J. Membrane Biol. 162, 225–232 (1998)

The Journal of
Membrane
Biology

Activators of Epithelial Na^+ Channels Inhibit Cytosolic Feedback Control. Evidence for the Existence of a G Protein-Coupled Receptor for Cytosolic Na^+

P. Komwatana, A. Dinudom, J.A. Young, D.I. Cook
Department of Physiology, University of Sydney, NSW 2006, Australia

Received: 1 October 1997/Revised: 24 December 1997

Abstract. We have previously shown that epithelial Na^+ channels in mouse mandibular gland duct cells are controlled by cytosolic Na^+ and Cl^-, acting, respectively, via G_o and G_i proteins. Since we found no evidence for control of epithelial Na^+ channels by extracellular Na^+ ($[Na^+]_o$), our findings conflicted with the long-held belief that Na^+ channel activators, such as sulfhydryl reagents, like para-chloromercuriphenylsulfonate (PCMPS), and amiloride analogues, like benzimidazolylguanidinium (BIG) and 5-N-dimethylamiloride (DMA), induce their effects by blocking an extracellular channel site which otherwise inhibits channel activity in response to increasing $[Na^+]_o$. Instead, we now show that PCMPS acts by rendering epithelial Na^+ channels refractory to inhibition by activated G proteins, thereby eliminating the inhibitory effects of cytosolic Na^+ and Cl^- on Na^+ channel activity. We also show that BIG, DMA, and amiloride itself, when applied from the cytosolic side of the plasma membrane, block feedback inhibition of Na^+ channels by cytosolic Na^+, while leaving inhibition by cytosolic Cl^- unaffected. Since the inhibitory effects of BIG and amiloride are overcome by the inclusion of the activated α-subunit of G_o in the pipette solution, we conclude that these agents act by blocking a previously unrecognized intracellular Na^+ receptor.

Keywords: Amiloride — Salivary gland — Na^+ current — Para-chloromercuriphenylsulfonate — Benzimidazolylguanidinium

Introduction

The activity of the Na^+ channels in the apical membranes of tight epithelia such as renal collecting ducts, colonic mucosa, amphibian skin, and salivary duct, is strictly controlled to ensure that the rate of Na^+ entry into the cytosol is matched to the rate at which Na^+ can be extruded by the Na^+-K^+-ATPase [23, 27] so as to hold cytosolic Na^+ constant. This so-called ''homocellular'' regulation has been extensively studied, although the mechanisms responsible for it are disputed. Suggested mechanisms for apical Na^+ channel inhibition include the binding of Na^+ to a hypothetical extracellular modifier site [10, 21, 28] or to an intracellular modifier site [3, 14, 24], as well as the action of other intracellular mediators, such as H^+ [12], free Ca^{2+} [9, 25] or cytosolic Cl^- [7, 8], the concentrations of which are influenced by the rate of Na^+ entry to the cytosol.

Recently, we have used whole-cell patch-clamp methods in mouse mandibular salivary duct cells to investigate the mechanisms by which Na^+ channels are controlled. We found that the activity of the channels is inhibited by increases in intracellular Na^+ [16] and Cl^- [7, 8] concentrations, acting, respectively, via the pertussis-toxin-sensitive G proteins, G_o and G_{i2} (or G_{i1}) [8, 16]. These effects were not mediated by changes in cytosolic pH or cytosolic-free Ca^{2+} [16]. In agreement with earlier single-channel studies in rat collecting ducts [22], we found no evidence for the control of Na^+ channel activity by an extracellular modifier site for Na^+ [15].

For over 20 years, agents such as the sulfhydryl reactive reagent, para-chloromercuriphenylsulfonate (PCMPS), and amiloride analogues, such as benzimidazolylguanidinium (BIG) and 5-N-dimethyl-amiloride (DMA), as well as amiloride itself in certain circumstances [1, 2, 4, 26], have been known to stimulate Na^+ transport across tight epithelia such as frog skin by increasing Na^+ channel activity [5, 19–21, 31], apparently by blocking normal processes of homocellular regulation [10]. These agents are thought to act by blocking the inhibitory action of extracellular Na^+ at an extracellular modifier site on the channel protein [11, 20, 21, 29], a

Correspondence to: D.I. Cook

belief that is clearly in conflict with our recent proposal [16] that homocellular regulation is due to feedback inhibition of Na^+ channels by cytosolic Na^+ and Cl^- [7, 8, 16]. In the hope of resolving this apparent conflict, we have now investigated the mechanisms by which PCMPS and BIG (as well as DMA and amiloride) can stimulate Na^+ channels and find that amiloride and related agents activate Na^+ channels by blocking a previously unsuspected G-protein coupled receptor for intracellular Na^+.

