed (Fig. 4*A*). Similarly, no outward currents were detected after replacing the cytoplasmic NaCl with 75 mM of either $CaCl_2$, $BaCl_2$ or $MnCl_2$/Ringer (Fig. 4 *B,C* and *D*). As shown in Fig. 4, the experimental *I-V* data obtained in the presence of NMDG or in the presence of the three different divalent cations could be fitted using the Goldman-Hodgkin-Katz constant-field equation. These calculations assume that the channel is perfectly selective for monovalent cations, when the monovalent cation concentration is 150 mM on the outside, and nominally 1 mM on the inside. For each condition shown in Fig. 4 (open circles), the resultant theoretical curve based on the limiting inward slope conductance is in good agreement with the experimental data. Therefore, the NMDG permeability of the M-1 nonselective cation channel is negligible, and there is no measurable permeability for divalent cations when present at the cytoplasmic side in a concentration of 75 mM.

Cation Versus Anion Selectivity

Figure 5 shows an experiment in which the cation versus anion selectivity of the channel was tested. The cytoplasmic salt concentration was lowered by changing the bath from NaCl/Ringer's to sucrose/Ringer's solution, which resulted in a shift of the reversal potential to the right. The data were fitted using the Goldman-Hodgkin-Katz equation. In seven similar experiments, as shown in Fig. 5, the reversal potential in sucrose/Ringer's solution averaged 47.9 ± 1.1 mV which corresponds to a permeability ratio of P_{cation}:P_{anion} of 59. This indicates that the M-1 nonselective cation channel is highly selective for cations over anions.

Activation by Cytoplasmic Calcium

We previously reported that the removal of cytoplasmic calcium reversibly abolishes channel activity of the M-1 nonselective cation channel (Ahmad et al., 1992). The response to calcium was further investigated in experiments as in that shown in Fig. 6. Many channels were open under control conditions in the presence of 1 mM cytoplasmic calcium but the channels closed rapidly and completely upon changing the bath solution to calcium-free Ringer's solution (nominally calcium-free and containing 1 mM EGTA). A subsequent stepwise increase of the cytoplasmic calcium revealed a threshold calcium concentration of 10^{-6} M at which channel activity resumed (n = 6). The inset of Fig. 6 summarizes the calcium dependence of the channel open probability for the experiment shown. The same threshold concentration was found in experiments in which the cytoplasmic calcium concentration was reduced in a stepwise manner.

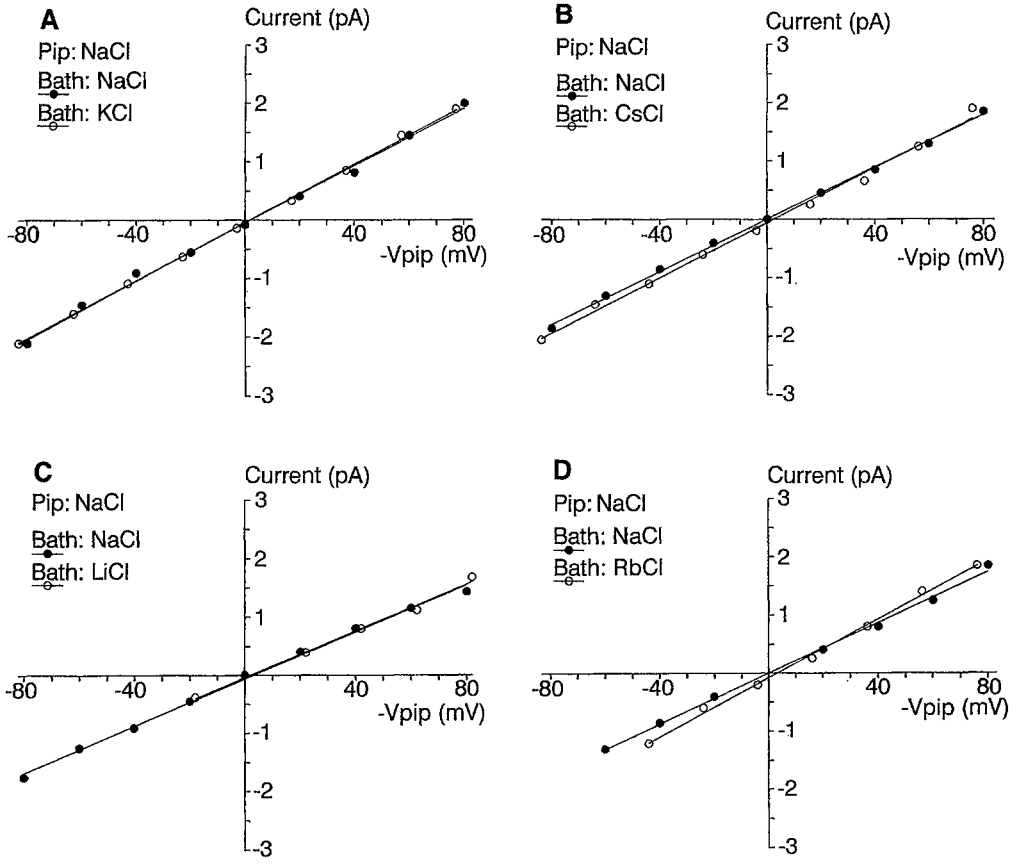

Fig. 3. Bi-ionic experiments with monovalent cations. The pipette contained 150/NaCl/Ringer's solution. Closed circles indicate initial single-channel *I-V* values obtained in symmetrical 150/NaCl/Ringer. Open circles indicate single-channel *I-V* values measured in the same inside-out patch after replacing 150 mM NaCl in the bath with (in mM): (*A*) 150 KCl, (*B*) 150 CsCl, (*C*) 150 LiCl, and (*D*) 150 RbCl.

Channel activity was still observed at a calcium concentration of 10^{-6} M, but was abolished at 10^{-7} M (n = 2).

