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Amiloride-sensitive Sodium Channels in Confluent M-1 Mouse Cortical Collecting Duct Cells

B. Letz[1], A. Ackermann[1], C.M. Canessa[2]*, B.C. Rossier[2], C. Korbmacher[1]

[1]Zentrum der Physiologie, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany
[2]Institut de Pharmacologie et de Toxicologie, Université de Lausanne, Switzerland

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Abstract. Confluent M-1 cells show electrogenic Na^+ absorption and possess an amiloride-sensitive Na^+-conductance (Korbmacher et al., *J. Gen. Physiol.* **102:**761–793, 1993). In the present study, we further characterized this conductance and identified the underlying single channels using conventional patch clamp technique. Moreover, we isolated poly(A)$^+$ RNA from M-1 cells to express the channels in *Xenopus laevis* oocytes, and to check for the presence of transcripts related to the epithelial Na^+ channel recently cloned from rat colon (Canessa et al., *Nature* **361:**467–470, 1993).

Patch clamp experiments were performed in 6–13-day-old confluent M-1 cells at 37°C. In whole-cell experiments application of 10^{-5} M amiloride caused a hyperpolarization of 24.9, SEM ± 2.2 mV (n = 35) and a reduction of the inward current by 107 ± 10 pA (n = 51) at a holding potential of –60 mV. Complete removal of bath Na^+ had similar effects, indicating that the amiloride-sensitive component of the inward current is a Na^+ current. The effect of amiloride was concentration-dependent with half-inhibition at 0.22 μM. The Na^+ current saturated with increasing extracellular Na^+ concentrations with an apparent K_m of 24 mM. Na^+ replacement for Li^+ demonstrated a higher apical membrane conductance for Li^+ than for Na^+. In excised inside-out (i/o) or outside-out (o/o) patches from the apical membrane, we observed single-channels which showed slow kinetics and were reversibly inhibited by amiloride. Their average conductance for Na^+ was 6.8 ± 0.5 pS (n = 15) and for Li^+ 11.2 ± 1.0 pS (n = 14). They had no measurable conductance for K^+. In o/o patches, channel activity was slightly voltage dependent with an open probability (NP_o) of 0.46 ± 0.14 and 0.16 ± 0.05 at a holding potential of –100 and 0 mV, respectively (n = 8, $P < 0.05$).

Using the two-microelectrode voltage-clamp technique, we assayed defolliculated stage V-VI *Xenopus* oocytes for an amiloride-sensitive inward current 1–6 days after injection with H_2O or with 20–50 ng of M-1 poly(A)$^+$ RNA. In poly(A)$^+$ RNA-injected oocytes held at –60 or –100 mV application of amiloride (2 μM) reduced the Na-inward current by 25.5 ± 4.6 nA (n = 25) while it had no effect in H_2O-injected oocytes (n = 19). Northern blot analysis of M-1 poly(A$^+$) RNA revealed the presence of transcripts related to the three known subunits of the rat colon Na^+ channel (Canessa et al., *Nature* **367:**463–467, 1994).

We conclude that the channel in M-1 cells is closely related to the amiloride-sensitive epithelial Na^+ channel in the rat colon and that the M-1 cell line provides a useful tool to investigate the biophysical and molecular properties of the corresponding channel in the cortical collecting duct.

Key words: Epithelial sodium channel — renal epithelial cell line — cortical collecting duct — amiloride — single-channel recordings — *Xenopus laevis* oocytes — Northern blot analysis

Introduction

An apical amiloride-sensitive sodium conductance is present in various sodium transporting epithelia including the renal distal tubule, urinary bladder, distal colon, sweat ducts and respiratory epithelial cells (for recent reviews *see:* Palmer, 1992; Horisberger, Canessa & Ros-

**Present address:* Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT

Correspondence to: C. Korbmacher

sier, 1993; Rossier et al., 1994; Benos et al., 1995). In all these epithelia, sodium ions enter the cell through the apical membrane and are pumped out basolaterally *via* the Na/K-ATPase. The apical sodium entry is believed to be the rate-limiting step for sodium reabsorption. The exact molecular nature and versatility of the single channels underlying the amiloride-sensitive sodium conductance in these tissues is still a matter of debate and several distinct channel subtypes may exist. A recent major breakthrough for the understanding of this putative channel family has been the molecular identification of the amiloride-sensitive epithelial sodium channel from rat colon by expression cloning in *Xenopus* oocytes (Canessa et al., 1993; Lingueglia et al., 1993). This channel consists of at least three different subunits (Canessa et al., 1994) and Northern blot analyses of the distribution of the rat colon sodium channel in other tissues have indicated that related channel transcripts are also present in kidney and lung (Canessa et al., 1993, 1994; O'Brodovich et al., 1993; Renard et al., 1995).

In the kidney, amiloride-sensitive sodium reabsorption is a characteristic feature of the cortical collecting duct (O'Neil & Boulpaep, 1979, 1982). Single-channel patch clamp recordings from principal cells of microdissected rat cortical collecting ducts (Palmer & Frindt, 1986, 1988) and from cultured rabbit cortical collecting duct cells (Ling et al., 1991) have demonstrated the presence of a low-conductance, highly-selective apical sodium channel which is sensitive to submicromolar concentrations of amiloride. The electrophysiological properties of this channel resemble those of the rat colon sodium channel expressed in *Xenopus* oocytes after coinjection with cRNAs of all three channel subunits (Canessa et al., 1994).

A suitable mammalian cell line to study the electrophysiological and molecular properties of the renal sodium channel has previously not been available. Such a cell line should provide a continuous source of readily available cells with minimal variability between samples. Recently, we have demonstrated that the M-1 cell line, which is derived from a single microdissected cortical collecting duct of a mouse transgenic for the early region of simian virus 40 (Stoos et al., 1991), preserves under tissue culture conditions morphological and functional properties typical for cortical collecting duct principal cells *in vivo*. In particular, we have shown that confluent M-1 cells express amiloride-sensitive sodium reabsorption and a highly sodium-selective apical membrane conductance (Korbmacher et al., 1993). The aim of the present study was to further characterize this conductance and to identify the underlying single channels using the patch clamp technique. Furthermore, we isolated poly(A)$^+$ RNA from M-1 cells to express the channel in *X. laevis* oocytes and to check for the presence of transcripts corresponding to the three known subunits of the rat colon sodium channel.

Materials and Methods

Cell Culture

The M-1 cell line was originally obtained from Dr. Fejes Tóth (Stoos et al., 1991). Cells were used from passage 10 through 32 and were handled as described previously (Korbmacher et al., 1993). 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 routinely grown on uncoated tissue culture dishes (Becton Dickinson, Plymouth, England) and maintained in a 5% CO_2 atmosphere at 37°C. PC1 culture medium (Hycor Biomedical, Irvine, CA) containing 5 μM dexamethasone and 15 μg/ml insulin was used and was supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For the first 24 hr after seeding the PC1 medium contained in addition 5% fetal calf serum (FCS) to promote initial cell attachment and growth. Subsequently the FCS was omitted and culture medium was exchanged twice a week. For patch-clamp experiments cells were seeded onto small pieces of glass coverslips and were used 6–13 days after seeding. At this stage, the M-1 cells had formed confluent monolayers with typical 'domes', as described previously (Korbmacher et al., 1993). We did not detect any differences in the electrophysiological responses of cells from 'domes' as compared to the responses of cells attached to the glass surface. Thus, the results from these experiments were pooled and are reported together.

Patch Clamp Technique

The conventional patch clamp technique was used to record whole-cell currents or single-channel currents in excised inside-out (i/o) or outside-out (o/o) membrane patches (Hamill et al., 1981) and experimental procedures were essentially the same as described previously (Korbmacher et al., 1993, 1995). Currents were amplified with an EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) using an ATARI computer system for data acquisition and analysis. Patch pipettes were pulled from Clark glass capillaries (Clark Electromedical Instruments, Pangbourne, UK) and had a resistance of 3–9 MΩ in NaCl/Ringer. Experiments were performed at 37°C in an elongated chamber (25 mm long, 5 mm 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 in the outflow path of the chamber. For i/o patches the trans-patch potential difference is defined as the negative holding potential ($-V_{pip}$), or cytoplasmic potential referred to pipette potential. For o/o patches and in the whole-cell configuration the cytoplasmic potential corresponds to the holding potential V_{pip}. In the whole-cell experiments, the membrane capacitance (C_m) and series resistance (R_s) were estimated by nulling capacitive transients using the automated EPC-9 compensation circuit. R_s averaged 28.3 ± 2.6 MΩ (n = 83) and was not compensated. Liquid junction (LJ) potentials occurring at the bridge/bath junction were measured using a 3 M KCl flowing boundary electrode and ranged from −5 to +4 mV. For data analysis, the V_{pip} values were corrected accordingly while the original traces shown in the figures are not LJ corrected. Upward current deflections correspond to cell membrane outward currents, i.e., movement of positive charge from the cytoplasmic side to the extracellular side. Data were recorded at 10 kHz bandwidth and stored on videotape after pulse code modulation (PCM 501, Sony, Japan). For data analysis, currents were filtered at 200 Hz with 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 kHz.