Materials and Methods

Cell Preparation

Isolated salivary duct cells were prepared by collagenase digestion of mandibular glands from male mice [6, 7]. The standard bath solution had the following composition (in mmol/l): NaCl (145), KCl (5.5), $CaCl_2$ (1.0), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), Na-*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. After establishing the whole-cell configuration in an isolated duct cell, we replaced the bath solution with a solution containing (in mmol/l): Na-glutamate (Na-glu) (145), NaCl (5.0), $MgCl_2$ (1.0), H-HEPES (10), glucose (10) and ethylene-glycol-bis(β-aminoethyl-ether)*N,N,N′,N′*-tetra-acetic acid (EGTA) (1.0); the pH was adjusted to 7.4 with NaOH. The pipettes were filled with solutions containing (in mmol/l): N-methyl-D-glucamine glutamate (NMDG-glu) and Na-glu (together totalling 150), $MgCl_2$ (1.0), H-HEPES (10), glucose (10) and EGTA (5.0); the pH was adjusted to 7.2 with *Tris* base or NaOH (7–22 mmol/l) as appropriate.

Patch-clamp Techniques

Previously described, standard whole-cell patch-clamp methods were used [6, 7]. Patch-clamp pipettes were pulled from borosilicate microhematocrit tubes (Modulohm, Hevik, Denmark) so as to have resistances of 1–3 MΩ. An Ag-AgCl pellet was used as the reference electrode and all potential differences were corrected for liquid junction potentials as appropriate [7]. An Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA) was used to measure whole-cell currents. To determine whole-cell *I-V* relations, a MacLab-4 data acquisition interface (ADInstruments, Sydney, Australia) attached to a Macintosh-IIci computer was employed to generate command voltages and to sample whole-cell currents. The amiloride-sensitive current was calculated as the difference between the whole-cell currents measured prior to and following the addition of 100 μmol/l amiloride to the bath solution. Whole-cell *I-V* relations were obtained by applying voltage pulses of 200-msec duration from a resting potential of 0 mV. Steady-state currents were calculated as the average current between 100 and 200 msec after the start of the voltage pulse. Chord conductances are calculated as the slope of the line joining the current at −80 mV and the reversal potential of the amiloride-sensitive or $NMDG^+$-sensitive current as appropriate.

Single-Channel Properties Measured Using Noise Fluctuation Analysis

Noise fluctuation analysis was carried out using methods described previously [15, 16]. In brief, the membrane potential was clamped at −80 mV during the application of 6-chloro-3,5-diamino-pyrazine-2-carboxamide (CDPC), a weak electroneutral Na^+ channel blocker, and the recorded whole-cell current was filtered at 500 Hz and sampled at 1000 Hz. For each 100-msec block of data, the mean current was determined and the current variance was calculated following high-pass filtering at 3 Hz to remove the DC component of the signal. The mean whole-cell Na^+ current (I_{Na}) was calculated by subtracting the CDPC-insensitive current, measured after prolonged exposure (>20 sec) to CDPC, from the mean whole-cell current for each block of data. The single-channel current was estimated by fitting the relation between mean Na^+ current (I_{Na}) and current variance (σ^2) with the equation

$$\sigma^2 = I_{Na}i - I_{Na}^2/N_o + \sigma^2_{\text{residual}}$$

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

$$N_T p = I_{Na}/i$$

where N_T is the number of channels available and p is the channel open probability. The single-channel conductance (γ) was estimated from the Goldman equation using the measured single-channel current (i) for a channel bathed symmetrically in solutions containing 157 mmol/l Na^+.

Chemicals

CDPC, EGTA, *Tris*, GTP-γ-S and HEPES were obtained from Sigma (St. Louis, MO), amiloride and 5-*N*-dimethylamiloride from RBI (Natick, MA), benzimidazolylguanidinium (BIG) and para-chloromercuriphenylsulfonate (PCMPS) from Aldrich (Castle Hill, Australia), and type IV collagenase from Worthington (Freehold, NJ). Recombinant myristoylated rat α-subunits of G_o, G_{i1} and G_{i2} were obtained from Calbiochem (Novato, CA) and activated as described by Lang and coworkers [17].

