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Control of the Amiloride-Sensitive Na^+ Current in Salivary Duct Cells by Extracellular Sodium

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Abstract. We have previously reported that intralobular salivary duct cells contain an amiloride-sensitive Na^+ conductance (probably located in the apical membranes). Since the amiloride-sensitive Na^+ conductances in other tight epithelia have been reported to be controlled by extracellular (luminal) Na^+, we decided to use whole-cell patch clamp techniques to investigate whether the Na^+ conductance in salivary duct cells is also regulated by extracellular Na^+. Using Na^+-free pipette solutions, we observed that the whole-cell Na^+ conductance increased when the extracellular Na^+ was increased, whereas the whole-cell Na^+ permeability, as defined in the Goldman equation, decreased. The dependency of the whole-cell Na^+ conductance on extracellular Na^+ could be described by the Michaelis-Menten equation with a K_m of 47.3 mmol/1 and a maximum conductance (G_{max}) of 2.18 nS. To investigate whether this saturation of the Na^+ conductance with increasing extracellular Na^+ was due to a reduction in channel activity or to saturation of the single-channel current, we used fluctuation analysis of the noise generated during the onset of blockade of the Na^+ current with 200 μmol/l 6-chloro-3,5-diaminopyrazine-2-carboxamide. Using this technique, we estimated the single channel conductance to be 4 pS when the channel was bathed symmetrically in 150 mmol/l Na^+ solutions. We found that Na^+ channel activity, defined as the open probability multiplied by the number of available channels, did not alter with increasing extracellular Na^+. On the other hand, the single-channel current saturated with increasing extracellular Na^+ and, consequently, whole-cell Na^+ permeability declined. In other words, the decline in Na^+ permeability in salivary duct cells with increasing extracellular Na^+ concentration is due simply to saturation of the single-channel Na^+ conductance rather than to inactivation of channel activity.

Key words: Amiloride — 6-Chloro-3,5-diaminopyrazine-2-carboxamide — Mouse mandibular glands

Introduction

Absorption of NaCl by salivary ducts is believed to have both electrogenic and nonelectrogenic components [8]. The electrogenic component involves Na^+ absorption across the apical membranes of the duct epithelial cells via an amiloride-sensitive Na^+ channel [6, 11, 30] with passive flow of Cl^- down its electrochemical gradient to maintain charge balance [2, 9, 24]. The electroneutral system involves paired Na^+-H^+ and K^+-H^+ exchangers in the apical membranes so that Na^+ is exchanged electroneutrally for K^+ [23, 34]. The relative importance of these two systems varies between the two species studied: the electrogenic system predominates in rabbit mandibular ducts whereas the electroneutral system predominates in rat mandibular ducts [8].

Microperfusion studies in rabbit mandibular extralobular ducts have demonstrated that ductal Na^+ transport is a saturating function of luminal Na^+ concentration [5]. Since much of the Na^+ transport by rabbit mandibular ducts is electrogenic, depending on apical Na^+ channels [6], it seemed likely that this saturation of ductal Na^+ transport arises from a decline in the apical membrane Na^+ permeability (P_{Na}) with increasing luminal Na^+ concentration, as has been reported in other Na^+-absorbing epithelia such as amphibian skin [17], toad bladder [15], colonic mucosa [37], kidney collecting duct [35] and urinary bladder [27]. The origin of this decline in apical Na^+ permeability with increasing mucosal Na^+ concentration, however, remains controversial [36]. Three dif-

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ferent mechanisms have been proposed: (i) saturation of the single-channel conductance by external Na^+ [32, 33]; (ii) inactivation of the apical Na^+ channels via an apical membrane receptor for external Na^+ (so-called ''self-inhibition'') [17, 25]; and (iii) inactivation of the apical Na^+ channels brought about by changes in cytosolic composition secondary to increased Na^+ influx across the apical membrane (so-called ''feedback inhibition'' or ''homocellular regulation'') [14, 16, 28, 29, 38]. Feedback inhibition of Na^+ channels (mechanism iii) has been extensively studied but the available experimental evidence for the existence of mechanism (i) and (ii) is contradictory.

In the present paper, we examine the dependence of the whole-cell Na^+ current on extracellular Na^+ in mouse mandibular intralobular duct cells. We find that the Na^+ conductance in these cells saturates with increasing extracellular Na^+ concentration due to saturation of the single-channel conductance. We find no evidence for inactivation (self-inhibition) of channel activity with increasing extracellular Na^+.

Materials and Methods

Cell Preparation

Male mice were killed by cervical dislocation. The mandibular glands were removed, finely chopped and incubated in a physiological salt solution containing 50 unit/ml collagenase (Worthington type IV, Freehold, New Jersey) for 40 min with intermittent trituration, until single cells were liberated. These were then washed and suspended in a NaCl-rich incubation solution having the following composition (in mmol/l): NaCl (145), KCl (5.5), $CaCl_2$ (1), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), the sodium salt of *N*-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (Na-HEPES, 7.5), H-HEPES (7.5) and glucose (10); the pH was adjusted to 7.4 with NaOH.

