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Cytosolic Na^+ controls an epithelial Na^+ channel via the G_o guanine nucleotide-binding regulatory protein

(amiloride/salivary gland/sodium current/G protein/pertussis toxin)

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ABSTRACT **In tight Na^+-absorbing epithelial cells, the rate of Na^+ entry through amiloride-sensitive apical membrane Na^+ channels is matched to basolateral Na^+ extrusion so that cell Na^+ concentration and volume remain steady. Control of this process by regulation of apical Na^+ channels has been attributed to changes in cytosolic Ca^{2+} concentration or pH, secondary to changes in cytosolic Na^+ concentration, although cytosolic Cl^- seems also to be involved. Using mouse mandibular gland duct cells, we now demonstrate that increasing cytosolic Na^+ concentration inhibits apical Na^+ channels independent of changes in cytosolic Ca^{2+}, pH, or Cl^-, and the effect is blocked by GDP-β-S, pertussis toxin, and antibodies against the α-subunits of guanine nucleotide-binding regulatory proteins (G_o). In contrast, the inhibitory effect of cytosolic anions is blocked by antibodies to inhibitory guanine nucleotide-binding regulatory proteins (G_{i1}/G_{i2}. It thus appears that apical Na^+ channels are regulated by G_o and G_i proteins, the activities of which are controlled, respectively, by cytosolic Na^+ and Cl^-.**

Like many tight epithelia, including renal collecting ducts, colonic mucosa, sweat ducts, and amphibian skin, the salivary duct epithelium transports Na^+ from the exterior to the interstitium by permitting the influx of Na^+ through amiloride-sensitive Na^+ channels in the apical membranes of the epithelial cells (1–3) and then pumping Na^+ into the interstitium using the Na^+-K^+-ATPase in the basolateral membranes (4). Epithelia of this type, including salivary ducts (5), regulate the rate of Na^+ influx across the apical membranes so as to match the basolateral extrusion rate and thereby maintain a stable cell volume and cytosolic Na^+ concentration, although the mechanism by which this so-called homocellular regulation or epithelial cross-talk occurs remains controversial (6, 7).

Early studies were interpreted as indicating that extracellular Na^+ regulated the activity of the apical Na^+ channels directly (8, 9), although an intracellular action of Na^+ had also been postulated (10). More recent electrophysiological studies have strengthened the view that the effects of extracellular Na^+ are not direct (11, 12), but are mediated through changes in cytosolic Na^+ that influence the activity of apical Na^+ channels indirectly by alterations in cytosolic pH (13) and free Ca^{2+} concentration (14, 15), arising, respectively, because of alterations in the activity of Na^+–H^+ and Na^+–Ca^{2+} exchangers. Studies using renal Na^+ channels reconstituted into lipid bilayers (16), on the other hand, have shown that increasing intracellular Na^+ decreases Na^+ channel activity, but only when the free Ca^{2+} bathing the cytosolic face of the channel is above 1 μmol/liter. Because this Ca^{2+} concentration is above the resting level in absorptive epithelia such as salivary ducts (17), the physiological relevance of the observation is unclear. Regulation of apical Na^+ channels seems also to be brought about by changes in cytosolic Cl^- concentration (18, 19). Given that in epithelial cells intracellular Cl^- is correlated with cell volume at constant extracellular osmolarity (20), this may provide a mechanism by which cell volume modulates Na^+ influx rate (18).

The present study demonstrates that cytosolic Na^+ can regulate Na^+ channels independent of changes in cytosolic pH, Ca^{2+}, and Cl^-, and explores the role of G proteins in mediating its action.

MATERIALS AND METHODS

Cell Preparation. Isolated cells were prepared by collagenase digestion of mandibular glands from male mice (3, 19). The standard bath solution had the following composition: 145 mmol/liter NaCl, 5.5 mmol/liter KCl, 1.0 mmol/liter $CaCl_2$, 1.2 mmol/liter $MgCl_2$, 1.2 mmol/liter NaH_2PO_4, 7.5 mmol/liter Na-*N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (Na-Hepes), 7.5 mmol/liter H-Hepes, and 10 mmol/liter glucose; the pH was adjusted to 7.4 with NaOH. After establishment of the whole-cell configuration, the bath was replaced with a solution containing 145 mmol/liter Na-glutamate, 5.0 mmol/liter NaCl, 1.0 mmol/liter $MgCl_2$, 10 mmol/liter H-Hepes, 10 mmol/liter glucose, and 1.0 mmol/liter EGTA; the pH was adjusted to 7.4 with NaOH. Exchanging the bath solution before establishment of the whole-cell configuration did not alter the results. The pipettes were filled with solutions containing NMDG-glutamate and NaCl (together totalling 150 mmol/liter), 1.0 mmol/liter $MgCl_2$, 10 mmol/liter H-Hepes, 10 mmol/liter glucose, and 5.0 mmol/liter EGTA; the pH was adjusted to 7.2 with Tris base or NaOH (7–14 mmol/liter) as appropriate.

Patch-Clamp Techniques. Standard whole-cell patch-clamp methods were used as previously described (3, 19). Patch-clamp pipettes were pulled from borosilicate microhematocrit tubes (Modulohm, Hevik, Denmark) so as to have resistances of 1–3 MΩ. A Ag-AgCl pellet was used as the reference electrode and all potential differences were corrected for liquid junction potentials as appropriate (19). 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) attached to a Macintosh IIci computer was used to generate command voltages and to sample whole-cell currents. The amiloride-sensitive current was measured as the difference between the whole-cell current before and following the addition of amiloride at 100 μmol/liter to the bath solution. Whole-cell *I–V* relations were obtained by applying voltage pulses of 800-ms duration from a resting potential of 0 mV. Steady-state currents were calculated as the average current between 700 and 800 ms after the start of the voltage pulse.