Results

Effect of PCMPS on Na^+ Channel Activity

In the present studies we used CDPC fluctuation analysis [15, 16] to measure simultaneously the amiloride-sensitive whole-cell current and the underlying single-channel current and channel activity (i.e., the number of channels, *N*, multiplied by their open probability, *p*) in single mouse mandibular granular duct cells held in the whole-cell patch-clamp configuration. We found that the presence of 1 mmol/l PCMPS in the bath solution had no effect on the whole-cell Na^+ current, the single-channel current or the activity of the Na^+ channels when the pipette solution contained NMDG-glu (Fig. 1), i.e., when both the Na^+ feedback and Cl^- feedback systems were inactive. We then confirmed that, as we have previously reported [16], increasing the intracellular Na^+ concentration to 72 mmol/l caused the whole-cell Na^+ current to decrease due to a marked decrease in channel activity (Fig. 1). Finally, we found that the inhibitory

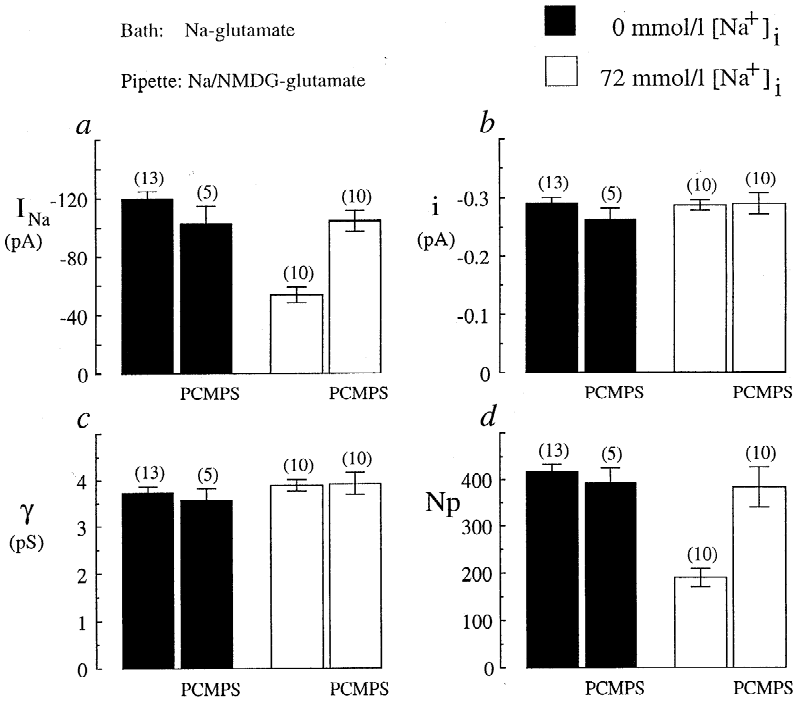

Fig. 1. Effect of extracellular PCMPS on the whole-cell CDPC-sensitive Na^+ current (panel *a*), and the single-channel current (*i;* panel *b*), the single-channel conductance (γ*;* panel *c*) and the channel activity (N_p; panel *d*) of Na^+ channels, measured by CDPC fluctuation analysis at a pipette potential of −80 mV with a pipette solution containing a Na^+-free, 150 mmol/l NMDG-glu solution (filled bars) or a mixture of 78 mmol/l NMDG-glu and 72 mmol/l Na-glu (open bars). The bath contained a Na-glu solution, together with 1 mmol/l PCMPS where indicated.

effect of this increase in intracellular Na^+ was completely overcome by the presence of 1 mmol/l PCMPS in the bath solution (Fig. 1).

The Effect of BIG on Na^+ Channel Activity

We also examined the effects of BIG on Na^+ channel activity. We first investigated whether BIG applied extracellularly stimulated Na^+ channels, but found that, if anything, it was inhibitory (Fig. 2). This conclusion is in accordance with noise analysis studies on frog skin which showed that BIG reduces the single-channel conductance [20]. The effects of BIG applied intracellularly via the pipette solution were then examined. We found that BIG, when applied intracellularly, stimulated the Na^+ current, with 1 mmol/l producing close to a maximum response (Fig. 3*e*). CDPC fluctuation analysis revealed that intracellular BIG (1 mmol/l) totally overcame the inhibitory effect of a high cytosolic Na^+ concentration on Na^+ channel activity, but had no effect on the activity of Na^+ channels when intracellular Na^+ was low (Fig. 3*a–d*).