Patch Clamp Methods

Standard patch clamp techniques were used [21]. Patch clamp pipettes were pulled from borosilicate microhaematocrit tubes (Modulohm, Hevik, Denmark) so as to have resistances of 1–3 MΩ and their tips were coated with Sylgard 184 (Dow Corning, Midland MI). The reference electrode was an Ag/AgCl pellet connected to the bath by a bridge filled with agar (10 mg/ml) dissolved in the NaCl-rich incubation solution (*see above*). All potential differences reported in this study have been corrected for junction potentials as appropriate [3, 12].

Following establishment of the whole-cell configuration, the solution bathing the cell was changed to a Na^+-rich (Cl^--poor) solution having the following composition (in mmol/1): Na-glutamate (145), NaCl (5), $MgCl_2$ (1.2), EGTA (1), H-HEPES (10) and glucose (10); the pH was adjusted to 7.4 with NaOH. Reductions in bathing solution Na^+ were achieved by equimolar substitution of Na-glutamate by *N*-methyl-D-glucamine-glutamate (NMDG-glutamate). In all experiments, the pipettes were filled with a nominally Na^+-free solution containing (in mmol/1): NMDG-glutamate (145), NMDG-Cl (5), $MgCl_2$ (1), H-HEPES (10), glucose (10) and EGTA (5); the pH was adjusted to 7.2 with Tris base.

An Axopatch-1D patch clamp amplifier (Axon Instruments, Foster City CA) was used to measure whole-cell currents. To determine *I-V* relations, a MacLab-4 data acquisition interface (ADInstruments, Sydney, Australia) attached to a Macintosh IIci computer was used to generate command voltages and sample the whole-cell currents.

All experiments were performed at room temperature (20–22°C).

Fluctuation Analysis

In the fluctuation analysis studies, 200 μmol/l 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC) dissolved in the appropriate bath solution was applied to the cells under investigation through a 30-gauge needle, the tip of which was positioned with the aid of a micromanipulator to be approximately 1.5 mm from the cell. Flow through the needle was regulated by a valve and driven by a hydrostatic pressure of 50 cm H_2O at a rate of approximately 50 μl/min. During the application of CDPC, the membrane potential was clamped at –80 mV. In preliminary experiments in which we applied 100 μmol/l amiloride using this technique, inhibition of the current was complete within 1 sec. Thus the slow time course of the onset of CDPC inhibition (*see* Fig. 6) is attributable to the kinetics of binding of the blocker to the channels, rather than to slow mixing of CDPC in the bath solution. For the fluctuation analysis, we used the entire period of onset of CDPC inhibition (i.e., from 8 to 24 sec in Fig. 6).

The whole-cell current signal was low-pass filtered at 500 Hz and recorded using a modified Sony PCM unit [12]. The mean current in each 100-msec data block was calculated from the whole-cell current sampled at 1,000 Hz, and the variance of the current in each 100-msec data block was calculated from the whole-cell current, which had been high-pass filtered at 3 Hz and sampled at 1,000 Hz. The mean whole-cell Na^+ current (I_{Na}) for each 100-msec block of data was calculated by subtracting the CDPC-insensitive current, measured after prolonged (> 20 sec) exposure of the cell to CDPC, from the mean whole-cell current. This use of the CDPC-sensitive component of the whole-cell current to estimate the total Na^+ current was based on our observation in 5 experiments that 200 μmol/l CDPC produced the same inhibition in inward current as 100 μmol/l amiloride. Finally, the single-channel current was estimated by fitting the relation between the mean Na^+ current (I_{Na}) and the variance of the current (σ^2) with the equation

$$\sigma^2 = I_{Na}\, i - \frac{I_{Na}^2}{N_o} + \sigma^2_{residual} \tag{1}$$

using, as free parameters, i the single-channel current, N_0 the number of channels open at the time of CDPC addition, and $\sigma^2_{residual}$, the residual current variance when all the Na^+ current is blocked.. The channel activity ($N_T p$) could then be calculated from the equation

$$N_T p = \frac{I_{Na}}{i} \tag{2}$$

where N_T is the total number of channels available, and p is the open probability.

This method of analysis is similar to that used by Ämmälä and coworkers [1] to determine the single-channel conductance of the K^+ channels activated by Ca^{2+} spikes in pancreatic β cells, except that we have examined current fluctuations during the onset of channel inhibition rather than during the onset of channel activation. The frequency range over which we measured the variance (3–500 Hz) was chosen to cover adequately the reported spectrum of CDPC-induced noise [22].

As discussed by Helman and Kizer [22], the spontaneous kinetics of epithelial Na^+ channels are characterized by a corner frequency of 0.01 Hz or lower [39], so the number of channels estimated from Eq. (1) is the number of channels that are open at the time of CDPC addition, N_o, rather than the total number of available channels, N_T. For this reason, we report channel activity, N_Tp, as estimated by Eq. (2), rather than separate estimates of channel numbers and channel open probability.