Effect of ATP and ADP

Several nonselective cation channels have been shown to be inhibited by millimolar concentrations of cytoplasmic ATP. Therefore, we tested the effect of ATP on the M-1 nonselective cation channel. At the start of each experiment, cytoplasmic calcium was removed to determine the current level at which all channels were closed. Readdition of cytoplasmic calcium fully reactivated the channels. As shown in Fig. 7, application of 1 mM ATP reversibly decreased channel activity by more than 50%. To quantify the effects of ATP (and of other substances used later on in this study), channel activities (NP_o) were calculated for each experimental period using amplitude histograms. The peaks of the amplitude histograms were used to calculate single-channel current amplitudes. A typical protocol is illustrated in Fig. 8 which shows an experiment in which the effect of 10^{-4} M ATP was tested. During the control period prior to the application of ATP, up to 11 channels were simultaneously open and NP_o was 10.2. In the presence of 10^{-4} M ATP NP_o fell to 6.39 but it recovered again to 8.75 following washout of ATP. The observed inhibition by ATP could either be a reduction in P_o or in N or a combined effect. However, the amplitude histograms did not allow a clear distinction between these possibilities. Fig. 9 (*A, B*) summarizes the results of similar experiments in which 10^{-4} M ATP or 10^{-3} M ATP was applied. The effect of ATP was concentration-dependent with an average decrease in NP_o of 23.1 ± 10% (n = 11) and 68.5 ± 10.6% (n = 9) for 10^{-4} M and 10^{-3} M ATP, respectively. Interestingly, ADP 10^{-3} M was even more potent than ATP in reducing NP_o with an average decrease of 92.9 ± 2.1% (n = 7, Fig. 9*C*). The inhibition by ADP suggests that the effect is not caused by channel phosphorylation but presumably by interaction with an inhibitory adenine nucleotide binding site on the channel protein which is relatively unselective since it can accommodate both ATP and ADP.

Effect of 8-Br-cGMP and cGMP-dependent Protein Kinase

We previously reported that 10^{-4} M cGMP reduced the open probability of the M-1 nonselective cation channel

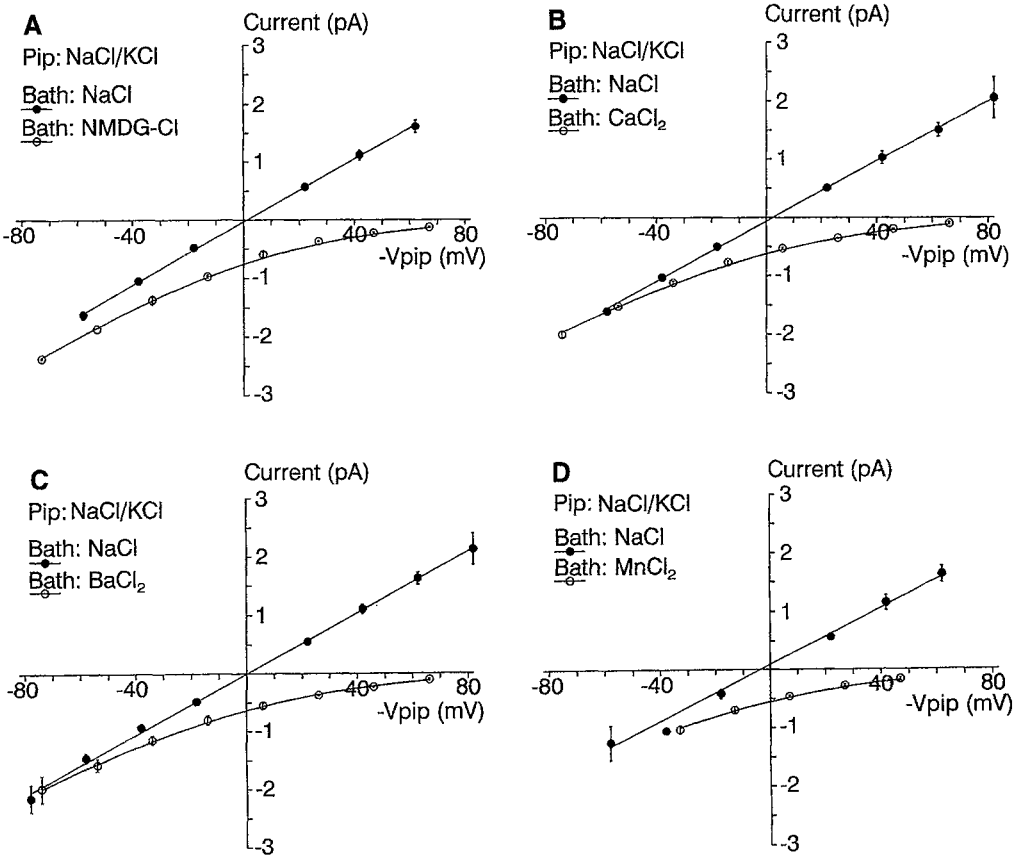

Fig. 4. Bi-ionic experiments with NMDG and divalent cations. The pipette contained KCl/NaCl/Ringer's solution. Closed circles indicate initial single-channel *I-V* values obtained in 150/NaCl/Ringer. Open circles indicate single-channel *I-V* values measured in the same inside-out patches after replacing 150 mM NaCl in the bath with (in mM): (*A*) 150 NMDG-Cl, (*B*) 75 $CaCl_2$, (*C*) 75 $BaCl_2$, or (*D*) 75 $MnCl_2$. Mean values from two to four experiments are given for each condition; vertical bars indicate SEM-values. The *I-V* data were fitted using the Goldman-Hodgkin-Katz equation for a monovalent cation concentration ratio of $[cation]_{in}/[cation]_{out}$ = 150 mM/1mM, and it was assumed that the channel was permeable only to monovalent cations (*see* text).

on average by 27% (Ahmad et al., 1992). However, the magnitude of the cGMP effect varied considerably from patch to patch. This could be due to the presence or absence of factors such as membrane bound protein kinases, phosphodiesterases, or certain substrates in the microenvironment of an excised patch.

In the present study, we used 8-Br-cGMP which is more resistant to hydrolysis by phosphodiesterase than cGMP (Butt et al., 1992). However, application of 10^{-4} M 8-Br-cGMP to the cytoplasmic patch surface also had rather variable effects ranging from a slight increase to a more than 50% decrease of NP_o. On average NP_o decreased by 11.9 ± 5.9% (n = 9), a value which was not statistically significant in paired *t* test. Increasing the concentration of 8-Br-cGMP to 10^{-3} M did not have a more pronounced effect (n = 3).

Exogenous cGMP-dependent protein kinase (10^{-8} M) has been shown to shut down nonselective cation channel activity in inner medullary collecting duct cells (Light, Corbin & Stanton, 1990). Therefore, we tested the effect of 10^{-7} M (n = 10) cGMP-dependent protein kinase in the presence of 10^{-5} M 8-Br-cGMP and 0.1 mM ATP on the M-1 nonselective cation channel. Under these conditions, which are similar to those used in the experiments on the inner medullary collecting duct channel (Light et al., 1990), cGMP-dependent protein kinase should be maximally active. However, channel activity was unaffected: NP_o decreased by 1.8 ± 7.9% (n = 10) which was not statistically significant. Thus, cGMP-PK does not seem to be involved in the regulation of the M-1 nonselective cation channel.