The Effect of PCMPS and BIG on Feedback Control by Cytosolic Anions

We then examined the effect of PCMPS on the inhibitory effects of cytosolic anions on Na^+ channel activity. In these experiments we used NO_3^- rather than Cl^- in the peptide solution to inhibit the Na^+ current because use of NO_3^- also eliminated the Cl^- current which, if present, would have interfered with our measurement of the Na^+ current [7, 8]. We found that 1 mmol/l PCMPS in the bath solution totally overcame the inhibitory effect of the inclusion of NO_3^- in the pipette solution (Fig. 4), whereas, in contrast, the presence of 1 mmol/l BIG in the pipette solution had no effect (Fig. 4).

The Sites of Action of PCMPS and BIG

Since our results showed that extracellular PCMPS activated Na^+ channels by preventing the inhibitory effects of cytosolic Na^+ and anions on Na^+ channel activity, it seemed likely that PCMPS acted at the only site common to the two feedback systems, the Na^+ channel itself. Furthermore, since PCMPS had no effect on Na^+ channel activity when these feedback systems were inactive, our findings suggested that PCMPS, rather than acting as a nonspecific stimulant of Na^+ channel activity, rendered the channels refractory to inhibition by G proteins.

We tested this hypothesis in two steps. We first demonstrated that inclusion of the activated recombinant α-subunits of G_o or G_{i2} in the NMDG-glu pipette solution inhibits the Na^+ channels (Fig. 5*a*). (In contrast, we also demonstrated that the activated α-subunit of G_{i1} was without effect (Fig. 5*a*), indicating that Cl^- feedback control of the Na^+ channels is mediated by G_{i2} not G_{i1}). We then showed that the activated recombinant α-subunit of G_o is unable to reverse the stimulatory effect of PCMPS when the pipette solution contains 72 mmol/l

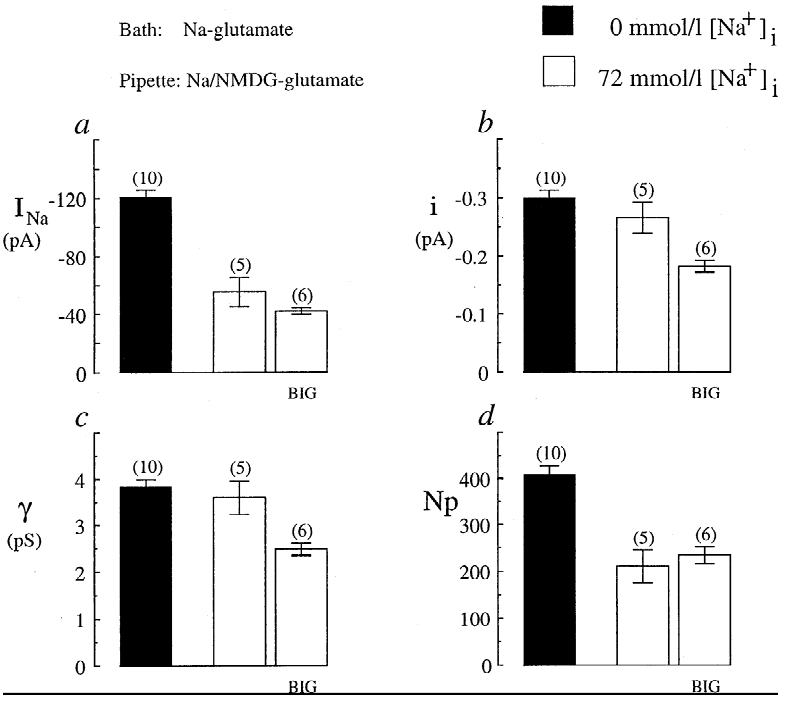

Fig. 2. Effect of extracellular BIG on the whole-cell CDPC-sensitive Na^+ current (panel *a*), and the single-channel current (*i;* panel *b*), the single-channel conductance (γ*;* panel *c*) and the channel activity (N_p; panel *d*) of Na^+ channels, by CDPC fluctuation analysis at a pipette potential of −80 mV with a pipette solution containing a Na^+-free, 150 mmol/l NMDG-glu solution (filled bars) or a mixture of 78 mmol/l NMDG-glu and 72 mmol/l Na-glu (open bars). The bath contained a Na-glu solution, together with 1 mmol/l BIG where indicated.