Analysis of *I-V* Relations

I-V relations were studied using 20-mV voltage pulses, each of 800-msec duration, delivered at voltages ranging between +140 and −120 mV, and the voltage pulses were separated by a 5-sec interval during which the cell potential was held at 0 mV. Steady-state currents were calculated by averaging the whole-cell current between 700 msec and 800 msec from the start of each voltage pulse. Zero-current (or reversal) potentials were estimated by fitting linear regression lines to the 4 data points nearest to the zero current potential. Inward conductances were calculated by fitting a linear regression to the data points between −60 and −120 mV.

The Na^+ permeability (P_{Na}) and the $NMDG^+$ permeability (P_{NMDG}) were estimated by fitting the Goldman equation to the full *I-V* relation of the amiloride-sensitive current (I_{Am}), assuming that the extracellular and intracellular compositions were identical to those of the bath and pipette solutions respectively. The form of the equation we used was

$$I_{Am} = P_{Na}\frac{VF^2/RT}{1-e^{(-VF/RT)}} \times [[Na^+]_o + (P_{NMDG}/P_{Na})[NMDG^+]_o - ([NA^+]_i + (P_{NMDG}/P_{Na})[NMDG^+]_i)\, e^{(-VF/RT)}] \quad (3)$$

where V is the command potential, $[X]_o$ and $[X]_i$ represent extracellular and intracellular concentrations of Na^+ or $NMDG^+$, and F/RT is 0.0364 mV^{-1} at 22°C.

Presentation of Data

In all figures, outward current, defined as positive charge leaving the pipette, is depicted as an upward deflection, and the potential differences are expressed as the potential of the pipette with respect to the bath. Results are presented as means ± SEM with the number of cells tested in parentheses. Statistical significance was assessed using unpaired Student's *t*-tests, and a probability level of $P < 0.05$ was taken as significant.

Materials

N-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), *N*-methyl-D-glucamine ($NMDG^+$), and 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC) were obtained from Sigma (St. Louis, MO). Amiloride was obtained from RBI (Natick, MA).

Results

In our initial studies, we used the protocol illustrated in Fig. 1 to measure the Na^+ current. In this protocol, we first measured the whole-cell *I-V* relation when the bath contained a Na^+-rich bath solution (Fig. 1*A*, filled circles). We then replaced the bath with a similar solution containing 100 μmol/l amiloride and remeasured the *I-V* relation (Fig. 1*A*, open circles). By subtracting the whole-cell records observed prior to the addition of amiloride from those observed following addition of the drug, we obtained whole-cell records and the steady-state *I-V* relation for the component of the whole-cell current attributable to amiloride-sensitive Na^+ channels (Fig. 1*B*). This amiloride-sensitive current (Fig. 1*B*) is not activated by voltage (Fig. 1, panel III), is inwardly rectifying (Fig. 1, panel III) and has a zero-current potential of +62.7 ± 3.4 mV ($n = 7$). We have previously shown this conductance to have a high selectivity for Na^+ over K^+ [11] and to be permeable to Li^+ [11], observations that are consistent with the possibility that epithelial Na^+ channels are responsible for the conductance.

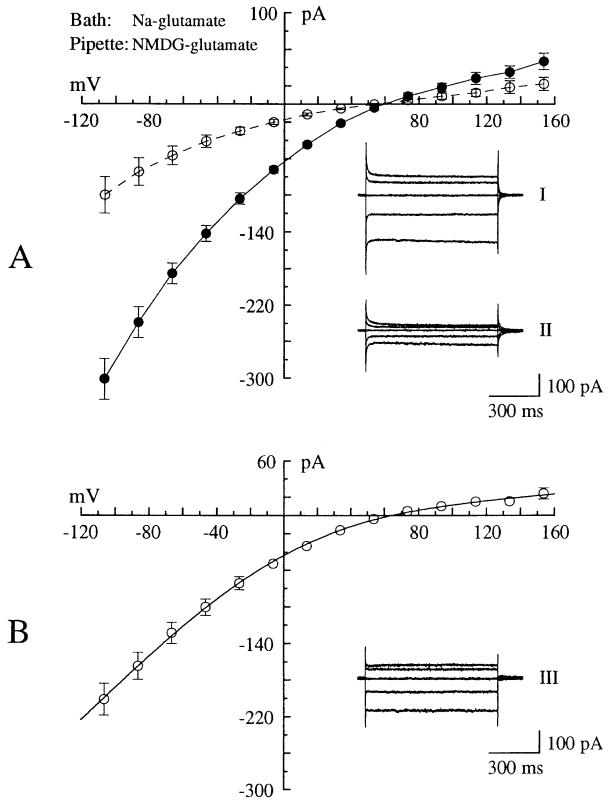

Fig. 1. The whole-cell Na^+ currents in salivary duct cells studied with NMDG-glutamate pipette solution. Panel *A* shows representative whole-cell recordings from a single cell for voltage-pulses of −120, −60, 0, +60 and +120 mV together with mean steady-state *I-V* relations recorded from 7 cells with a 158 mmol/l Na^+-containing bath (filled circles and I) and following the addition of 100 μmol/l amiloride to the bath solution (open circles and II). Panel *B*, shows representative recordings from a single cell of the whole-cell Na^+ current (III) and the mean steady-state *I-V* relation of the Na^+ current calculated by subtraction of the two sets of data in panel *A*. Each steady-state *I-V* relation shows mean ± SEM of measurements on 7 cells.