Effect of Flufenamic Acid

The nonsteriodal anti-inflammatory drug flufenamic acid is a potent blocker of nonselective cation channels (Gögelein et al., 1990). We tested the effect of flufenamic acid on the M-1 nonselective cation channel. Fig. 10*A* shows an experiment in which application of 10^{-4} M flufenamic acid reduced nonselective cation channel activity by more than 90%. On average NP_o decreased by

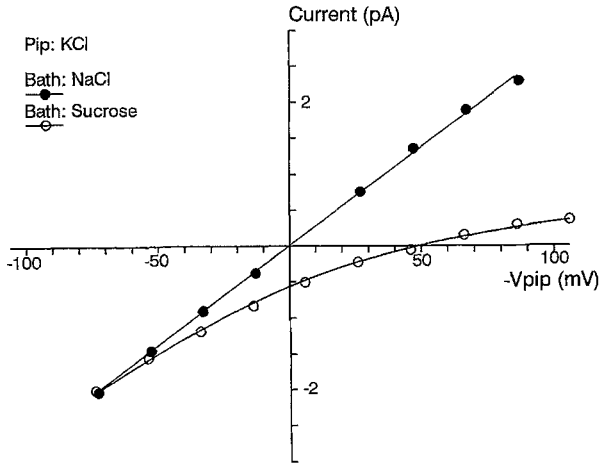

Fig. 5. Salt dilution experiments to test cation versus anion selectivity. Filled circles represent initial single-channel *I-V* values obtained in an inside-out patch excised in NaCl/Ringer's solution with KCl/Ringer as pipette solution. Open circles indicate the *I-V* values from the same patch after changing the bath solution to sucrose/Ringer. The data were fitted using the Goldman-Hodgkin-Katz equation for a monovalent cation concentration ratio of $[cation]_{in}/[cation]_{out}$ = 20 mM/150 mM, and an anion concentration ratio of $[Cl]_{in}/[Cl]_{out}$ = 19 mM/149 mM. The least-squares fit of the data yields a reversal potential of $-V_{pip}$ = 48.8 mV, in good agreement with the predicted value of 50.9 mV for a channel perfectly selective for monovalent cations.

58.8 ± 8.2% (n = 15). Closer inspection of the data revealed a voltage dependence of the flufenamic acid block. At hyperpolarizing voltages (between −80 and −30 mV) 10^{-4} M flufenamic acid decreased NP_o by 80.0 ± 7.8% (n = 8, Fig. 10*B*) while at depolarizing voltages (between +50 and +70 mV) it decreased NP_o by only 34.6 ± 8.3% (n = 7, Fig. 10*C*). The degree of inhibition was significantly different between hyperpolarization and depolarization ($P < 0.001$, unpaired *t*-test).

Effect of Amiloride

A nonselective cation channel in the inner medullary collecting duct has been reported to be inhibited in the presence of 0.5 μM extracellular amiloride at hyperpolarizing voltages (Light et al., 1988). Amiloride effectively penetrates biological membranes and is believed to interact with the pore of the Na-selective channel from the outside (Garty & Benos, 1988). However, cytoplasmic application of 10^{-4} M amiloride did not significantly affect the open probability of M-1 nonselective cation channels (n = 7). In additional experiments amiloride was included in the pipette solution. Amiloride is positively charged and an amiloride block should therefore be more pronounced at hyperpolarizing voltages. However, channel activity was maintained in the presence of 10^{-4} M extracellular amiloride even at hyperpolarizing potentials (n = 4). These experiments demonstrate that the M-1 nonselective cation channel is not amiloride-sensitive.

Multiple Conductance States

Close inspection of the current trace in Fig. 11 shows that small current transitions are superimposed on the various levels of large transitions. In this experiment, the current amplitudes of the small transitions could be resolved at various holding potentials. The *I-V* relationships of the small and the large transitions are shown in the lower part of Fig. 11. The single-channel conductance calculated for the small transitions was 8.4 pS and for the large transitions 21.4 pS. Since the pipette solution was Na_2SO_4/K_2SO_4/Ringer and the bath solution was NaCl/Ringer, the reversal potential of close to 0 mV indicates that the channels underlying the large and the small transitions are nonselective cation channels.

Multiple conductance states were only occasionally observed in our experiments and the small superimposed current transitions were difficult to analyze since they usually occurred together with several large channel transitions. Moreover, the amplitudes of the small conductance states were quite variable and ranged from about one-fourth to two-thirds of the amplitude of the large conductance states (*data not shown*). While in the majority of patches channel transitions corresponding to the "usual" 20–28 pS nonselective cation channel were predominant, in some experiments only small transitions were seen initially after excision. In the course of such experiments, the pattern of channel activity could change spontaneously from small to large transitions with or without additional subconductance levels. Small current transitions were sensitive to calcium removal and application of ATP in the same way as the large transitions (*data not shown*). Taken together our data indicate that the multiple conductance states are related to the same nonselective cation channel.

Discussion

The nonselective cation channel which we have observed in inside/out membrane patches of M-1 cells is activated by elevation of the cytosolic calcium concentration above 1 μM and is strongly inhibited by cytosolic ATP in the physiological concentration range. These features indicate that the M-1 nonselective cation channel differs from the majority of channels listed in the Introduction, and that it belongs to a separate family of nonselective cation channels, members of which have been described in various preparations (Sturgess, Hales & Ashford, 1987; Paulais & Teulon, 1989; Cook et al., 1990; Champigny et al., 1991; Popp & Gögelein, 1992; Thorn & Petersen, 1992; Van den Abbeele, Huy & Teulon,

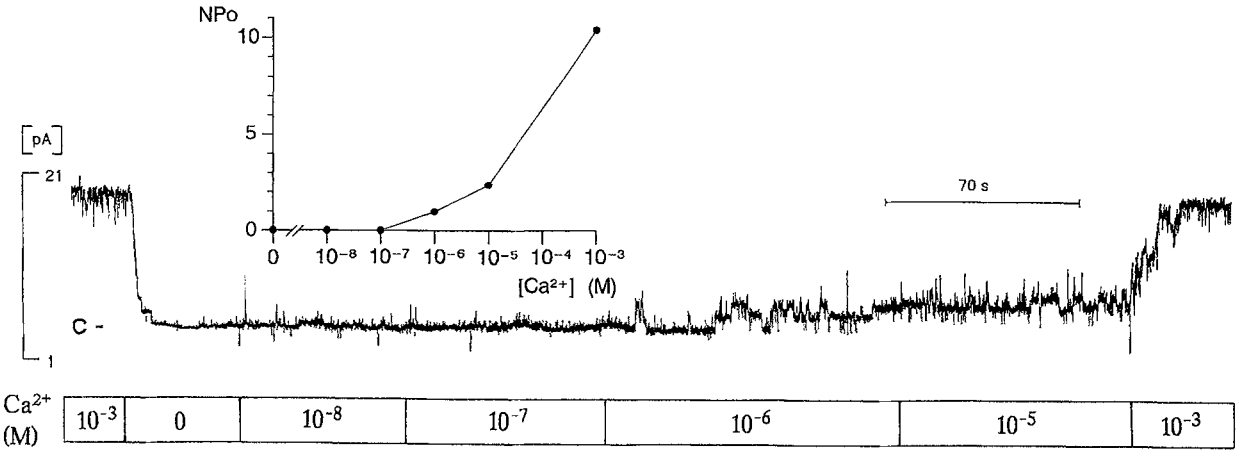

Fig. 6. Activation by cytoplasmic calcium. Nonselective cation channel activity in an inside-out patch excised in NaCl/Ringer's solution with Na_2SO_4/K_2SO_4/Ringer as pipette solution at a trans-patch potential of $-V_{pip} = 49$ mV. Cytoplasmic calcium concentration was varied as indicated below the current trace. Upward current deflections correspond to channel openings, the symbol (c -) indicates the current level at which all channels are closed. The inset shows a plot of channel activity (NP_o) versus calcium concentration.