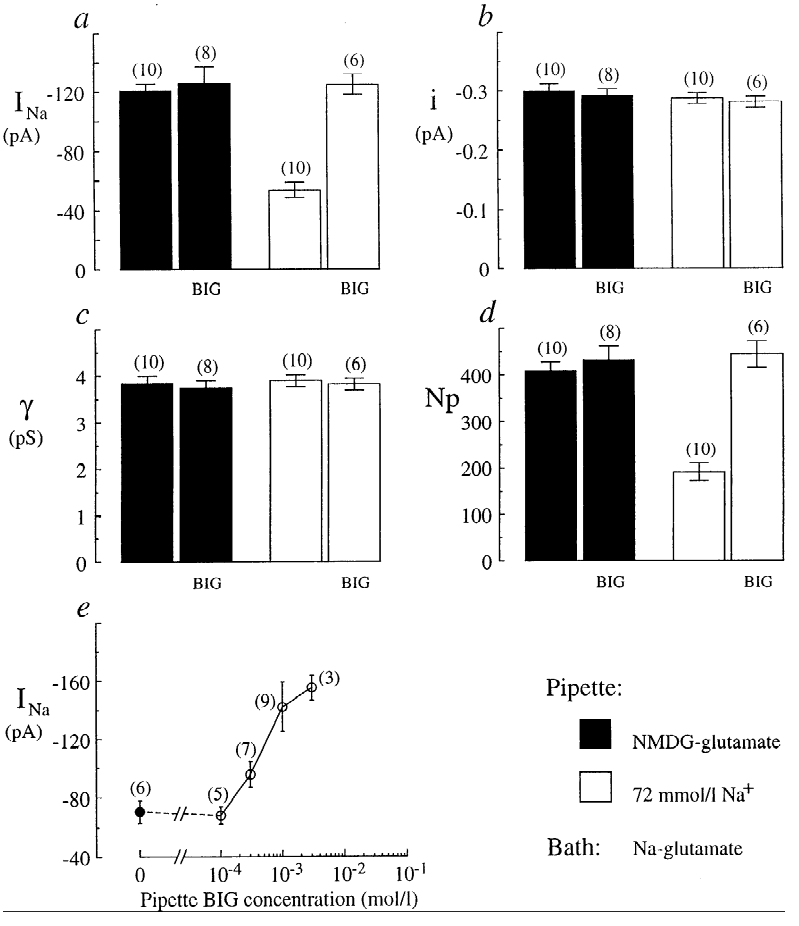

Fig. 3. Effect of intracellular BIG (1 mmol/l) on the whole-cell CDPC-sensitive Na^+ current (panel *a*), and the single-channel current (*i;* panel *b*), the single-channel conductance (γ*;* panel *c*) and the channel activity (*Np;* panel *d*) of Na^+ channels, measured at a pipette potential of −80 mV by CDPC fluctuation analysis at a pipette potential of −80 mV with a pipette solution containing a Na^+-free, 150 mmol/l NMDG-glu solution (filled bars) or a mixture of 78 mmol/l NMDG-glu and 72 mmol/l Na-glu (open bars). The concentration-response relation for the effect of BIG on the Na^+ current (measured by $NMDG^+$ substitution for Na^+) is shown in panel *e*. In all experiments, the bath contained a Na-glu solution.

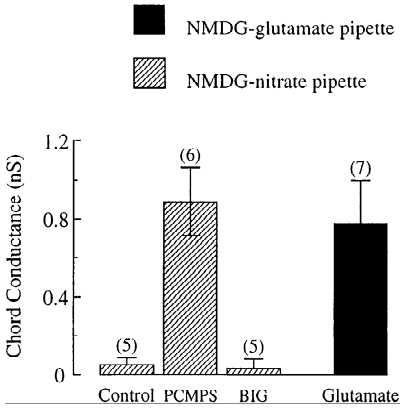

Fig. 4. Effect of extracellular PCMPS (1 mmol/l) and intracellular BIG (1 mmol/l) on the whole-cell amiloride-sensitive Na^+ conductance measured when the pipette contained NMDG-NO_3 (hatched bars). The amiloride-sensitive Na^+ conductance measured when the pipette contained a Na^+-free NMDG-glu solution (filled bar) is included for ease of comparison. The bath contained a Na-glu solution.