The computer software for data analysis was written by A. Rabe in our laboratory. Channel activity was estimated from binned current amplitude histograms as the product NP_o, where N is the number of channels and P_o is single-channel open probability. Single-channel current amplitudes for *I-V* plots were estimated from amplitude histograms or by measuring and averaging several individual current transitions. Data are given as mean values ±SEM, significances were evaluated by the appropriate version of Student's *t*-test.

Solutions and Chemicals

The composition of the solutions is given in mM. For the patch clamp experiments, the standard bath solution was NaCl-solution: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (adjusted to pH 7.5 with NaOH). Other bath solutions were: KCl-solution (140 KCl, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.5 with KOH), LiCl-solution (145 LiCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.5 with KOH), and NMDG-Cl-solution (145 N-methyl-D-glucamine-Cl, 1 $CaCl_2$, 1 $MgCl_2$ 10 HEPES, adjusted to pH 7.5 with KOH). All bath solutions contained 5 mM glucose. As pipette solution for whole-cell and o/o experiments we routinely used KCl/EGTA-solution (140 KCl, 5 NaCl, 1 $CaCl_2$, 1 EGTA, and 10 HEPES adjusted to pH 7.5 with KOH). In a few experiments, pipette solutions were supplemented with 1–2 mM Mg-ATP or with a combination of 1 mM ATP and 0.3 mM Na-GTP. The presence of ATP and GTP did not significantly affect the electrophysiological response to amiloride. Occasionally, NMDG-gluconate-solution (135 NMDG-gluconate, 10 NMDG-Cl, 5 NaCl, 1 Mg-ATP, 1 EGTA, 10 HEPES, adjusted to pH 7.5 with KOH) or Li_2SO_4/EGTA-solution (65 Li_2SO_4, 15 LiCl, 65 sucrose, 1 EGTA, 1 $MgSO_4$, 10 HEPES adjusted to pH 7.5 with Tris) was used as pipette solution. For i/o patches the pipette solution was either LiCl-solution or Li_2SO_4-solution (60 Li_2SO_4, 25 LiCl, 60 sucrose, 10 HEPES adjusted to pH 7.5 with Tris). Amiloride-hydrochloride was obtained from Sigma (Deisenhofen, Germany).

Oocyte Expression Studies

Total RNA was isolated from confluent M-1 cells by using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski & Sacchi (1987). Subsequently poly(A)$^+$ RNA was purified from total RNA *via* an oligo(dT)-cellulose-column (Life Technologies, Berlin, Germany). Using the two-microelectrode voltage-clamp technique (TURBO Tec 01C amplifier, NPI Electronic, Tamm, Germany) we assayed defolliculated stage V-VI *Xenopus* oocytes for an amiloride-sensitive inward current 1–6 days after injection with H_2O or with 20–50 ng of M-1 poly(A)$^+$ RNA. Experimental procedures for obtaining and injecting the oocytes were essentially as described previously (Kroll et al., 1989). The isolated and injected oocytes were kept in a modified Barths' saline (88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 10 U/ml sodium penicillin, 10 μg/ml streptomycin sulphate, 15 HEPES adjusted to pH 7.6 with Tris) at 18°–20°C. During the experiments, oocytes were routinely bathed in frog-NaCl-solution (115 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES adjusted to pH 7.4 with Tris). To test ion selectivity, the bath NaCl was replaced by an equal amount of LiCl, KCl or NMDG-Cl. Inward currents were continuously recorded at a holding potential of −60 or −100 mV.

Northern Blot Analysis

Isolation of poly(A)$^+$ RNA from rat tissues was performed as described previously (Jaisser et al., 1993) and Northern blot analysis was performed as described (Jaisser et al., 1993) according to standard procedure (Sambrook et al., 1989). Northern blot analyses were performed using 3 μg poly(A)$^+$ RNA obtained from rat kidney cortex, rat kidney medulla or confluent M-1 cells. The M-1 poly(A)$^+$ RNA samples were from the same batches which were also functionally assayed in oocyte expression studies. Hybridizations were done with specific probes for the three known subunits of the rat colon sodium channel (rENaC) (Canessa et al., 1994) using a 1-2150 bp α subunit (α-rENaC) cDNA probe, a 1-1985 bp β subunit (β-rENaC) cDNA probe and a 3-2035 bp γ subunit (γ-rENaC) cDNA probe. Probes were labeled with ^{32}P dCTP. Final washes of the blot were done with 1.0 × SSC, 1% SDS at 56°C for 30 min (Sambrook et al., 1989). Autoradiograms were exposed for 1 to 10 days.

Results

Whole-cell Recordings in Confluent M-1 Cells

In M-1 cells which had formed an epithelial monolayer and displayed 'domes' giga-ohm seals were obtained in about one out of five attempts. Following giga-ohm seal formation, conventional whole-cell patch configurations were achieved in about 50% of the attempts. The cell membrane capacitance (C_m) averaged 13.2 ± 0.6 pF (n = 83) which indicates that the cells under investigation were not electrically coupled to the neighboring cells in the monolayer (Korbmacher et al., 1993). Within the first minute after achieving the whole-cell configuration with KCl/EGTA-solution as pipette solution and NaCl-solution in the bath, the apical membrane voltage (V_m) averaged −31.2 ± 1.3 mV (n = 100), and whole-cell inward currents averaged −152 ± 11 pA (n = 51) at a holding potential of −60 mV. In some experiments, giga-ohm seals could be maintained from several minutes up to more than half an hour. This made it possible to perform solution exchanges and to apply drugs while continuously monitoring V_m or whole-cell currents in voltage clamp (*VC*) or in zero current clamp (*CC*) mode, respectively.

Effects of Amiloride and Sodium Removal

In the experiments shown in Fig. 1, the effect of complete extracellular sodium replacement by the impermeant cation NMDG (N-methyl-D-glucamine) and the effect of application of 10^{-5} M amiloride were tested. The upper trace represents a continuous recording of V_m in CC mode. Both experimental maneuvers reversibly hyperpolarized the apical membrane by about 30 mV, indicating that the cells possessed an amiloride-sensitive sodium conductance. In similar experiments, the hyperpolarization induced by sodium removal or by 10^{-5} M amiloride averaged 22.8 ± 4.5 mV (n = 5) and 24.9 ± 2.2 mV (n = 35), respectively. The lower trace in Fig. 1 depicts a corresponding experiment carried out in VC mode. Whole-cell currents were continuously recorded

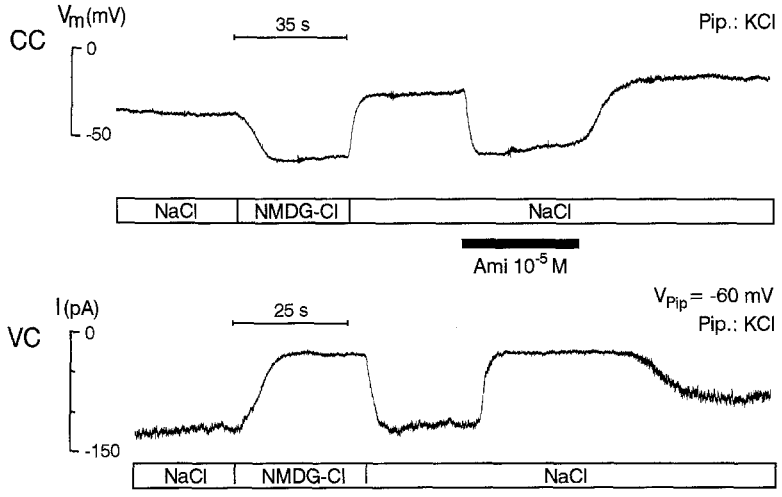

Fig. 1. Effect of sodium removal and amiloride on apical membrane voltage (V_m) and whole-cell current (I) of confluent M-1 cells. Upper trace: continuous recording of V_m in zero current clamp (CC) mode. Lower trace: continuous recording of whole-cell current in voltage clamp (VC) mode at a holding potential of −60 mV. Pipette contained KCl/EGTA-solution, bath solutions are indicated below the traces.

at a holding potential of −60 mV. The inward whole-cell current was largely reduced upon extracellular sodium removal and recovered back to its original level after readdition of sodium. Application of 10^{-5} M amiloride had about the same inhibitory effect as sodium removal. On average sodium removal and 10^{-5} M amiloride reduced inward currents by 114 ± 28 pA (n = 15) and 107 ± 10 pA (n = 51), respectively. This indicates that most of the sodium inward current is sensitive to amiloride. In further experiments, we investigated the concentration-dependence of the amiloride effect. As shown in Fig. 2, amiloride inhibits the inward current with an IC_{50} of 0.22 μM. This indicates that the apical sodium conductance of confluent M-1 cells is highly sensitive to amiloride which is in good agreement with the in vivo situation in the cortical collecting duct.