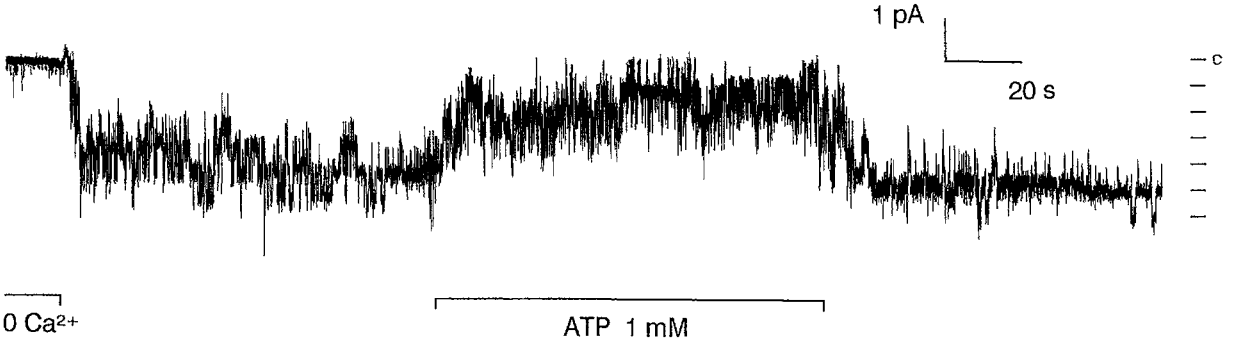

Fig. 7. Inhibition by cytoplasmic ATP. Nonselective cation channel activity in an inside-out patch excised in NaCl/Ringer's solution with Na_2SO_4/K_2SO_4/Ringer as pipette solution at a trans-patch potential of $-V_{pip} = -51$ mV. The duration of the cytoplasmic application of 1 mM ATP and the initial absence of cytoplasmic calcium are indicated by the bars below the current trace.

1994; Chraibi et al., 1994; Ono et al., 1994). The nonselective cation channels in all these preparations require at least micromolar calcium concentrations at the cytosolic surface of inside-out patches for activation, and are inhibited by cytosolic ATP concentrations in the millimolar range. Thus, a common problem with all members of this group is how these channels could be activated in a normal living cell. This pertains also to the M-1 cells in which the normal intracellular calcium concentration is 135 nM (Korbmacher et al., 1993*b*) which is well below the threshold concentration of 1 μM necessary for channel activation in excised patches. Moreover, we know that normal intracellular ATP levels of collecting duct cells range from 2 to 3 mM (Uchida & Endou, 1988). These concentrations should suppress channel activity even if the cytosolic calcium concentration rose above threshold. Hence the normal intracellular milieu does not favor channel activation, and this probably explains why these channels are normally quiescent and cannot be observed in cell-attached or whole-cell patch-clamp experiments. This is also in agreement with our previous whole-cell study on M-1 cells which did not reveal a significant contribution of a nonselective cation conductance to the overall whole-cell conductance under resting conditions (Korbmacher et al., 1993*c*). This raises the question of the physiological significance of these channels.

Indeed, the physiological role of any of the calcium-activated and ATP-inhibited nonselective cation channels described in the literature, is only poorly understood. In particular, their mode of activation under physiological conditions is in most cases unknown. However, it has recently been demonstrated, both in cell-attached and whole-cell patch clamp experiments in mouse pancreatic acinar cells, that calcium activated, ATP-inhibited nonselective cation channels can be stimulated by physiological concentrations of cholecystokinin, and it has been suggested that the stimulated chan-