Na^+ (Fig. 5*b*). Similarly, we showed that the activated recombinant α-subunit of G_{i2} is unable to reverse the stimulatory effect of PCMPS when the pipette solution contains NO_3^- (Fig. 5*b*). These findings are consistent with our hypothesis that PCMPS acts down-stream of G_o and G_{i2}.

On the other hand, since BIG was only effective in inhibiting the Na^+, but not the Cl^-, feedback system, it appeared that it acted at some point on the Na^+ feedback system not shared with the Cl^- feedback system, but it was unclear whether it was acting upstream or down-stream of the only known mediator of this pathway, G_o. We examined whether inclusion of the activated α-subunit of G_o in the pipette solution could overcome the stimulatory effect of BIG (Fig. 5*c*). We found that it could. Furthermore, BIG could not reverse the inhibition produced by the inclusion of a submaximal concentration of activated $G\alpha_o$ (0.02 μmol/l) in the NMDG-glutamate pipette solution. We found that the amiloride-sensitive Na^+ conductance in the presence of activated 0.02 μmol/l $G\alpha_o$ alone (144.4 ± 31.0 pS, $n = 4$) was not significantly different from the conductance in the presence of activated 0.02 μmol/l $G\alpha_o$ plus 1 mmol/l BIG (166.5 ± 50.5 pS, $n = 5$). This excludes the possibility that BIG and G_o could be competing for the same site on the Na^+ channels. Thus we can conclude that BIG blocks the Na^+ feedback pathway at a point up-stream of G_o, presumably at a previously unrecognized receptor for cytosolic Na^+.

The Mechanism by which Amiloride and DMA can Stimulate Na^+ Channels

Several amiloride derivatives, such as DMA, as well as low concentrations of amiloride itself, have been reported paradoxically to stimulate epithelial Na^+ channels in a variety of tissues [1, 2, 4, 19, 20, 26]. The structural similarity between amiloride (and DMA) and BIG [10] suggested to us that the paradoxical effects of amiloride and DMA would be explicable if, as we postulate for BIG, they prevented the binding of Na^+ to an intracellular Na^+ receptor. When we investigated this possibility (Fig. 6), we found that inclusion of DMA in a pipette solution containing 72 mmol/l Na^+ did indeed lead to a concentration-dependent increase in the amiloride-sensitive Na^+ current (Fig. 6*b*), the effect being complete at a DMA concentration of 100 μmol/l. On the other hand, DMA had no effect when applied in a Na^+-free pipette solution containing only NMDG-glu or NMDG-NO_3 (Fig. 6*a*). Similarly, the inclusion of 3 μmol/l amiloride in the pipette solution overcame the inhibitory effect of the presence of Na^+ (Fig. 6*a*), but did not overcome the inhibitory effect of NO_3^- (Fig. 6*a*). As we saw previously in the case of BIG, this effect of amiloride was overcome by the inclusion of activated G_o in the pipette solution (Fig. 6*a*).

Discussion

This study resolves the conflict between our findings on the mechanisms of homocellular regulation in salivary duct cells and the long-held view that Na^+ channel activators work by inhibiting an extracellular modifier site for Na^+. It shows that PCMPS acts extracellularly to block the sensitivity of the Na^+ channels to activated G proteins, and so interrupts both the Na^+ and the Cl^- feedback systems (Fig. 7). BIG, DMA, and amiloride, however, act intracellularly and interrupt the Na^+, but not the Cl^-, feedback system (Fig. 7). Our present model thus provides an explanation for the paradoxical ability of amiloride and its derivatives to stimulate epithelial Na^+ channels [29]. It also explains the previously inexplicable observation in toad skin [29] that parachloromercuribenzoate, an analogue of PCMPS, and amiloride, which is normally a blocker of Na^+ channels, are both able to stimulate Na^+ channel activity when these channels have been inactivated by increasing intracellular Na^+ [1]. The different mechanisms of action of PCMPS and amiloride analogues such as BIG may also explain the variability in the reported activity of the two classes of compound [18, 20], since amiloride analogues would be expected only to be effective when the Na^+ feedback was dominant, whereas PCMPS and related compounds would stimulate Na^+ channel activity when feedback inhibition by either cytosolic Na^+ or by cytosolic Cl^- was operating. Furthermore, neither class of compound would be expected to stimulate Na^+ channels under conditions when the cytosolic Na^+ and Cl^- feedback systems were not operating or were ineffective.