Ion Selectivity and Sodium Dependence

In experiments as those shown in Fig. 3, we investigated the cation selectivity of the inward conductance. The upper trace in Fig. 3 represents a continuous V_m recording and the lower trace a continuous current recording from two corresponding experiments. Changing the bath solution from NaCl to LiCl increased the whole-cell inward current (Fig. 3, lower trace) and accordingly induced a depolarization of the apical membrane (Fig. 3, upper trace). After LiCl exposure, the steady-state membrane potential in NaCl was slightly higher than at the start of the experiment (Fig. 3, upper trace). A similar effect was observed in 4 out of 7 such experiments. We do not know whether this is an effect of LiCl or whether this is related to a slow spontaneous variation of the membrane potential which may occur in these cells under whole-cell recording conditions as reported previously (Korbmacher et al., 1993). Nevertheless, the fast initial membrane potential response to LiCl and the corresponding current change clearly indicate that the apical membrane is more permeable for lithium than for sodium. In contrast, changing from NaCl-solution to KCl-solution reduced the inward current (Fig. 3, lower trace) and caused a membrane hyperpolarization (Fig. 3, upper trace). In this experiment, the KCl-induced hyperpolarization appears to be slightly smaller than the hyperpolarization induced by amiloride. This may indicate that a small apical K^+-selective conductance exists in confluent M-1 cells or that some KCl reaches the basolateral membrane during exposure to KCl bath solution. This assumption is supported by the observation that after the initial hyperpolarization the cells subsequently depolarized in the prolonged presence of KCl bath solution. In similar experiments, the initial hyperpolarization induced by amiloride or KCl averaged −20 ± 5 mV and −18 ± 5 mV, respectively (n = 6, not significantly different in paired t-test). The hyperpolarization induced by KCl and the corresponding reduction of the inward current clearly indicate that the apical membrane is less permeable for potassium than for sodium. Moreover, in the presence of KCl bath solution application of amiloride 10^{-5} M had no significant effect on V_m (n = 6) or on the inward current (n = 9). Taken together, these results indicate that the amiloride-sensitive whole cell conductance is highly selective for sodium over potassium and that lithium is slightly more permeable than sodium. In further experiments, we investigated the dependence of the whole-cell inward current on the concentration of extracellular sodium. A continuous current trace from one of these experiments is shown in Fig. 4*A*. Whole-cell inward currents were minimal in the absence of bath sodium and increased with increasing extracellular sodium concentrations. Results of 11 such experiments are summarized in Fig. 4*B* and a Michaelis-Menten fit of the data reveals a K_m for extracellular sodium of 24 m*M*.

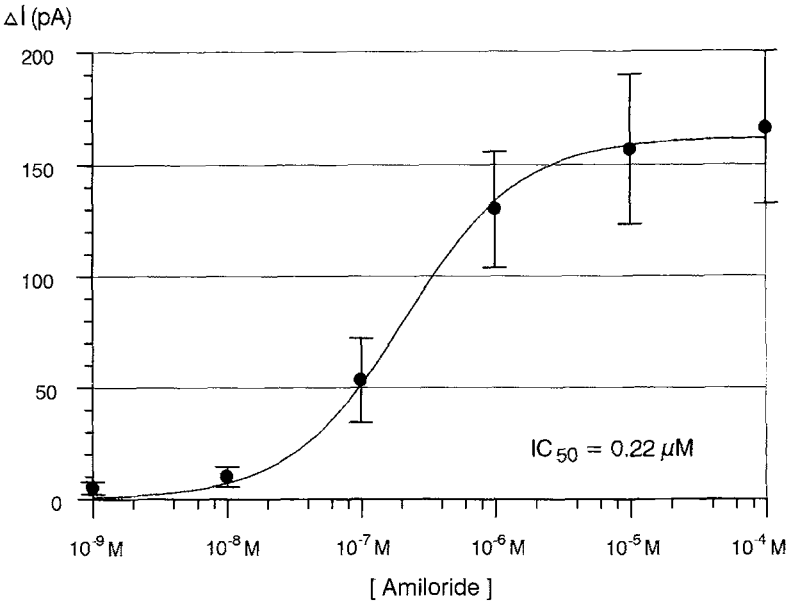

Fig. 2. Concentration-dependence of amiloride effect on whole-cell inward currents. Whole-cell currents were recorded as described in Fig. 1 and various amiloride concentrations were tested. Results of 6 experiments are summarized and the average decrease (mean ±SEM) of the inward current (ΔI) induced by amiloride is plotted *vs.* the amiloride concentration. A Michaelis-Menten fit of the data reveals an IC_{50} of 0.22 μM.

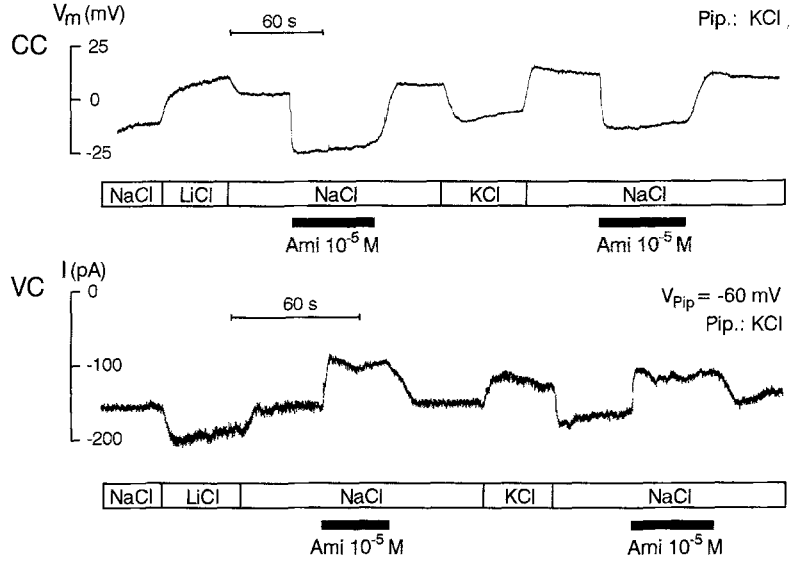

Fig. 3. Cation selectivity of the apical inward conductance. Continuous V_m recording (upper panel) and corresponding whole-cell current (I) recording (lower panel) obtained from two separate experiments performed as in Fig. 1. Bath solutions were changed as indicated below the traces.