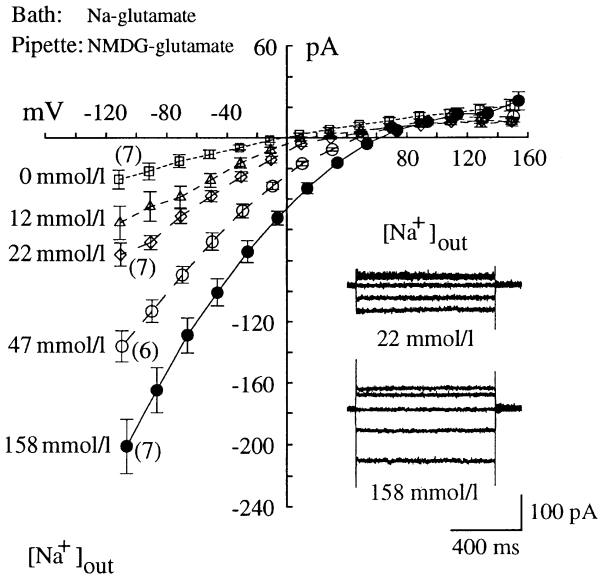

Fig. 2. Dependence of the steady-state *I-V* relation of the whole-cell Na^+ current (measured as shown in Fig. 1) on the extracellular Na^+ concentration. The lines are smooth curves with no theoretical significance. Also shown are representative whole-cell current records.

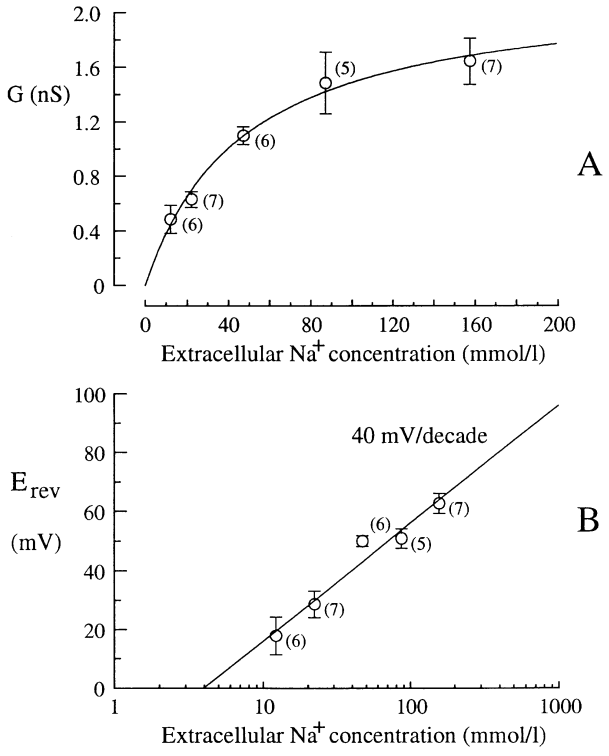

Fig. 3. Panel *A.* Plot of the inward conductance (*G*) of the whole-cell Na^+ current as a function of extracellular Na^+. The line is a least squares fit of the Michaelis-Menten equation. Panel *B.* Semilogarithmic plot of the zero-current potential of the amiloride-sensitive whole-cell current as a function of extracellular Na^+. The straight line is the line of best fit.

When we used this protocol to measure the whole-cell Na^+ current in cells bathed in solutions in which the Na^+ concentration ($[Na^+]_o$) had been varied by equimolar replacement with $NMDG^+$ (Fig. 2), we found that the inward Na^+ current declined with decreasing $[Na^+]_o$ (Fig. 2). Plotting the inward Na^+ conductance as a function of $[Na^+]_o$ revealed a saturating relation (Fig. 3*A*) which, when fitted by the Michaelis-Menten equation, gave a maximum inward conductance (G_{max}) of 2.18 ± 0.27 nS ($n = 31$) and a K_m of 47.3 ± 14.6 mmol/l ($n = 31$). In the nominal absence of Na^+, both extracellularly and intracellularly, we were still able to measure an amiloride-sensitive conductance of 0.16 ± 0.02 nS ($n = 7$). This was small compared to the inward conductance measured with 158 mmol/l Na^+ in the bath solution (1.65 ± 0.17 nS, $n = 7$), but its presence suggests that $NMDG^+$ has a significant permeability through the amiloride-sensitive channels. Plotting the zero-current potential of the amiloride-sensitive current as a function of the logarithm of $[Na^+]_o$ (Fig. 3*B*), revealed a straight line relation with a slope of +40 mV/decade change in $[Na^+]_o$. This observation also is consistent with the postulate that the amiloride-sensitive current is partially carried by $NMDG^+$.