Our present finding that the activated α-subunit of

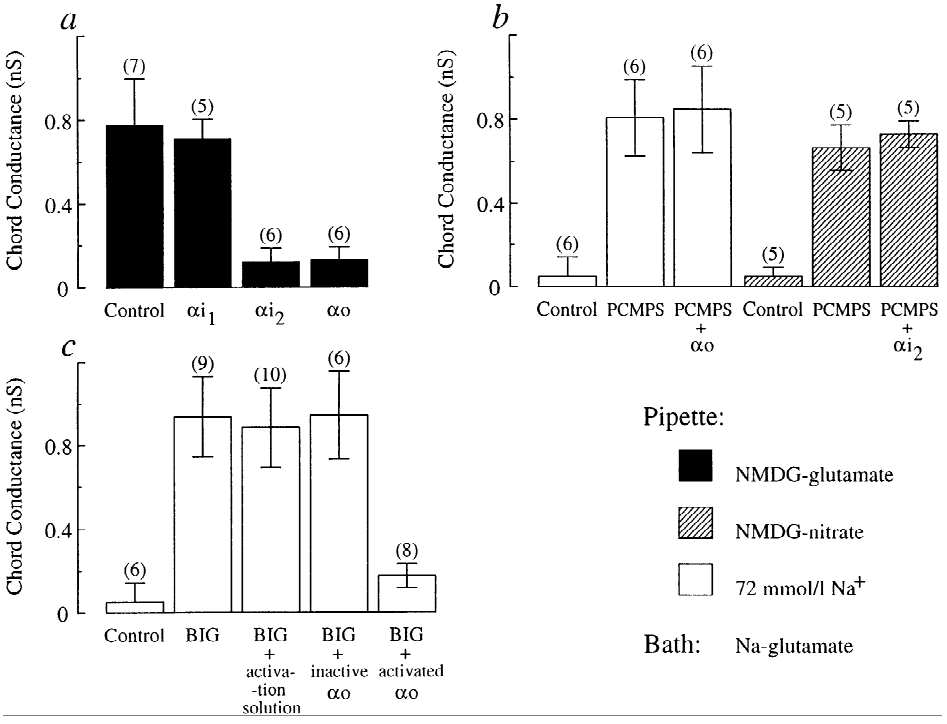

Fig. 5. Panel *a.* Effects of the inclusion in the pipette solution of activated G protein α-subunits (0.2 μmol/l) on the amiloride-sensitive Na^+ conductance measured at −80 mV. The control pipette solution was Na^+-free NMDG-glu. Panel *b.* Effects of the inclusion of activated α-subunits (0.2 μmol/l) in the pipette solution on the stimulation of the amiloride-sensitive Na^+ conductance by extracellular PCMPS (1 mmol/l). In experiments examining the effects of $G\alpha_o$, the pipette solution contained 72 mmol/l Na^+ whereas in experiments examining the effects of $G\alpha_{i2}$, the pipette solution contained an NMDG-NO_3 solution. Panel *c.* Effects of the inclusion of activated α-subunits of G_o in the pipette solution on the stimulation by intracellular BIG (1 mmol/l) of the amiloride-sensitive Na^+ conductance. In all experiments in panel *C*, pipette solutions containing 78 mmol/l NMDG-glu plus 72 mmol/l Na-glu were used, together with: (i) 1 mmol/l BIG; (ii) 1 mmol/l BIG plus the G protein activation solution, but with no added $G\alpha_o$; (iii) 1 mmol/l BIG plus unactivated $G\alpha_o$ (0.2 μmol/l); (iv) 1 mmol/l BIG plus activated $G\alpha_o$ (0.2 μmol/l).