Single-channel Recordings in Outside-out Patches

In about one out of ten attempts, we were able to successfully excise an o/o patch at the end of a whole-cell recording. Current traces from such an o/o patch are shown in Fig. 5*A* at different extracellular sodium concentrations. Single channel current transitions with up to four channel levels can be seen. In all four recordings, the single-channel current amplitudes are smaller than 1 pA. However, it can be seen that the single-channel current amplitudes increased with increasing extracellular sodium concentrations. In Fig. 5*B*, complete *I-V* plots obtained in the presence of four different extracellular sodium concentrations are shown. The data points derived from the current traces shown in Fig. 5*B* are indicated in the *I-V* plots. Goldman-Hodgkin-Katz curves correspond well with the experimental data (Fig. 5*A*) and demonstrate that the single channels are sodium-selective. Figure 5*C* summarizes the results from two such o/o patch experiments. For different holding potentials, single-channel current amplitudes were plotted *vs.* the extracellular sodium concentration and the K_m-values for sodium were estimated. The calculated K_m-values were all in the same range and appeared to be voltage independent. The average K_m of 34 mM is in good agreement with the K_m of 24 mM found in the whole-cell

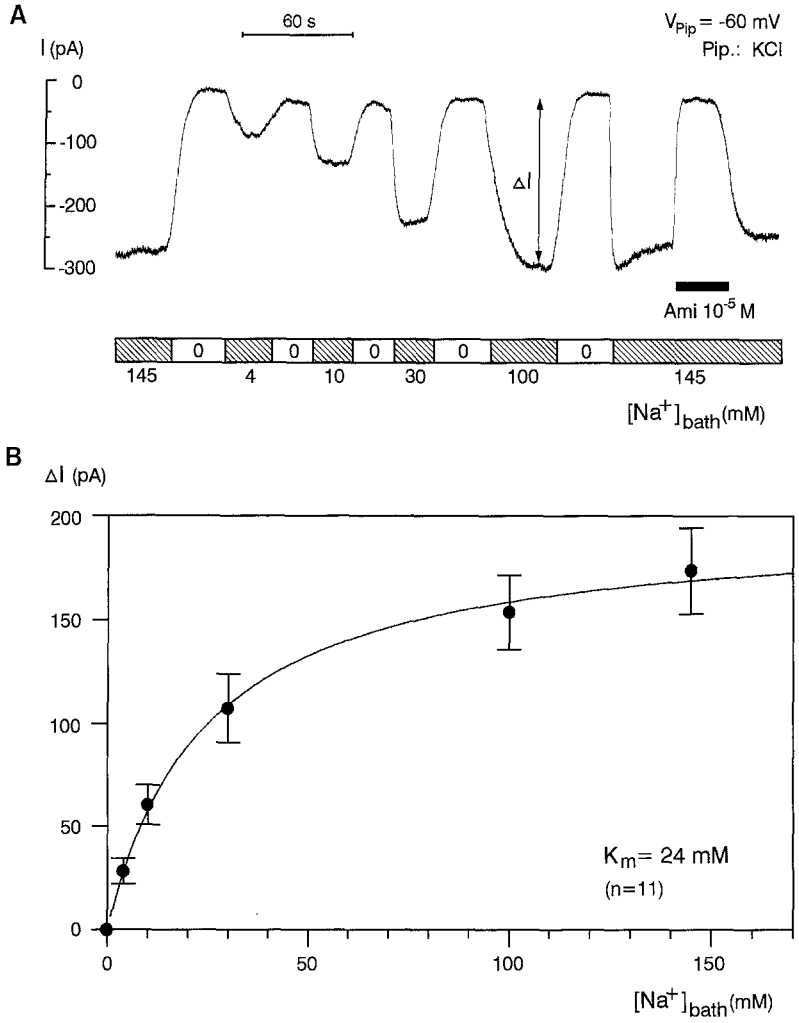

Fig. 4. Sodium-dependence of the inward conductance. (*A*) Continuous whole-cell current recording performed as in Fig. 1. Inward currents were recorded in the presence of different bath sodium concentrations as indicated below the trace. The current difference (ΔI) between the inward current in the complete absence of sodium (replaced by NMDG) and in the presence of various sodium concentrations was estimated as indicated by the arrow. (*B*) Summary of 11 such experiments where ΔI is plotted *vs.* the bath sodium concentration ($[Na^+]_{bath}$). A Michaelis-Menten fit of the data reveals a K_m for extracellular sodium of 24 mM.

experiments (*see above*). Figure 6 demonstrates the effect of amiloride on a single-channel recorded in another o/o patch. In this experiment the patch pipette was filled with NMDG-gluconate solution and the bath contained NaCl-solution. Under these conditions, the inward single-channel currents observed at a holding potential V_{pip} = –48 mV are carried by sodium. Application of 10^{-5} M amiloride decreased the mean open time from 36.2 msec to 2.7 msec and slightly increased the mean closed time from 47.3 msec to 54.9 msec thereby reducing NP_o from 0.489 to 0.001. This effect was reversible upon washout of amiloride. On average, 10^{-5} M amiloride reduced NP_o of sodium channels in o/o patches from 0.55 ± 0.18 to 0.08 ± 0.02 (n = 9, $P < 0.001$). This corresponds to an inhibition of about 85%, which is in good agreement with the inhibitory effect of 10^{-5} M amiloride on the whole-cell currents (*see above*). Thus, the single channels identified in the o/o patches are highly sensitive to amiloride.

Voltage-Dependence

In those channel recordings from o/o patches in which NP_o-values could be determined for several seconds (on average for 20 ± 12 sec) at various holding potentials, we looked for a voltage dependence of NP_o. This analysis is hampered by the fact that NP_o-values varied considerably from patch to patch and could spontaneously change in the same recording at a constant holding potential. These rather variable kinetics are a known feature of epithelial sodium channels (for review: Horrisberger et al., 1993). Nevertheless, a slight voltage dependence appeared to be present in the majority of patches with channels having the tendency of being more active at hyperpolarizing potentials as compared to depolarizing potentials. In a total number of 8 analyzed patches, NP_o averaged 0.46 ± 0.14 at a holding potential of –100 mV, and was significantly larger than the NP_o of 0.16 ± 0.05 determined at 0 mV ($P < 0.05$).

Single-channel Recordings in Inside-out Patches

Cell-attached patches were usually silent but in a number of excised i/o patches from apical membranes of confluent M-1 cells we detected small conductance single channels. However, the majority of i/o-patches had no