It has been generally accepted that the voltage-dependence of the amiloride-sensitive Na^+ current in epithelia can be adequately described by the Goldman equation [17, 25, 33]. When, however, we attempted to fit the Goldman equation to the *I-V* relations of the amiloride-sensitive current assuming that only Na^+ was permeable (i.e., P_{NMDG} set to zero in equation 3), we were unable to obtain acceptable fits (*see* Fig. 4, broken lines). Allowing P_{NMDG} to assume a positive value, however, yielded fits that were markedly improved, particularly in describing the *I-V* relation accurately at extreme voltages. The fits still deviated, however, from the experimental data at potentials close to the zero current potential (Fig. 4, unbroken lines).

Plotting the permeabilities for Na^+ (P_{Na}) and $NMDG^+$ (P_{NMDG}) calculated using the Goldman equation (Eq. 3), showed that P_{Na} decreased with increasing $[Na^+]_o$ from $(8.6 \pm 1.8) \times 10^{-10}$ cm^3/sec ($n = 6$) at 12 mmol/l $[Na^+]_o$, to $(3.1 \pm 0.3) \times 10^{-10}$ cm^3/sec ($n = 7$) at 158 mmol/l $[Na^+]_o$. The permeability to $NMDG^+$ was small ($(0.16 \pm 0.03) \times 10^{-10}$ cm^3/sec, $n = 6$ at 12 mmol/l $[Na^+]_o$) and showed no consistent trend with increasing Na^+ (Fig. 5).

We then decided to determine whether the saturation of the whole-cell Na^+ conductance with increasing $[Na^+]_o$ (Fig. 3*A*) and the accompanying decline in P_{Na} (Fig. 5) were due to saturation of the single-channel conductance or to inactivation of the single-channel activity.

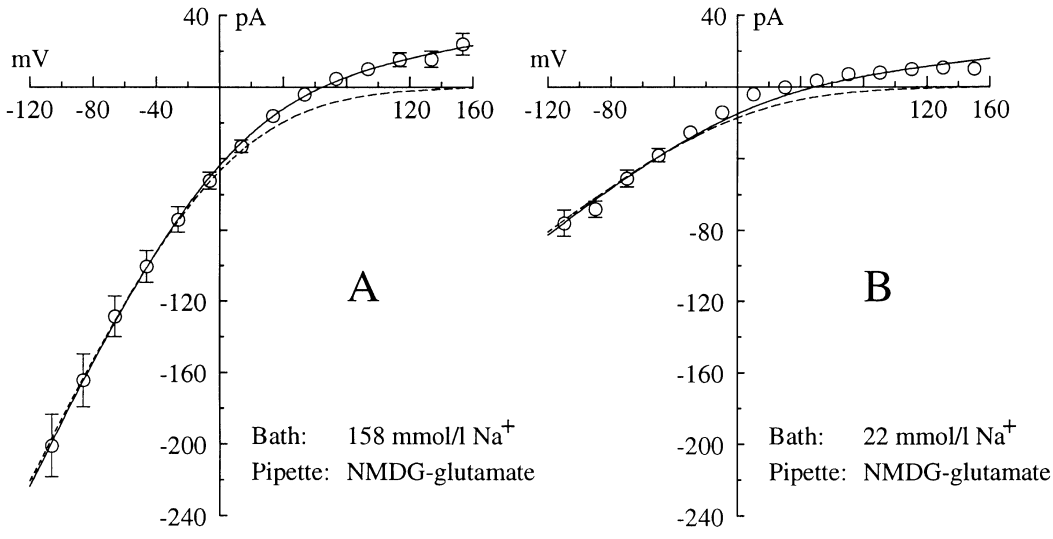

Fig. 4. Steady-state *I-V* relations obtained with 158 mmol/l Na^+ (Panel *A*) and 22 mmol/l Na^+ (Panel *B*) in the bathing solutions. In each case, the data have been fitted with the Goldman equation (*see* Methods) assuming only Na^+ is permeable (broken lines) and with the Goldman equation, assuming both Na^+ and $NMDG^+$ are permeable (unbroken lines). Each point represents the mean ± SEM of 7 experiments.

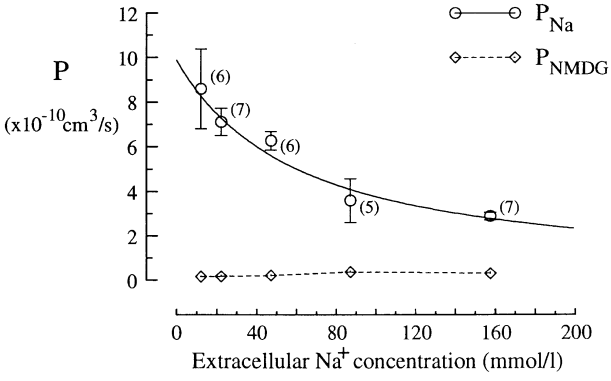

Fig. 5. Plot of the whole-cell Na^+ permeability (P_{Na}) and $NMDG^+$ permeability (P_{NMDG}) as functions of the extracellular Na^+ concentration. P_{Na} (open circles) and P_{NMDG} (open diamonds) were calculated by fitting the Goldman equation to the whole-cell *I-V* relations for the amiloride-sensitive current shown in Fig. 2. The smooth curve is a least squares fit of the reciprocal of the Michaelis-Menten equation. The broken line has no theoretical significance.