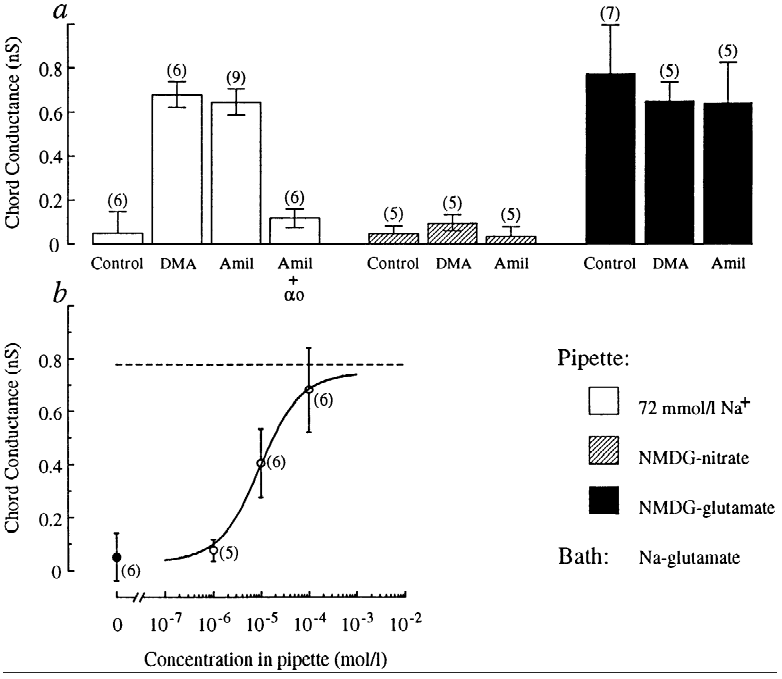

Fig. 6. Panel *a.* Effect of the inclusion of 100 μmol/l DMA or 3 μmol/l amiloride in the pipette solution on the chord conductance of the amiloride-sensitive current. Panel *b.* Concentration-response relation for the effects of DMA on the chord conductance of the amiloride-sensitive current when the pipette solution contained 72 mmol/l Na^+. The broken line indicates the mean chord conductance observed when the Na-glu pipette solution was used.

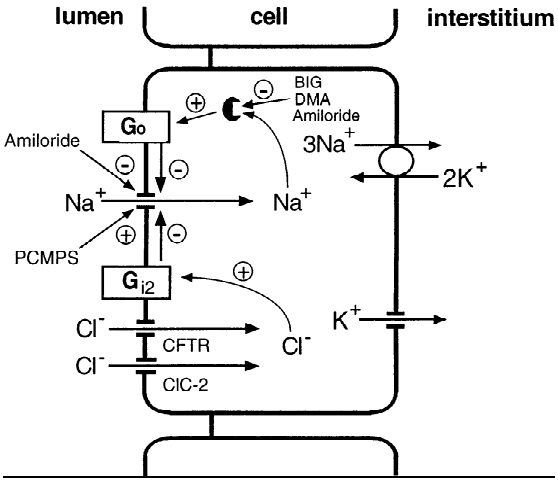

Fig. 7. Model for feedback regulation of salivary duct Na^+ channels by cytosolic Na^+ and Cl^- acting via G proteins, including the proposed sites of action for PCMPS, acting externally on the Na^+ channel itself, and BIG, DMA and amiloride, acting internally on the proposed Na^+ receptor.

G_o inhibits the Na^+ channels gives further support to the identification of G_o as the mediator of the Na^+ feedback system. This identification had previously been based on the observed sensitivity of the Na^+ feedback system to GDP-β-S, to pertussis toxin, and to antibodies directed against the C- and N-terminals of the α-subunit of G_o [16] and the high level of expression of G_o in salivary ducts [30], but we had not demonstrated that G_o could actually inhibit the Na^+ channels. This gap in our argument has now been filled. Furthermore, our finding that the Na^+ channels are inactivated by the α-subunit of G_{i2}, whereas the α-subunit of G_{i1} is without effect, resolves the uncertainty in our previous antibody studies [16] that prevented us from determining whether the anion feedback pathway was mediated by G_{i1} or by G_{i2}.

Importantly, our finding that activated G_o overcomes the stimulatory effects of intracellular BIG and of intracellular amiloride, indicates that the effect of intracellular Na^+ is not due to a nonspecific biophysical effect on the G protein, as has been suggested for the effects of Cl^- on G protein activity [13]. It also rules out any direct effect of BIG and amiloride on the Na^+ channels themselves. Given that they are all monovalent cations, the most likely mode of action of BIG, amiloride and structurally related Na^+ channel stimulators is by inhibiting an intracellular receptor site for Na^+.

This project was supported by the National Health and Medical Research Council of Australia and the Australian Kidney Foundation. Dr. D.I. Cook is a Fellow of The Medical Foundation of the University of Sydney and Dr. A. Dinudom was supported by a Postdoctoral Fellowship from The Medical Foundation of the University of Sydney. The authors also wish to thank Professor E. Frömter for his advice and encouragement in the early stages of this project.

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