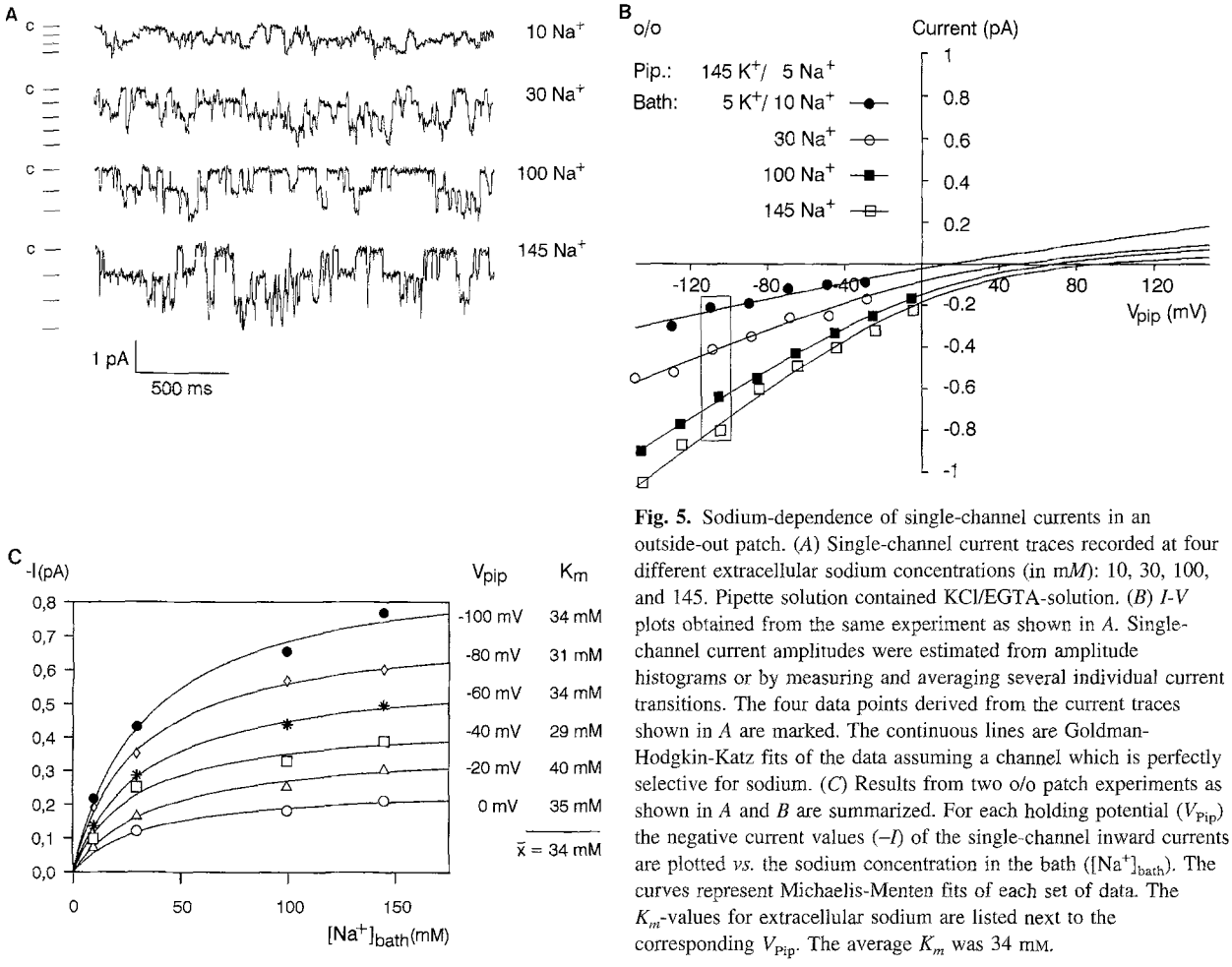

Fig. 5. Sodium-dependence of single-channel currents in an outside-out patch. (*A*) Single-channel current traces recorded at four different extracellular sodium concentrations (in m*M*): 10, 30, 100, and 145. Pipette solution contained KCl/EGTA-solution. (*B*) *I-V* plots obtained from the same experiment as shown in *A*. Single-channel current amplitudes were estimated from amplitude histograms or by measuring and averaging several individual current transitions. The four data points derived from the current traces shown in *A* are marked. The continuous lines are Goldman-Hodgkin-Katz fits of the data assuming a channel which is perfectly selective for sodium. (*C*) Results from two o/o patch experiments as shown in *A* and *B* are summarized. For each holding potential (V_{Pip}) the negative current values ($-I$) of the single-channel inward currents are plotted *vs.* the sodium concentration in the bath ($[Na^+]_{bath}$). The curves represent Michaelis-Menten fits of each set of data. The K_m-values for extracellular sodium are listed next to the corresponding V_{Pip}. The average K_m was 34 mM.

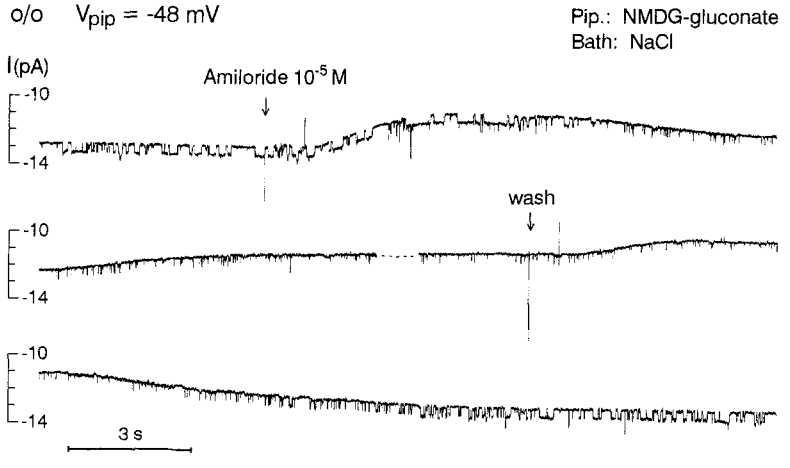

Fig. 6. Effect of amiloride on single-channel sodium currents in an outside-out patch. Continuous recording of single channel activity from an o/o patch with NMDG-gluconate as pipette solution and NaCl-solution in the bath. Holding potential was $V_{Pip} = -48$ mV throughout the recording. Under these experimental conditions, the single-channel inward currents correspond to sodium currents. Application of amiloride 10^{-5} M and washout are indicated by arrows. The shifts of the current baseline after amiloride application and after washout are probably caused by seal perturbations during the solution exchange.

or only short-lived channel activity with channel 'run down' shortly after excision. One of the few experiments in which channel activity persisted for a prolonged period of time is shown in Fig. 7. The pipette was filled with Li_2SO_4 solution and the bath contained initially LiCl solution. Single-channel activity was blocked by the cytoplasmic application of 10^{-4} M amiloride. Amiloride is thought to block sodium channels from the extracellular side and it is conceivable that the lipophilic amiloride effectively diffuses through the patch membrane and reaches sufficient extracellular concentrations to block the channel from the outside. The rather slow recovery of channel activity observed after washout of cytoplasmic amiloride is compatible with an amiloride

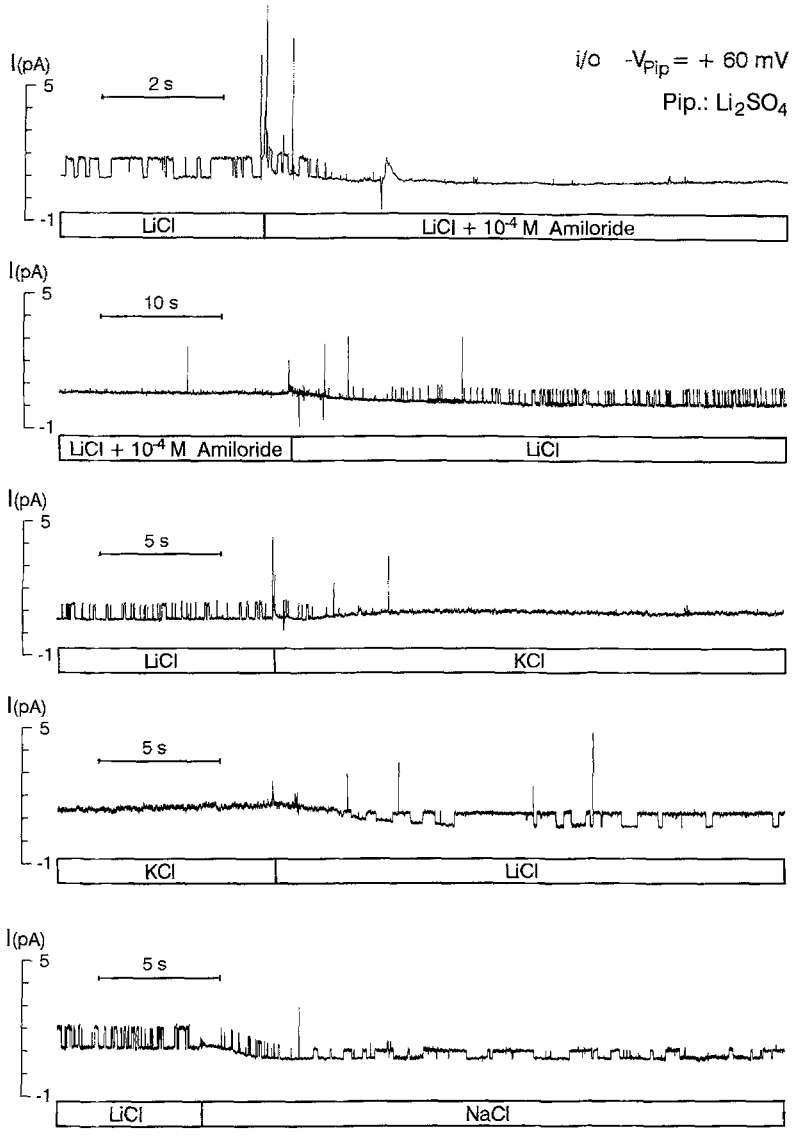

Fig. 7. Cation selectivity of the amiloride-sensitive Na-channel in an inside-out patch. Five sequential current traces from the same i/o patch are shown starting 300 sec, 380 sec, 440 sec, 500 sec and 690 sec after patch excision (from top to bottom). Bath solution changes are indicated below the traces; no changes were performed during the time intervals between the five traces shown. Pipette solution was Li_2SO_4. The holding potential was $-V_{Pip}$ = +60 mV throughout the experiment.

block from the outside (note the different time scale for the second trace in Fig. 7). However, we cannot rule out the possibility of an additional cytoplasmic inhibitory binding site for amiloride. In the presence of cytoplasmic KCl-solution, the single-channel outward currents disappeared which demonstrates that the channels have no measurable permeability for potassium (3rd and 4th trace in Fig. 7). During the 160 sec-time interval between the 4th and the last trace single-channel current amplitudes increased and channel kinetics became faster. These changes developed gradually (*not shown*) and were probably caused by an increase in the bath temperature which was not perfectly controlled during this experiment. Changing from LiCl solution to NaCl solution (last trace in Fig. 7) reduced the single-channel current amplitudes which indicates that the channels are more permeable for lithium than for sodium. Note that Fig. 8*A* depicts single-channel *I-V* curves from the same i/o patch as shown in Fig. 7. Under asymmetrical conditions for chloride, with Li_2SO_4 solution in the pipette and LiCl solution in the bath, the reversal potential of close to 0 mV indicates that the single-channel currents are indeed carried by lithium. Linear regression of the data reveals a single-channel conductance of 9.7 pS for lithium. In the presence of NaCl bath solution, outward currents were significantly reduced. Using a Goldman-Hodgkin-Katz fit of the data, the single-channel conductance for sodium was estimated to be 6.4 pS. With KCl-solution on the cytoplasmic side no more outward currents were detected and the reversal potential estimated from the Goldman-Hodgkin-Katz fit of the inward current data was shifted far to the right. Taken together the *I-V* curves confirm our conclusion that potassium ions do not permeate the channel and that the channel has a higher single-channel conductance for lithium than for sodium. These findings are consistent with the whole-cell data.