We did this by applying fluctuation analysis to the whole-cell current during the onset of inhibition by the electroneutral weak Na^+ channel blocker, CDPC (*see* Methods). As shown in Fig. 6*A*, the onset of inhibition by 200 μmol/l CDPC (between 8 sec and 24 sec in Fig. 6*A*) is accompanied by an increase in whole-cell current noise. This increase in current noise is evident once the current signal has been high-pass filtered (Fig. 6*B*), and results in a marked increase in the variance of the whole-cell current during the onset of CDPC inhibition (Fig. 6*C*). Plotting the variance of the current (Fig. 6*C*) as a function of the mean CDPC-sensitive current (*see* Methods), revealed a parabolic relation (Fig. 6*D*) which, when fitted by Eq. (1), revealed a single-channel current of –0.33 pA. From this estimate of single-channel current, we estimated (Eq. 2) the channel activity (N_Tp) as 380.2. In 8 experiments of this type, performed on cells bathed in 158 mmol/l Na^+ solution, we estimated that the single-channel current was –0.31 ± 0.01 pA, and the channel activity was 396.7 ± 19.1. Using the Goldman equation, we calculated the single-channel current to be equivalent to a single-channel conductance of 4.0 ± 0.14 pS ($n = 8$) for a channel bathed symmetrically in 158 mmol/l Na^+ solutions.

We then used fluctuation analysis to measure the single-channel currents and channel activities when the bath contained 7, 22 or 87 mmol/l Na^+ (Fig. 7). We found that the CDPC-sensitive component of the whole-cell current was a saturating function of $[Na^+]_o$ (Fig. 7*A*), with a K_m of 24.6 ± 7.8 mmol/l ($n = 22$) and a maximum current of –132.3 ± 11.1 pA ($n = 22$). Furthermore, the single channel current was a saturating function of $[Na^+]_o$ (Fig. 7*B*) which was described by the Michaelis-Menten equation with a K_m of 17.7 ± 4.3 mmol/l ($n = 22$) and a maximum single channel current of –0.32 ± 0.02 pA ($n = 22$). The channel activity (N_Tp) was not affected by $[Na^+]_o$ (Fig. 7*C*).

We used an alternative approach to test further whether Na^+ channel activity is directly regulated by extracellular Na^+. Fuchs and coworkers [17] have shown that the response of the short-circuit current in amphibian skin to a rapid, step-increase in the Na^+ concentration bathing the apical membrane, exhibits a transient overshoot. This has been interpreted to indicate

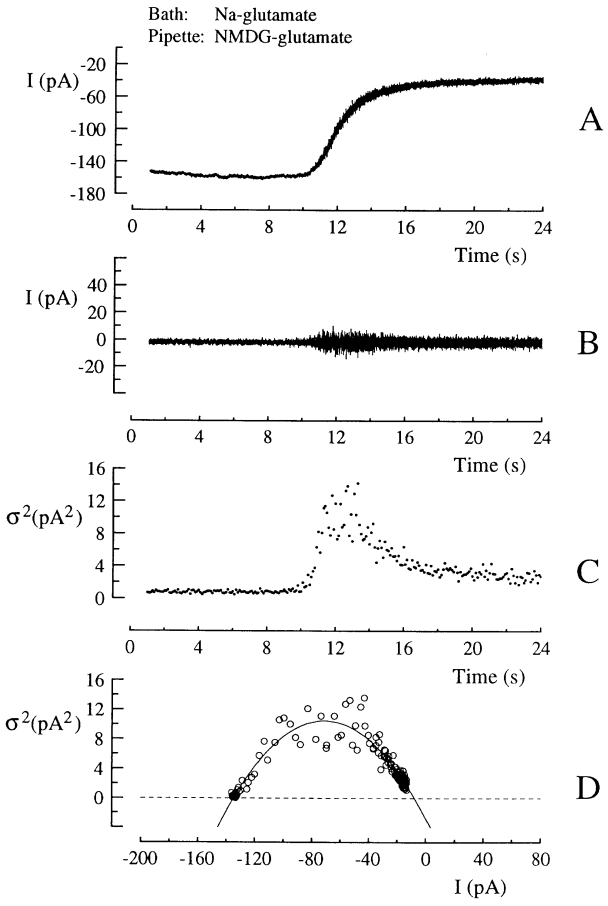

Fig. 6. Panel *A.* Time course of the effect of 200 μmol/l CDPC on the whole-cell current of a duct cell bathed in an Na-glutamate solution containing 158 mmol/l Na^+ with an NMDG-glutamate pipette solution. The holding potential was −80 mV. Panel *B.* The record in *A* following high-pass filtering at 3 Hz. Panel *C.* Variance (σ^2) of the whole-cell current calculated from the high-pass filtered current. Panel *D.* Graph showing the relation between the current variance and the mean whole-cell Na^+ current (calculated as described in Methods). The unbroken line is a least-squares fit of equation (1) to the data.