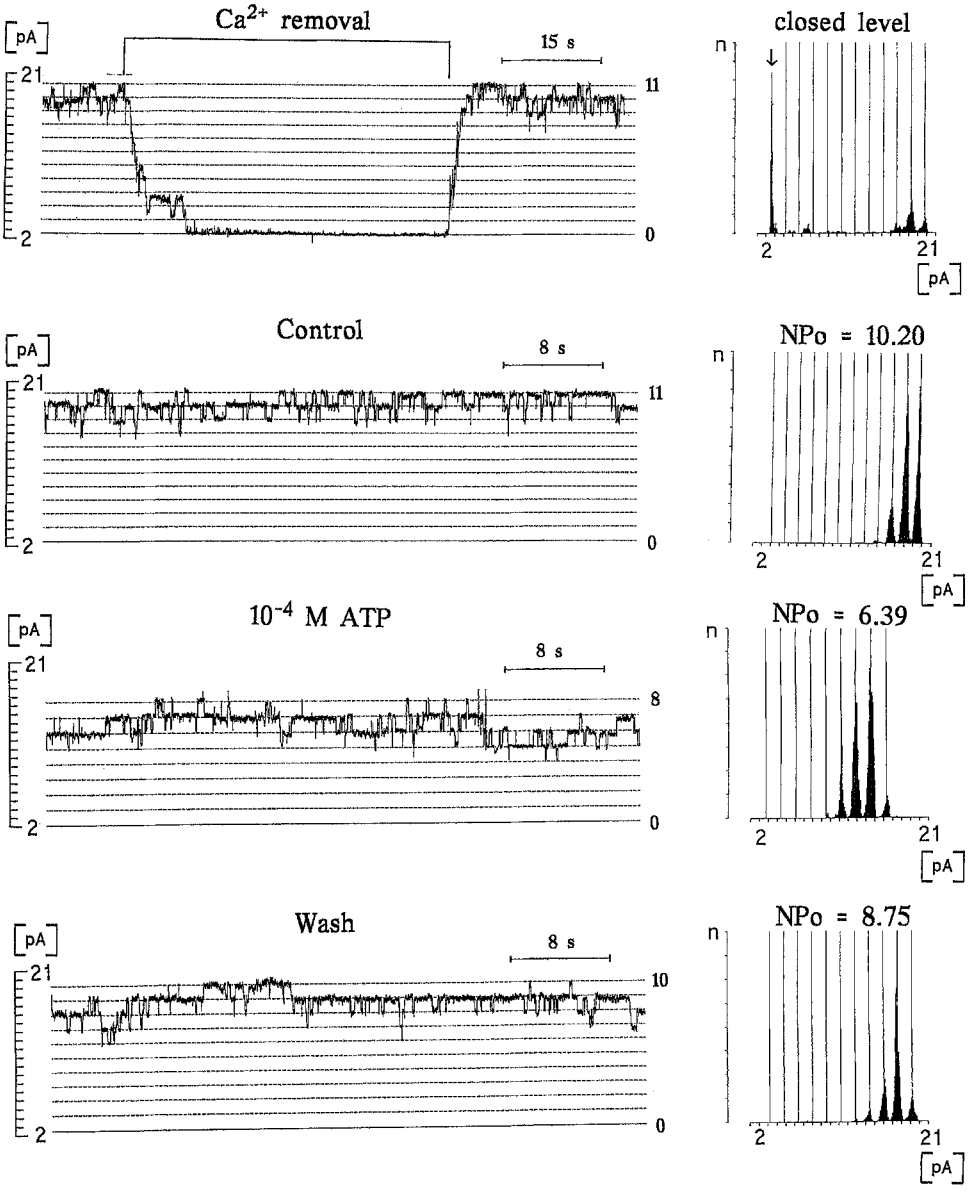

Fig. 8. Quantification of the reversible effect of ATP on channel activity (NP_o) using amplitude histograms. Four sequential current traces are shown that were obtained from an inside-out patch at a trans-patch potential of $-V_{pip}$ = 69 mV. Bath solution was NaCl/Ringer; the pipette contained Na_2SO_4/K_2SO_4/Ringer's solution. The top trace demonstrates a calcium removal maneuver to determine the current level at which all channels are closed. The second, third and fourth traces show nonselective cation channel activity under control conditions, in the presence of 10^{-4} M ATP, and after wash out of ATP, respectively. The corresponding amplitude histograms and NP_o values are shown to the right of each trace.

nels may serve in fluid secretion (Thorn & Petersen, 1992). Furthermore, it has recently been shown that nonselective cation channels are activated in vascular endothelial cells under the influence of pathophysiologically relevant concentrations (5 μM) of histamine (Nilius, 1990; Nilius et al., 1993). Moreover, stimulation of a calcium-activated nonselective cation channel has recently been observed in cell-attached patches of distal lung epithelium under influence of the β_2-agonist terbutaline. Interestingly, this activation was associated with an increased calcium sensitivity of the channels by a factor of 100 to 1000 that persisted when the membrane patches were excised (Tohda et al., 1994). Taken together, these findings suggest that calcium-activated nucleotide-sensitive nonselective cation channels are normally quiescent but may be activated under special physiological and pathophysiological conditions.

Physiological Role of the Collecting Duct Nonselective Cation Channel

Nonselective cation channels have previously been described by others on the apical membrane of confluent

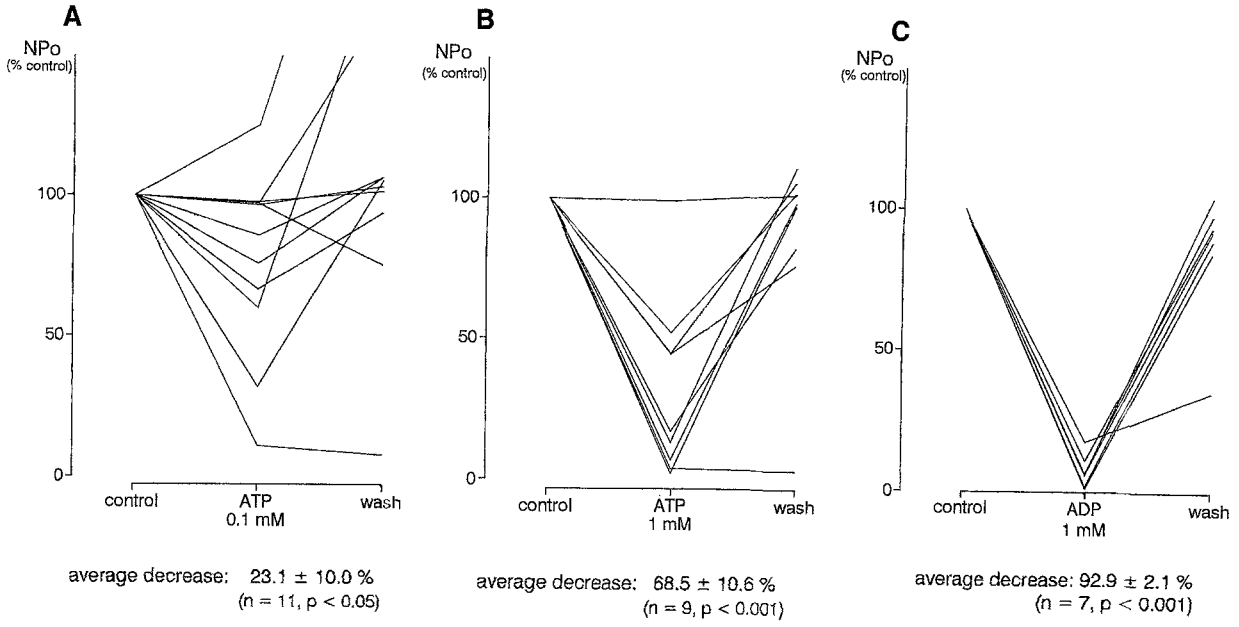

Fig. 9. Effect of adenine nucleotides on NP_o. Summary of results from experiments in which the effect of (*A*) 0.1 mM ATP, (*B*) 1 mM ATP, and (*C*) 1 mM ADP were tested. Experiments were performed according to the protocol illustrated in Figs. 7 and 8. NP_o was calculated for each experimental period and NP_o values were normalized to the control NP_o (% control). Each line represents a single experiment; arithmetic means ± SEM are given below each panel. Statistical significance was evaluated using the paired *t*-test.

cortical collecting duct cells grown on permeable support (Laskowski et al., 1990; Ling et al., 1991) and also in microdissected cortical collecting ducts from rat (Palmer & Frindt, 1988) and mouse (Chraibi et al., 1994) in the apical and basolateral membrane, respectively. Moreover, we have occasionally observed nonselective cation channels in i/o patches from the apical membrane of confluent M-1 cells (*data not shown*), which form a polarized epithelium with tight junctions and express a highly Na-selective amiloride-sensitive whole-cell conductance (Korbmacher et al., 1993*c*). Thus, we may safely assume that nonselective cation channels are present in cortical collecting duct cells under physiological conditions and are not a peculiarity of special tissue culture growth conditions. However, the physiological function and mechanism of activation of a calcium-activated nucleotide-sensitive nonselective cation channel in collecting duct epithelium is not readily evident. Because of the involvement of the collecting duct in sodium absorption, and because collecting duct function is finely controlled by different hormones (Breyer & Ando, 1994), it has been speculated that nonselective cation channels might be involved in transepithelial sodium absorption or might serve as calcium entry pathway in the signal transduction process following hormonal stimulation. Moreover, the nonselective cation channels may contribute to volume regulatory processes which are known to play an important role in this nephron segment.