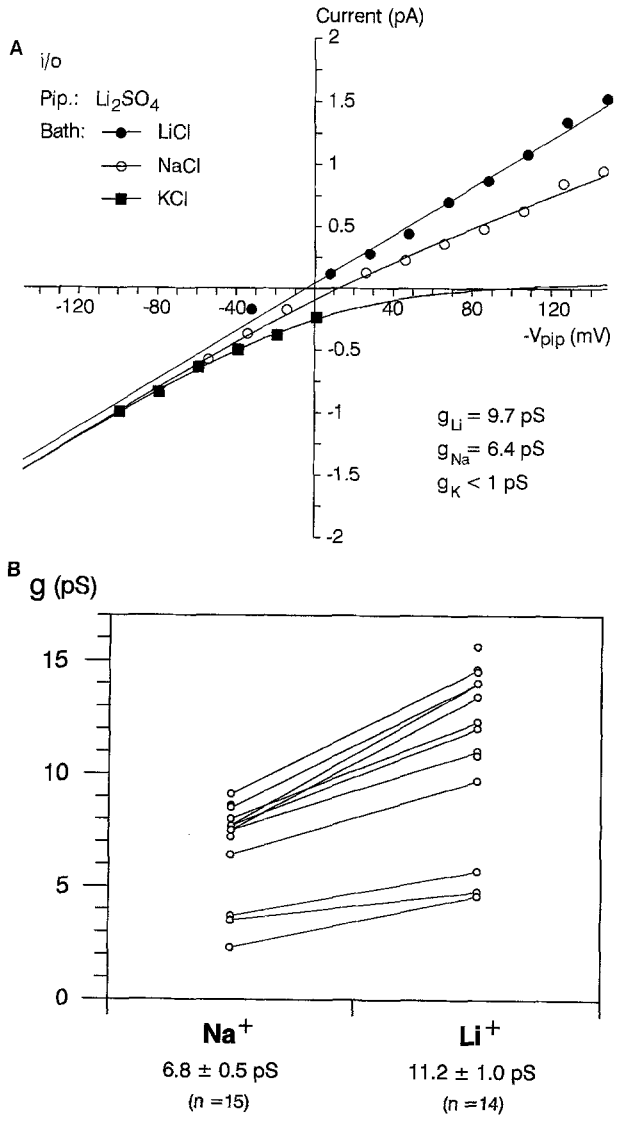

Fig. 8. Single-channel *I-V* plots and single-channel conductances of amiloride-sensitive Na-channels in inside-out and outside-out patches. (*A*) Single-channel *I-V* curves from the same i/o patch as shown in Fig. 7 in the presence of LiCl-, NaCl- and KCl-solution in the bath. Linear regression of the data in LiCl-solution reveals a single-channel conductance of 9.7 pS for lithium. The curves in the presence of NaCl-solution and KCl-solution are Goldman-Hodgkin-Katz fits which reveal a single-channel conductance for sodium of 6.4 pS and a negligible single-channel conductance for potassium. (*B*) Summarizes single-channel conductance values obtained from 6 i/o and 10 o/o apical membrane patches. In 15 out of the 16 experiments amiloride was tested and channel inhibition by amiloride was confirmed. Conductance values for lithium and sodium obtained in the same patch are connected by a straight line.

Single Channel Kinetics

In a total of 16 o/o and i/o patches in which amiloride-sensitive sodium-selective single channels were observed, the number of apparent channels per patch ranged from 1 to 4 with an average of 1.9 ± 0.3 channels per patch. NP_o varied considerably from patch to patch ranging from 0.03 to 1.24 with a mean NP_o of 0.33 ± 0.08 (n = 15) at a hyperpolarizing potential of −60 mV. In those experiments in which only one channel level was present throughout the recording, we attempted an analysis of channel kinetics during prolonged periods of channel activity at a constant holding potential of −60 mV. In nine analyzed experiments, mean open times ranged from 0.03 sec to 0.47 sec and averaged 0.16 ± 0.05 sec while the mean closed times ranged from 0.11 sec to 2.92 sec and averaged 0.51 ± 0.30 sec.

Variable Single Channel Conductances and Multiple Conductance States

Figure 8*B* summarizes single-channel conductance values obtained from 6 i/o and 10 o/o apical membrane patches. In 15 out of the 16 experiments, amiloride was tested and channel inhibition by amiloride was confirmed. The single-channel sodium conductances ranged from 2.3 pS to 9.1 pS. In a few recordings, small transitions in the 2–4 pS range and larger transitions in the 6–10 pS range were present simultaneously. These multiple conductance states were difficult to analyze since they usually occurred in patches with several channel levels. Moreover, in the course of such experiments, the smaller transitions usually disappeared spontaneously. All channels tested for both ions had a higher conductance for lithium than for sodium. The average single-channel conductance for sodium was 6.8 ± 0.5 pS (n = 15) and for lithium 11.2 ± 1.0 pS (n = 14). The wide range of single-channel conductances and the presence of multiple conductance states suggest that the underlying channels may have various functional states and may be composed of more than one channel subunit.

Channel Expression in Oocytes and Northern Blot Analysis

Figure 9 shows a current recording from a *X. laevis* oocyte held at −60 mV holding potential four days after it had been injected with 50 ng of M-1 poly(A)$^+$ RNA. In the presence of frog-NaCl-solution application of 2 μM amiloride reduced the inward current by more than 50 nA. In contrast, in the presence of frog-KCl-solution, amiloride has no effect. After changing back to NaCl-frog solution the amiloride response could again be elicited. These findings indicate that the inward current inhibited by amiloride was sodium selective. As can be seen in Fig. 9, changing from frog-NaCl-solution to frog-KCl-solution reduced the inward current in this experiment and the effect was reversible after changing back to NaCl-frog solution. A similar reduction of the inward current was observed in 3 out of 5 such experiments

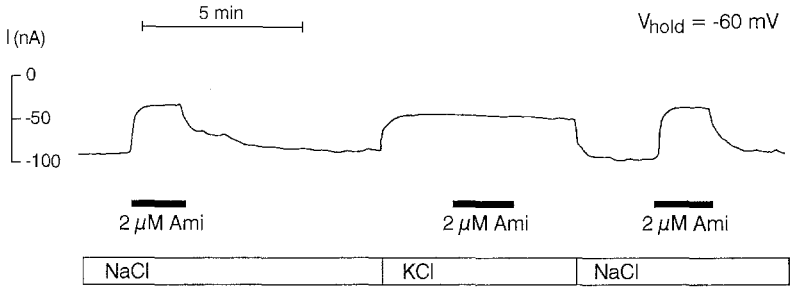

Fig. 9. Expression of amiloride-sensitive sodium currents in *X. laevis* oocytes. Current (*I*) recording from a *X. laevis* oocyte held at −60 mV holding potential four days after injection with 50 ng of M-1 poly(A)$^+$ RNA. Application of 2 μM amiloride and solution exchanges are indicated below the trace.

while in the other 2 experiments the inward currents actually increased in the presence of KCl-frog solution. Replacing NaCl in the bath by KCl may have two opposing effects: In the presence of KCl inward current *via* the sodium selective ion channels is abolished, while on the other hand the high extracellular potassium concentration favors a potassium inward current *via* endogenous potassium-selective ion channels known to be present in *X. laevis* oocytes (Dascal, 1987). Depending on which of the two effects is dominant, one may observe either a decrease or an increase of the total inward current after changing to KCl in the bath, as observed in our experiments. Regardless of whether KCl by itself decreased or increased the total inward current, application of amiloride always failed to have an effect in KCl-frog solution (n = 5). In contrast, in the presence of NaCl-frog solution 2 μM amiloride reduced the sodium inward current at a holding potential of −60 or −100 mV on average by 25.5 ± 4.6 nA in 25 oocytes one to six days after injection with 20–50 ng of M-1 poly(A)$^+$ RNA. No effect of amiloride was observed in water-injected control oocytes (n = 19). In four out of four experiments M-1 poly(A)$^+$ RNA-injected oocytes displayed larger amiloride sensitive currents in the presence of LiCl as compared to the amiloride-sensitive currents in NaCl. The current ratio averaged about 1.4. This indicates that the expressed channels are more permeable to Li than to Na, which is consistent with the findings in the patch clamp experiments on M-1 cells. Taken together, these experiments demonstrate that injection of poly(A)$^+$ RNA isolated from confluent M-1 cells leads to functional expression of amiloride-sensitive sodium channels in *X. laevis* oocytes. We hypothesized that the M-1 cells contain transcripts which may resemble those coding for the epithelial sodium channel of rat colon (Canessa et al., 1994). Therefore the M-1 poly(A)$^+$ RNA was tested in Northern blot experiments as shown in Fig. 10. Probing a blot of M-1 poly(A)$^+$ RNA with specific cDNA-probes for the three subunits of the rat colon sodium channel (rENaC) revealed specific bands of an expected size for the α-, β-, and γ- subunit of the channel. A strong band was observed for α, a weaker for β and a very weak for γ, but clearly all transcripts are present in M-1 cells.