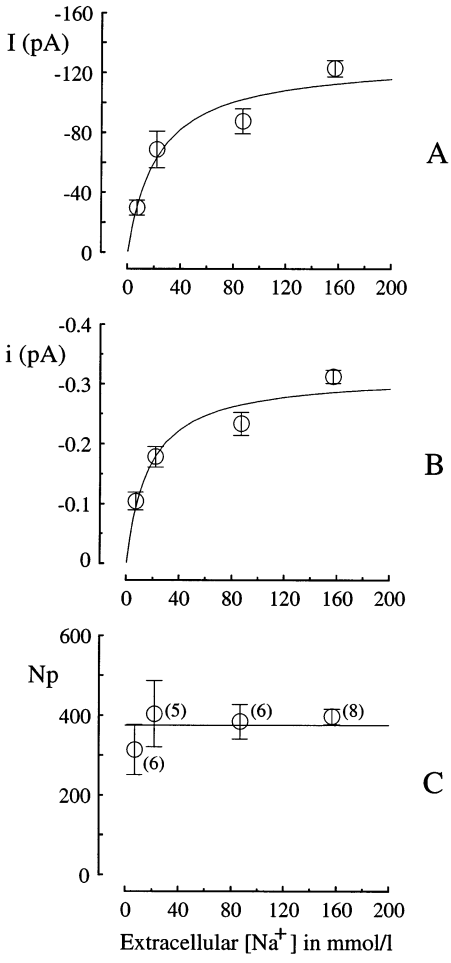

Fig. 7. Dependency of a single-channel parameters on extracellular Na^+ as determined by fluctuation analysis (*see* Fig. 6). Panel *A.* Dependency of the whole-cell Na^+ current (*I*) in these experiments on extracellular Na^+. Panel *B.* Dependency of the single-channel current (*i*) on extracellular Na^+. Panel *C.* Dependency of the channel activity (*Np*) on extracellular Na^+.

that the saturation of the steady-state current observed with increasing apical Na^+ arises from inactivation of the activity of the Na^+ channels rather than from saturation of the conductance pathway [17]. The rabbit mandibular excretory duct also shows a transient overshoot in the response of its transepithelial potential to step changes in luminal Na^+ [5]. Consequently, we decided to examine whether a step increase in $[Na^+]_o$ was accompanied by a transient overshoot in the whole-cell current in our preparation. In 5 experiments in which we changed $[Na^+]_o$ rapidly (<200 msec) from zero to 158 mmol/l, we failed to observe a consistent overshoot in the whole-cell current at −80 mV. The tracing that most convincingly showed an overshoot is depicted in Fig. 8. The mean overshoot, however, was only 7.2% ± 1.6 (n = 5) of the peak increase in current of 75.5 ± 14.9 pA (n = 5).

Discussion

We have found that the whole-cell Na^+ conductance in mouse mandibular intralobular duct cells is a saturating function of $[Na^+]_o$ (Fig. 3). This saturation in whole-cell current is associated with a reduction in P_{Na} as $[Na^+]_o$ increases (Fig. 5), and is due to saturation of the single-channel current (Fig. 7*B*) rather than to a decrease in channel activity (Fig. 7*C*).

The single-channel conductance we have estimated for the amiloride-sensitive Na^+ channels in salivary duct cells (4 pS) is similar in size to that measured in single-channel studies and in noise analysis studies on the highly-selective, amiloride-sensitive Na^+ channel seen in other tight epithelia (reviewed in [31]). This suggests that the amiloride-sensitive Na^+ channels in salivary ducts, despite their relative insensitivity to amiloride

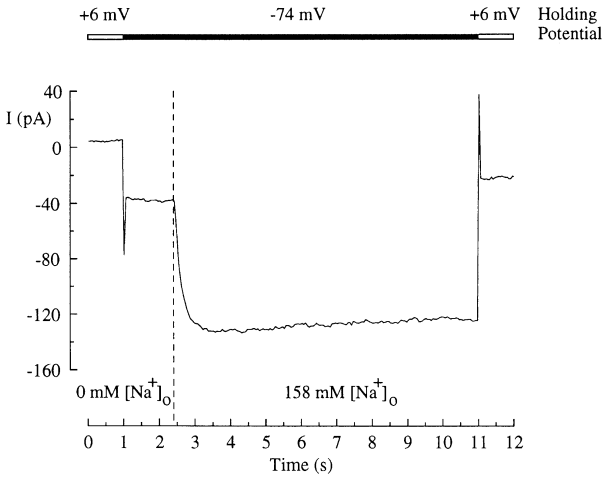

Fig. 8. Effect of a rapid (<200 msec) increase in extracellular Na^+ from 0 mmol/l to 158 mmol/l (marked by the vertical broken line) on the inward current measured at −74 mV.