Sodium Absorption

Amiloride-sensitive sodium reabsorption is a characteristic transport function of the renal collecting duct epithelium (O'Neil & Boulpaep, 1979, 1982) and small conductance, amiloride-sensitive channels which are highly selective for sodium are believed to be the major route for sodium entry across the apical membrane (Garty & Benos, 1988; Palmer, 1992). In addition, it has been suggested that nonselective cation channels may contribute to amiloride-sensitive sodium reabsorption in the collecting duct, since the nonselective cation channel in inner medullary collecting duct cells has been reported to be sensitive to 0.5 μM extracellular amiloride (Light et al., 1988). Our studies clearly demonstrate that the M-1 nonselective cation channel is neither affected by cytoplasmic application of 10^{-4} M amiloride, nor by the presence of 10^{-4} M extracellular amiloride, even at hyperpolarizing potentials which should favor a voltage-dependent amiloride block. We have previously shown that confluent M-1 cells express amiloride-sensitive whole-cell currents which are highly selective for sodium (Korbmacher et al., 1993*c*). The presence of an amiloride-sensitive sodium selective whole-cell current, and the absence of amiloride inhibition of the M-1 nonselective cation channel, argue against a contribution of nonselective cation channels to amiloride-sensitive sodium reabsorption in cortical collecting duct cells.

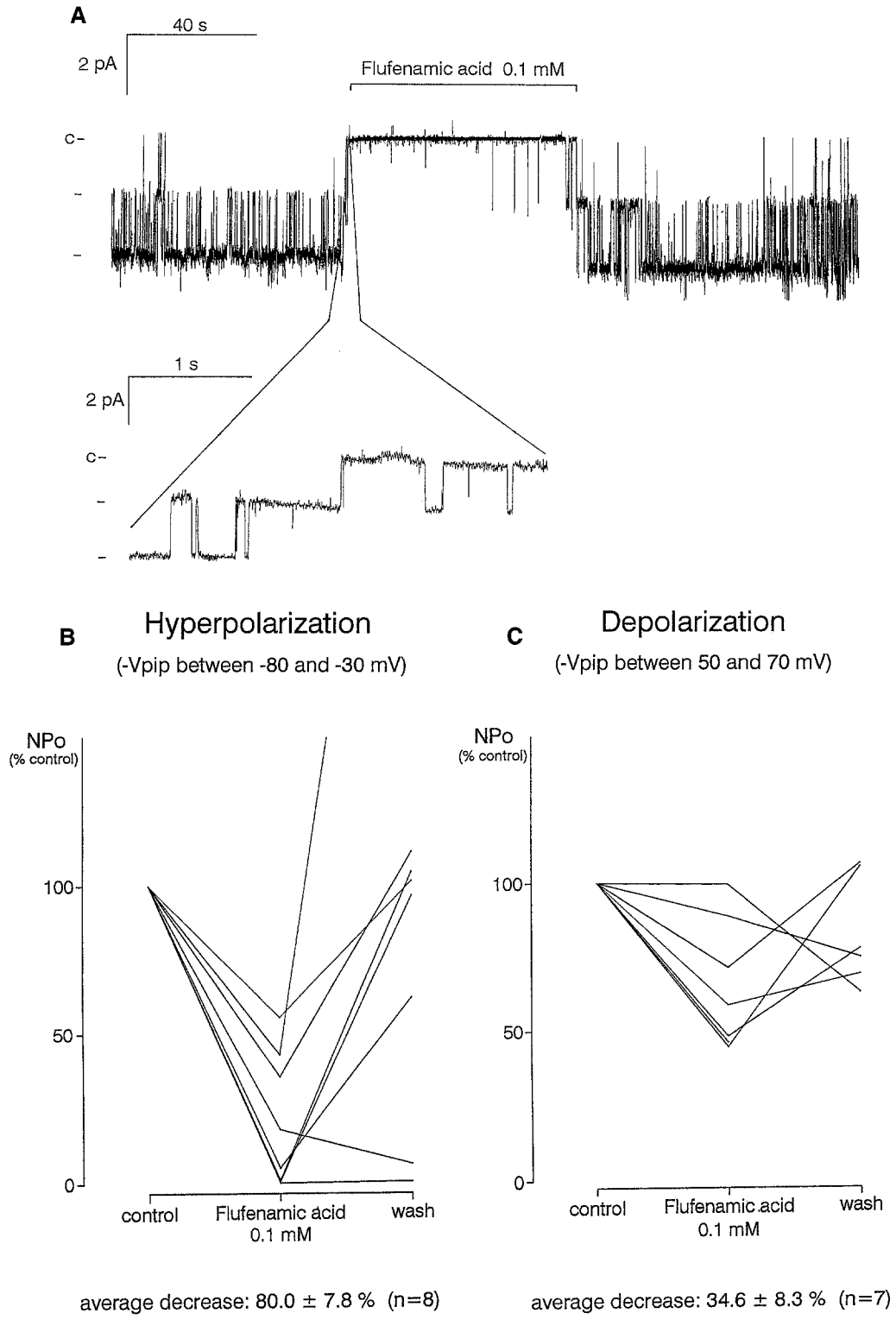

Fig. 10. Voltage-dependent block by flufenamic acid. Flufenamic acid (0.1 mM) was applied to the cytosolic surface of inside-out patches. (*A*) Current recording from an inside-out patch held at $-V_{pip} = -80$ mV. Pipette contained KCl/Ringer's solution; bath contained NaCl/Ringer. Flufenamic acid was present during the time indicated by the horizontal bar. The inset shows the onset of the flufenamic acid block on an extended time scale. Results from similar experiments to that shown in (*A*) are summarized in (*B*) and (*C*). The effect of flufenamic acid was tested at (*B*) hyperpolarizing patch potentials ($-V_{pip}$ between −80 and −30 mV), or at (*C*) depolarizing patch potentials ($-V_{pip}$ between 50 and 70 mV). Each line represents a single experiment; the average decrease in NP_o is given below each panel as arithmetic mean ± SEM. Statistical significance was evaluated using the paired *t*-test.