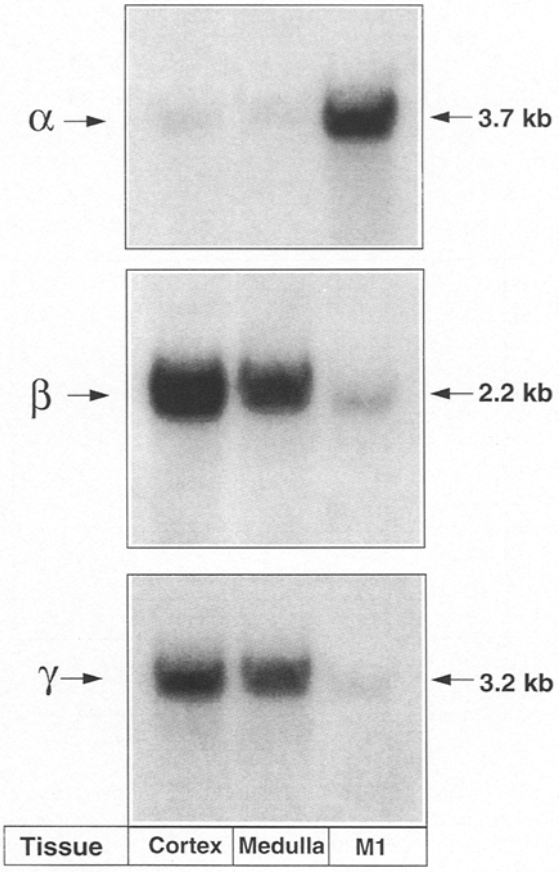

Fig. 10. Northern blot analysis of α rENaC, β rENaC and γ rENaC expression in rat kidney cortex, medulla and in confluent M-1 cells. poly(A)$^+$ RNA (3 μg) was electrophoresed on a 0.8% denaturing glyoxal agarose gel and transferred to a nylon membrane. The membrane was hybridized with random primed ^{32}P-labelled dCTP full length rENaC cDNAs (*see* Material and Methods). The size of the full length rENaC cDNA probes for the different subunits is indicated on the right. The same RNA-blot was used for all three hybridization reactions. Blot probed with α or β rat cDNA probes (upper and middle panel) was exposed one day. Blot probed with γ rat cDNA probe was exposed 10 days (lower panel).

Transcripts for all three subunits were also present in rat renal cortex and rat renal medulla which is consistent with the results previously reported (Canessa et al., 1994).

Discussion

In the present study, we have characterized the amiloride-sensitive whole-cell conductance of confluent M-1 cells and have identified the underlying apical sodium channels in single-channel recordings. The whole-cell conductance and the single channels are highly sensitive to amiloride and highly selective for sodium over potassium. Moreover, the single-channel conductance of these channels is 'low'. Thus, according to the classification of Palmer (1992) and Benos et al. (1995) the channel in M-1 cells may be considered as a 'type Na(5) channel' or a 'H-type channel', respectively. Channels belonging to this group have previously been identified by single-channel patch-clamp recordings in A6 cells grown on filters (Hamilton & Eaton, 1986), in principal cells of microdissected rat cortical collecting ducts (Palmer & Frindt, 1986), in rabbit proximal tubule (Gögelein & Greger, 1986), in toad urinary bladder (Frings et al., 1988), in primary cultures of rabbit cortical collecting duct cells (Ling et al., 1991), and also in lung epithelial cells (Voilley et al., 1994). The classification by Palmer stresses the low single-channel conductance of about 5 pS and the high selectivity for sodium over potassium, while the classification of Benos emphasizes the high sensitivity to amiloride ('H' for highly sensitive). Channels are considered as highly sensitive to amiloride if half maximal inhibition is achieved at a blocker concentration of less than 1 μM (Benos et al., 1995). The half maximal inhibitory concentration of 0.22 μM observed in M-1 cells is well within the typical range of 0.1 to 0.5 μM reported for other systems (Palmer, 1992).

In our whole-cell recordings at a holding potential of −60 mV, about two-thirds of the observed inward current was carried by sodium. This sodium-dependent inward current of about 100–150 pA/cell could be completely blocked by amiloride and was selective for sodium over potassium. This indicates that apical sodium entry occurs exclusively *via* amiloride-sensitive sodium channels and no other sodium conductance is present in the apical membrane of confluent M-1 cells. It has previously been suggested that nonselective cation channels may contribute to sodium reabsorption (*for review:* Korbmacher & Barnstable, 1993). However, we have identified a nonselective cation (NSC) channel in M-1 cells which is not inhibited by amiloride (Ahmad et al., 1992; Korbmacher et al., 1995). Moreover, this NSC channel is normally quiescent. It can be activated by cellular shrinkage during exposure to hypertonic extracellular solutions and may play a role during volume regulation (Volk et al., 1995). Our results suggest that the NSC channel is not involved in transepithelial sodium transport and that the amiloride-sensitive highly selective sodium channels are solely responsible for apical sodium entry during sodium reabsorption in the cortical collecting duct. The situation may be different in the inner medullary collecting duct where amiloride-sensitive nonselective cation channels have been observed (Light et al., 1988; Ono et al., 1994). To date, it is not clear how these nonselective cation channels are related to the sodium-selective channels described in the present study.

In vivo, the activity of the amiloride-sensitive sodium channels is tightly regulated depending on the need of sodium reabsorption, and aldosterone is believed to be the main modulator of sodium channel activity (Pácha et al., 1993). If dietary sodium intake is high, aldosterone levels are low and sodium channels in the cortical collecting duct are inactive. In contrast, if sodium intake is low and aldosterone levels rise sodium channels become active. Accordingly, no significant amiloride-sensitive whole-cell conductance was detectable in microdissected cortical collecting ducts from rats on a normal diet containing a sufficient amount of sodium (Frindt et al., 1990). Under these conditions sodium reabsorption is downregulated. In contrast, in cortical collecting ducts of animals fed a low-salt diet, whole-cell sodium currents of about 200 pA/cell were measured (Palmer, Antonian, & Frindt, 1993). Interestingly, these stimulated whole-cell currents are in the same order of magnitude as those measured in the M-1 cells under standard tissue culture conditions. The M-1 cells are grown in PC-1 tissue culture medium which contains 5 μM dexamethasone which in this high concentration is likely to also have mineralocorticoid effects. This could explain the high level of baseline stimulation observed in the M-1 cells. Future experiments will have to address the question in more detail which of the tissue culture medium components are responsible for the baseline stimulation of the sodium currents in M-1 cells.

The sodium channels described in rabbit and rat cortical collecting duct cells are highly selective for sodium over potassium which is consistent with our finding that the single channels in M-1 cells had no measurable potassium permeability. The single-channel conductances for sodium averaged 3.7 ± 0.8 pS (Ling et al., 1991) and 4.9 ± 0.2 pS (Palmer & Frindt, 1986) in rabbit and rat, respectively. These values are slightly smaller than the average single-channel sodium conductance of 6.8 ± 0.5 pS measured in the M-1 cells. We cannot rule out that species differences between rat, rabbit and mouse may play a role for these minor differences. However, the lower conductances in rabbit and rat were measured at room temperature, while the measurements in M-1 cells were performed at 37°C. Thus, a temperature effect is the most likely explanation for the slightly larger conductance values found in the M-1 cells. This assumption is consistent with recent findings of Palmer and colleagues who reported two different lithium conductance values for the channel in rat cortical collecting duct with

about 7 pS and 13 pS at room temperature and at 37°C, respectively (Frindt et al., 1993). In the M-1 cells the average single-channel conductance for lithium was 11.2 ± 1.0 pS at 37°C and is in good agreement with the lithium conductance of the rat channel at the same temperature. The slight preference for lithium over sodium of the rat cortical collecting duct channel also compares favorably with our findings in M-1 cells, where each channel tested for both ions had a higher permeability for lithium than for sodium with an average lithium to sodium single-channel conductance ratio of about 1.6. This preference for lithium over sodium appears to be another characteristic feature of this type of epithelial sodium channel and has also been found in the toad urinary bladder (Palmer, 1982).