[11], are closely related to the high-selectivity group of epithelial Na^+ channels, which includes those in renal cortical collecting duct, amphibian skin and toad bladder [4, 19]. This possibility is also supported by reports that all three of the known subunits of the epithelial Na^+ channel are expressed in salivary ducts [13] and that the apical membrane Na^+ permeability of salivary ducts is controlled by aldosterone [20].

Our finding that the single-channel current saturates with increasing $[Na^+]_o$ is in agreement with previous single-channel studies on rat renal cortical collecting duct [32, 33]. Noise analysis studies on whole tissues, however, have not revealed saturation of the single-channel conductance with increasing $[Na^+]_o$ [7, 28, 38]. The reason for the difference in findings between the single-channel and the whole-cell patch-clamp studies, on the one hand, and the whole-tissue studies, on the other, is unclear. One possible explanation could be that the whole-tissue studies may have been performed under conditions in which saturation would not be demonstrable. Van Driessche and Lindemann [38], for example, only increased apical Na^+ to 60 mmol/l, and the use of choline as a Na^+ substituent by Christensen and Bindslev [7] may have increased the K_m for Na^+ to such an extent that saturation would not have been observed (*c.f.* [33]). These criticisms, however, do not apply to a noise-analysis study carried out on rabbit colon [28] which also failed to demonstrate saturation. Another possible explanation is that the differing findings with respect to saturation could be attributable to the existence of subtypes of epithelial Na^+ channels [19]. The findings that the high amiloride affinity Na^+ channels in rat collecting ducts and the low amiloride affinity Na^+ channels in salivary ducts both show saturation indicate, however, that subtypes would not correspond with the usual classifications of Na^+ channels based on their affinity for amiloride [19]. Finally, it may be that the whole-cell and single channel methods alter the biophysical properties of the Na^+ channel by an unknown mechanism, possibly by perturbing the intracellular or membrane environment of the channels. Given the markedly different relation between the channel protein and the patch pipette in these two techniques, this seems unlikely.

From our studies, it is not possible to say whether $NMDG^+$ passes through the same channels as the Na^+ current. $NMDG^+$ may, for example, be passing through a subpopulation of channels with properties similar to those of the amiloride-sensitive, low-selectivity Na^+ channels in fetal lung epithelium that have been reported to have a substantial permeability to $NMDG^+$ [18]. Amiloride-sensitive, high selectivity channels such as those that predominate in salivary duct cells are not usually considered to be permeable to $NMDG^+$, although the finding that $NMDG^+$ and Na^+ compete for a site within the Na^+ channel in rat cortical collecting duct cells, is consistent with $NMDG^+$ having a small but significant permeability through these channels [33]. Our finding that P_{Na} depends on extracellular Na^+, whereas P_{NMDG} does not, cannot be used to argue that $NMDG^+$ is passing through a different channel type to the bulk of the Na^+. This is because, in our experiments, the estimate of $P_{N\text{-}MDG}$ was largely determined by the *I-V* relation at positive pipette potentials that reduce the concentration of Na^+ in the pore and so minimize the effect of extracellular Na^+ on the channel properties.

Our failure to observe any change in Na^+ channel activity with changes in $[Na^+]_o$ is consistent with single-channel studies on cell-attached patches from cortical collecting duct cells showing that increasing pipette Na^+ concentration is not accompanied by any change in channel open probability [32]. It suggests, at least for salivary duct cells, that Na^+ channel activity is not controlled by an apical membrane receptor for extracellular Na^+ of the type suggested previously for amphibian skin [17].

The experiments presented here do not exclude the possibility that changes in $[Na^+]_o$ may alter Na^+ channel activity with a slower time course than that examined here (i.e., 30 min or longer). They also do not exclude the possibility that increasing $[Na^+]_o$ may lead to a reduction in channel open probability that is balanced by an increase in Na^+ channel numbers so as to leave channel activity unchanged. These possibilities do not, however, invalidate the major conclusion of this paper that self-inhibition is not a significant short-term physiological regulator of Na^+ transport in salivary duct cells.

Since the cytosolic composition in our whole-cell studies is more or less fixed by the pipette composition, our failure to observe a marked transient component in the response to step changes in extracellular Na^+ (Fig. 8) suggests that the occurrence of marked transients following step changes in luminal Na^+ seen in in-vitro perfusion

studies on salivary ducts [5] may be attributable to changes in cytosolic composition secondary to increased Na^+ influx across the apical membrane. We do not yet have data on the intracellular mediators of this cytosolic regulatory mechanism. Our findings in salivary duct cells that increasing intracellular Cl^- inhibits the Na^+ conductance [10, 12] and the observation that the intracellular Cl^- concentration depends on the rate of Na^+ influx across the apical membrane of the duct cells [26] suggest that cytosolic Cl^- may play an important role in this process. We have also found that intracellular Na^+ may directly control the activity of the Na^+ channels in duct cells (P. Komwatana, A. Dinudom, J.A. Young and D.I. Cook, *submitted*). Further experiments will thus be required to establish whether Cl^-, Na^+ or some other intracellular constituent is principally responsible for homocellular regulation of electrogenic Na^+ transport in duct cells.

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