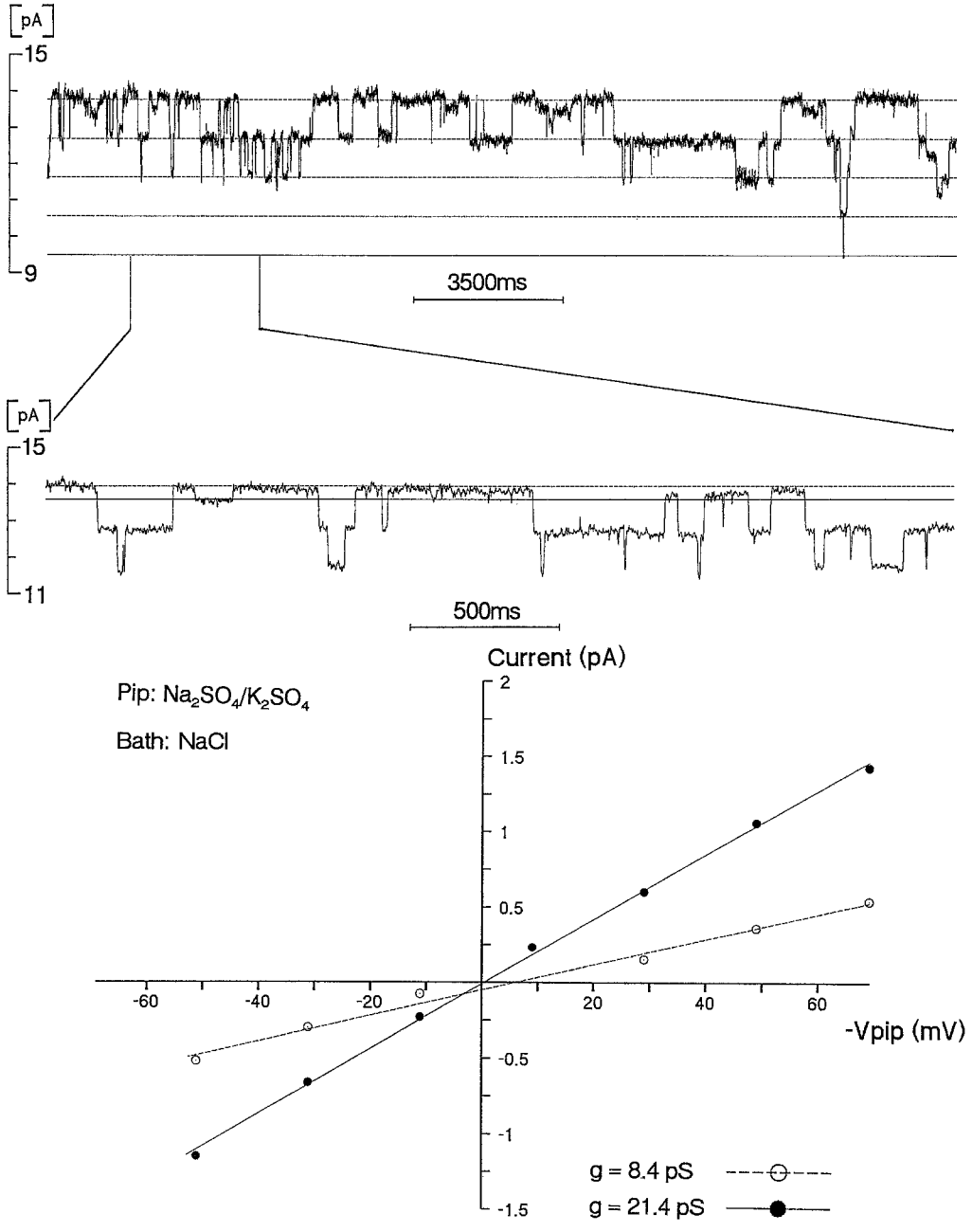

Fig. 11. Channel subconductance states. The current trace at the top shows channel activity at $-V_{pip}$ = 49 mV. Pipette contained Na_2SO_4/K_2SO_4/Ringer's solution and the bath contained NaCl/Ringer. The middle trace portrays on an expanded time scale small as well as large transitions. The *I-V* plots for each conductance are shown below. The large transitions have a conductance of 21.4 pS (filled circles); the small transitions have a conductance of 8.4 pS (open circles).

Calcium Entry

In renal epithelial cells, calcium entry mechanisms seem to be important for calcium transport (Friedmann & Gesek, 1993) and for calcium-dependent signaling mechanisms involved in the regulation of salt and water transport. Hormones like AVP, PGE_2, EGF, muscarinic cholinergic agents and bradykinin have been shown to increase intracellular calcium in various cortical collecting duct preparations (Breyer, 1991). In addition, intracellular calcium measurements using fura-2 have demonstrated that the peptide hormone endothelium stimulates calcium entry in microdissected mouse cortical collecting tubules as well as in cultured M-1 mouse cortical collecting duct cells (Naruse et al., 1991; Korbmacher et al., 1993*b*). The channels mediating calcium entry have not yet been identified but it has been speculated that nonselective cation channels might be involved.

Although in our present study we have not been able to detect any single-channel currents carried by divalent cations this does not rule out the possibility that the M-1 nonselective cation channels have a subpicosiemens conductance for calcium. Even such a small single channel calcium conductance may be of physiological importance, since whole-cell calcium currents of only about 2 pA suffice to alter $[Ca]_i$ at a rate of 100 nM s^{-1} (Neher, 1992). Nevertheless, we may conclude that the nonselective cation channel is not a major calcium entry pathway for M-1 cells.

Volume Regulation

Since collecting duct cells in vivo are exposed to a wide range of extracellular osmolality, potent volume regulatory mechanisms are needed for the maintenance of cell volume and epithelial transport (Sun & Hebert, 1989; Natke, 1990). Since Chan and Nelson (1992) have reported that extracellular hypertonicity stimulates a nonselective cation conductance in lung alveolar epithelial cells, we have recently looked for a similar effect in M-1 cells. Indeed, using the whole-cell patch clamp technique we could demonstrate that extracellular hypertonicity stimulates a nonselective cation conductance in M-1 cells and that the underlying channels are identical to the channels characterized in the present study in excised inside-out patches (Volk et al., 1994; Korbmacher et al., 1994). From these data, which will be reported in detail in a separate publication (*submitted*), we conclude that the calcium-activated nucleotide-sensitive channels may play an important role in volume regulation during cellular shrinkage. Interestingly, the nonselective cation channels do not seem to respond directly to mechanical stress, since preliminary experiments in which we applied additional suction to the patch pipette did not result in a change of channel activity (*unpublished observation*).