Another typical feature of the epithelial sodium channel is its slow gating behavior with long-lived open and closed states. At 22–23°C mean open and mean closed times were reported which ranged from 2 to 3 sec and 0.4 to 5 sec, respectively (Palmer & Frindt, 1986; Ling et al., 1991). The gating behavior of the M-1 sodium channel was considerably faster. This may again be an effect of temperature since our experiments were performed at 37°C. Indeed, Palmer and coworkers have recently compared channel kinetics in the rat cortical collecting duct at room temperature and at 37°C and found considerably faster kinetics at 37°C with a mean open time of 0.15 sec and a mean closed time of 0.44 sec (Frindt et al., 1993). These values resemble those found in the M-1 cells and support our assumption that the faster kinetics in M-1 cells are a result of the higher temperature.

The voltage dependence of channel activity is not an impressive property of most epithelial sodium channels described to date. However, a modest activation by hyperpolarizing potentials has been reported for the sodium channels both in rabbit (Ling et al., 1991) and rat cortical collecting duct cells (Palmer & Frindt, 1988). In the latter channel, open probability was reported to increase with hyperpolarization by an average of 2.3 %/mV with substantial variability from patch to patch. This degree of voltage dependence is in good agreement with our observations in M-1 cells. Under physiological conditions, an increase of the current through the sodium channels will tend to depolarize the apical membrane and hence decrease P_o. Thus, the observed voltage dependence may serve as a negative feedback loop in the regulation of the sodium channel activity.

In M-1 cells, investigation of the extracellular sodium dependence of the whole-cell and the single-channel currents revealed similar K_m-values of 24 and 34 mM, respectively. This, together with a similar amiloride sensitivity and lithium over sodium selectivity, supports the conclusion that the identified single channels are indeed the channels underlying the whole-cell currents. Moreover, these K_m-values are in excellent agreement with the K_m of 25 mM reported for the single channels in the rat cortical collecting duct (Palmer & Frindt, 1988). However, K_m-values ranging from 10 to 75 mM (Palmer et al., 1990; Palmer & Frindt, 1986) have also been reported which suggests that the K_m for extracellular sodium may be regulated in these channels.

Can the single channels observed in the i/o and o/o patches account for the sodium whole-cell currents? While sodium-selective whole-cell currents could routinely be measured in the whole-cell configuration, it was much more difficult to detect and identify single channels in excised membrane patches. Single-channel activity was found in less than 10% of the patches, and at present we do not know why most of the patches were silent. The observation that in some patches a brief initial period of channel activity was followed by spontaneous channel 'rundown' indicates that the majority of silent patches probably contained inactive channels. Possibly, channel inactivation occurs as a consequence of seal formation which may disturb the complex interaction between plasma membrane and cytoskeleton within the patch (Milton & Caldwell, 1990). This would explain the difficulty of finding active sodium channels in cell-attached and in excised membrane patches and the relative ease of recording sodium currents from whole-cell membranes in which the cytoskeleton below the cell membrane is largely unaffected by the seal formation. Recent reports stress the importance of cytoskeleton elements in the regulation of sodium channel activity (Smith et al., 1991; Cantiello et al., 1991; Prat et al., 1993). Another possibility to explain the absence of detectable single-channel transitions in the majority of excised patches would be the presence of single channels with single-channel conductances in the subpicosiemens range (Laskowski et al., 1990). Such channels would have single-channel current amplitudes below the resolution limit of our recording technique. However, application of up to 10^{-4} M cytoplasmic amiloride had no effect on the overall conductance of silent i/o patches (*data not shown*) which argues against the presence of such very low conductance sodium channels in M-1 cells. In those patches in which we could clearly identify single-channel transitions of sodium channels, we observed on average 2 channels per patch. Thus, if we assume a patch-membrane area of 1 μm^2 and an apical cell surface area of about 200 μm^2 we should expect 400 channels per cell provided that the sodium channels are distributed evenly in the apical membrane. At a holding potential of –60 mV, 400 sodium channels with a single channel conductance of 7 pS and a mean open probability of 0.5 would carry a sodium inward current of about 80 pA which would be well within the range of currents actually observed in M-1 cells. These considerations demonstrate that it is conceivable that the identified single channels can account for the whole-cell currents observed in our whole-cell recordings.

Our oocyte expression studies demonstrated that poly(A)$^+$ RNA isolated from M-1 cells induced an amiloride-sensitive sodium conductance after injection into *X. laevis* oocytes. Similar expression of amiloride-sensitive sodium fluxes or currents was previously achieved by injecting *X. laevis* oocytes with poly(A)$^+$ RNA derived from A6 cells (Hinton & Eaton, 1989), toad urinary bladder (Asher et al., 1992), and respiratory epithelia (Kroll et al., 1989). Using this technique for expression cloning, Canessa and colleagues (Canessa et al., 1993) and Lingueglia and colleagues (Lingueglia et al., 1993) have independently isolated a sodium channel cDNA clone from rat distal colon. In the meantime, it has become clear that the amiloride-sensitive sodium channel in the rat colon is composed of at least three different subunits which are called α-rENaC, β-rENaC, and γ-rENaC, respectively (Canessa et al., 1994). Single-channel recordings from oocytes coinjected with cRNA of all three subunits revealed single channels with conductances of 4.6 pS or 7.7 pS in the presence of 100 mM Na^+ or Li^+, respectively (Canessa et al., 1994). The channels had slow kinetics with long open and closed times lasting for several seconds. These measurements in the oocytes were performed at room temperature. If we take temperature-related differences into consideration (*see above*) the overall single-channel properties of the expressed colon sodium channel are in good agreement with the single-channel properties of the channels observed in the M-1 cells. Therefore, it was no surprise that transcripts related to all three subunits of the colon channel could be detected in the M-1 cells by Northern analyses.

Interestingly, the single-channels observed in M-1 cells had a relatively wide range of single-channel conductances and occasionally showed subconductance states. In contrast, the single-channel conductances observed in oocytes injected with the three subunits of the colon channel showed only little variability from experiment to experiment and subconductance states were usually not present (L. Schild, *personal communication*). As far as we know, single-channel recordings from the native rat colon sodium channel are not yet available. Therefore, we do not know whether the single-channel conductance of the native colon channel shows a similar variability as observed in M-1 cells, or whether it is as stable as in the oocyte expression system. We speculate that the rather wide range of single-channel current amplitudes and the presence of subconductance states observed in the M-1 cells may indicate, that the sodium channels are assembled with a variable stoichiometry of their subunits. Alternatively, M-1 cells may express additional channel subunits which may be responsible for slightly different biophysical properties of the colon and the renal sodium channel. There is ample precedence in the literature that ion channels change their biophysical properties depending on the presence or absence of additional homologous channel subunits (Chen et al., 1993; Krapivinski et al., 1995). The question, whether additional renal subunits of the sodium channel exist, is particularly interesting in the light of the recent finding that mutations of the β-subunit of the epithelial sodium channel are linked to a rare autosomal dominant form of hypertension, the Liddle-syndrome (Liddle, 1963; Botero-Velez et al., 1994; Shimkets et al., 1994; Schild et al., 1995). Minor mutations or genetic variations in one or more of the channel subunits of the renal sodium channel may be involved in more common forms of arterial hypertension.

In conclusion, we have characterized the electrophysiological properties of an amiloride-sensitive highly-selective sodium channel in M-1 cells and have demonstrated that M-1 cells express transcripts which are related to the three known subunits of the epithelial sodium channel previously cloned from rat colon (Canessa et al., 1994). The M-1 cells may serve as a useful tissue culture model to search for renal specific sodium channel subunits and to further study the electrophysiology, molecular biology, and regulation of the epithelial sodium channel in the cortical collecting duct.

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