Further Characterization of the Channel

Inhibition by Flufenamic Acid

Highly specific blockers for nonselective cation channels are not yet available. However, derivatives of diphenylaminecarboxylate (DPC), such as flufenamic acid, not only inhibit chloride channels but have also been shown to block nonselective cation channels (Gögelein & Pfannmüller, 1989; Gögelein et al., 1990). Flufenamic acid acts by increasing the channel's closed time, i.e., it induces a slow channel block rather than a flicker block (Siemer & Gögelein, 1992). This is consistent with our observations in M-1 cells. In addition, we demonstrate that flufenamic acid blocks the channel in a voltage-dependent manner. At pH 7.5 flufenamic acid is anionic. Therefore, the simplest explanation of the voltage-dependent block is that the flufenamate binding site is inside the pore and partway across the transmembrane electric field (Woodhull, 1973). Another interpretation of the voltage-dependent block is that flufenamic acid has a higher affinity for a particular state of the channel which is favored when the membrane is hyperpolarized. To our knowledge a voltage-dependence of the flufenamic acid block has not yet been described in any other preparation.

Role of cGMP

We recently reported that M-1 mouse cortical collecting duct cells express a gene related to the nonselective cation channel of the photoreceptor (Ahmad et al., 1992; Korbmacher & Barnstable, 1993). Cyclic nucleotide-gated nonselective cation channels, such as the photoreceptor channel, are directly activated by cGMP (Kaupp 1991). In contrast, in our previous study we observed on average a 27% reduction of the M-1 channel open probability in the presence of 10^{-4} M cGMP (Ahmad et al., 1992). In the present study, the average effect of 10^{-4} M 8-Br-cGMP did not reach statistical significance. Yet, the slight inhibitory trend observed appears consistent with our previous observations with cGMP. In any case, the fact that neither cGMP nor 8-Br-cGMP significantly stimulate the M-1 nonselective cation channel clearly distinguishes this channel from the photoreceptor channel. Moreover, both channels are affected differently by divalent cations. Whereas the photoreceptor is blocked by divalent cations, the M-1 channel requires cytoplasmic calcium for channel activation. There are, however, a number of similarities between the photoreceptor channel and the nonselective cation channel in M-1 cells. These include a similar single-channel conductance, a linear single-channel *I-V* relationship, and an increase in open probability at more depolarized voltages. Thus, at present it remains unclear whether the expression of the gene is related to the ion channel activity seen in patch clamp experiments.

Role of cGMP-PK

The nonselective cation channel in the inner medullary collecting duct is inhibited by cGMP by a dual mechanism. Cytoplasmic application of 10^{-4} M cGMP reduced channel open probability directly by about 40% (Light et al., 1989; Light et al., 1990) while simultaneous application of cGMP and cGMP-PK almost completely shut down channel activity (Light et al., 1990). In contrast, in excised patches of M-1 cells we did not detect any channel inhibition upon application of cGMP-dependent protein kinase in the presence of 10^{-5} M 8-Br-cGMP and 0.1 mM ATP. Considering in addition the different respon-

siveness to amiloride and calcium (Light et al., 1988), it is likely that the channels of M-1 cells and inner medullary collecting duct cells belong to different groups of nonselective cation channels.

Channel Substates

The M-1 nonselective cation channels can show multiple conductance states which are difficult to resolve. The observed subconductance amplitudes were quite variable and ranged from about one fourth to two thirds of the amplitude of the large conductance states and it is not yet clear how many different discrete subconductance states may exist, whether all of them reflect conductance sublevels of the same nonselective cation channel, or whether different nonselective cation channel subtypes exist in M-1 cells. To our knowledge there is only one report in the literature on multiple conductance states of a calcium-activated nonselective cation channel (von Tscharner et al., 1986). However, several subconductance states have been detected in single-channel recordings from photoreceptor channels (Zimmerman & Baylor, 1986; Hanke, Cook & Kaupp, 1988; Bennett et al., 1989). Interestingly, a recent report (Chen et al., 1993) on the cloning of a new subunit of the cyclic nucleotide-gated cation channel in retinal rods indeed suggests that the native photoreceptor channel has a hetero-oligomeric structure and that the subunit composition may be responsible for different biophysical properties of the channel, including subconductance levels. Thus, our observation of subconductance states suggests that the M-1 nonselective cation channel may also have a hetero-oligomeric subunit composition.

Conclusions

We have characterized a nonselective cation channel in inside-out patches of M-1 mouse cortical collecting duct cells. The single-channel properties described in the present study permit a functional classification of the channel. First, the M-1 nonselective cation channel seems to be different from the nonselective cation channel described in inner medullary collecting duct cells (Light et al., 1988, 1990) since the M-1 channel is neither regulated by cGMP-dependent protein kinase nor inhibited by amiloride. The lack of amiloride sensitivity of the M-1 nonselective cation channel argues against a physiological role in sodium reabsorption. Second, while the channel does not discriminate between the monovalent cations Na^+, K^+, Rb^+, Cs^+ and Li^+, it has no measurable single channel conductance for divalent cations. This argues against a major role of the channel as calcium entry mechanism. Third, the calcium activation and the lack of a stimulatory cGMP effect distinguish the M-1 nonselective cation channel from the photoreceptor channel (Kaupp, 1991). This functional distinction is of interest because the M-1 cells express a gene related to the photoreceptor channel (Ahmad et al., 1992). If the expressed gene is related to the nonselective cation channel activity seen, it would be interesting to determine whether a different subunit composition is responsible for the difference in channel function. Finally, the combination of calcium activation, sensitivity to cytosolic ATP as well as ADP, and inhibition by flufenamic acid indicate that the M-1 channel belongs to an emerging family of calcium-activated and nucleotide-sensitive nonselective cation channels. Members of this family seem to be expressed in a variety of tissues including insulin-secreting cells (Sturgess et al., 1987), outer hair cells of the cochlea (Van den Abbeele et al., 1994), endothelial cells (Popp & Gögelein, 1992) as well as epithelial cells (Maruyama & Petersen, 1982; Gögelein & Pfannmüller, 1989; Paulais & Teulon, 1989; Cook et al., 1990; Champigny et al., 1991; Thorn & Petersen, 1992; Siemer & Gögelein, 1992, 1993; Chraibi et al., 1994; Ono et al., 1994). The normal intracellular milieu with low calcium concentrations and high adenine nucleotide concentrations does not favor channel activity under resting conditions. However, different hormonal stimuli and signal transduction pathways may be responsible for channel activation under certain physiological and pathophysiological conditions (Thorn & Petersen, 1992; Nilius, 1990; Nilius et al., 1993; Siemer & Gögelein, 1993; Tohda et al., 1994). An exciting new finding has come from recent whole-cell experiments in M-1 cells which indicate that the calcium-activated and nucleotide-sensitive nonselective cation channel described in the present study is activated by extracellular hypertonicity. These results indicate that in the collecting duct normally quiescent, nonselective cation channels may be important for volume regulation during cellular shrinkage (Volk et al., 1994; Korbmacher et al., 1994).

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