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Abbreviations: G_o, guanine nucleotide-binding regulatory protein; G_i, inhibitory guanine nucleotide-binding regulatory protein.

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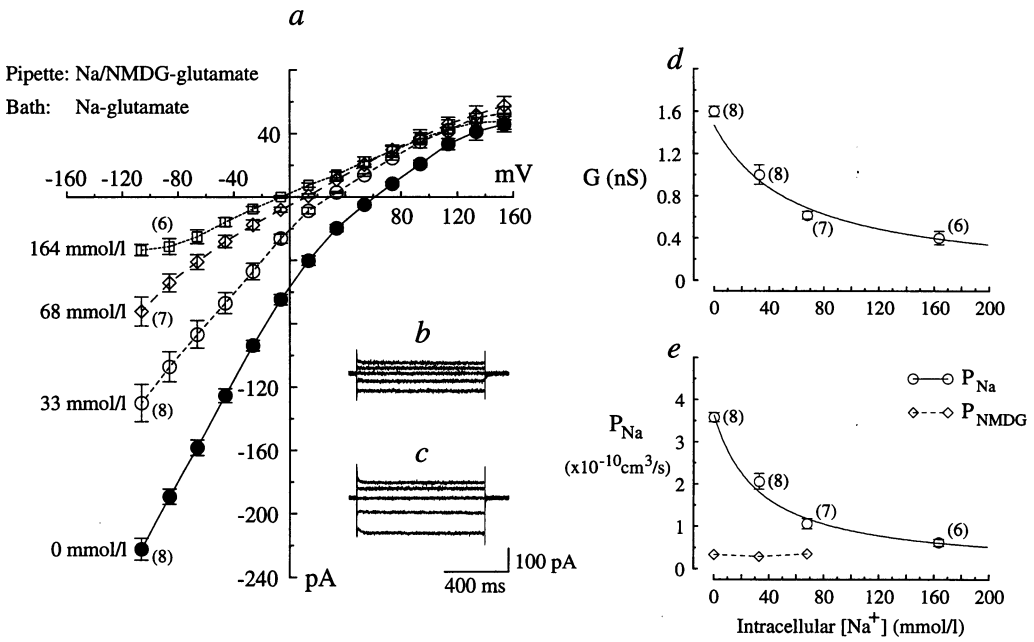

FIG. 1. (*a*) *I–V* relation of the amiloride-sensitive Na^+ conductance in mouse intralobular granular duct cells measured at pipette Na^+ concentrations of 0, 33, 68, and 164 mmol/liter. (*Insets*) Representative voltage-clamp recordings of the amiloride-sensitive Na^+ current with 68 mmol/liter (*b*) and zero (*c*) Na^+ in the pipette solution. (*d*) Plot of the inward Na^+ conductance (measured at pipette potentials between −60 mV and −120 mV) as a function of pipette Na^+ concentration. (*e*) Plot of the Na^+ permeability (P_{Na}) and the $NMDG^+$ permeability (P_{NMDG}) as a function of pipette Na^+ concentration. Each point represents the mean ± SEM with the number of separate experiments shown in parentheses.

Estimation of Ionic Permeabilities. The Na^+ permeability (P_{Na}) and the $NMDG^+$ permeability (P_{NMDG}) were estimated by fitting the Goldman equation to the *I–V* relation of the amiloride-sensitive current ($I_{amiloride}$). The form of the equation we used was

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

where V denotes the command potential, the subscripts o and i denote extracellular and intracellular concentrations of Na^+ and $NMDG^+$, respectively, and F/RT is equal to 0.0364 mV^{-1} at 22°C.

Single-Channel Properties Measured Using Noise Fluctuation Analysis. During the application of the weak electroneutral Na^+ channel blocker, 6-chloro-3,5-diamino-pyrazine-2-carboxamide (CDPC), the membrane potential was clamped at −80 mV. The whole-cell current was then filtered at 500 Hz and sampled at 1000 Hz. For each 100-ms 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 s) 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

$$\sigma^2 = I_{Na}\, i - (I^2_{Na}/N_o) + \sigma^2_{residual} \quad [2]$$

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

$$N_T p = I_{Na}/i \quad [3]$$

where N_T is the number of channels available and p is the 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 Na^+ at 157 mmol/liter.

Chemicals and Antibodies. GDP-β-S was obtained from Boehringer Mannheim and pertussis toxin from Calbiochem. Pertussis toxin was stored as a 111 μg/ml stock solution and was activated just before use by incubation for 15 min at 35°C with dithiothreitol at 5 mmol/liter, and then diluted to 500 ng/ml in aliquots of pipette solution to which nicotinamide adenine nucleotide at 1 mmol/liter had been added (21). Antibodies directed against the C termini of the α subunits of G_{i1}/G_{i2}, G_{i3}, and G_{i3}/G_o (G_i, inhibitory guanine nucleotide-binding regulatory protein; G_o, guanine nucleotide-binding regulatory protein) were obtained from Calbiochem and antibodies against the N terminal of the α-subunit of G_o were obtained from DuPont/NEN. These antibodies were chosen because antibodies directed against the C termini of the α-subunits of G proteins (22) and against the N terminus of the α-subunit of G_o (23) are known to interfere with signaling mediated by these G proteins. Each antibody was included in the pipette solution in a concentration of 1 in 200 (vol/vol). CDPC, EGTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate (BAPTA), Tris, and Hepes were obtained from Sigma, amiloride from Research Biochemicals (Natick, MA), and collagenase (type IV) from Worthington.

RESULTS AND DISCUSSION

Dependence of Amiloride-Sensitive Na^+ Current on Cytosolic Na^+ Concentration. As we have previously reported (3, 12), when mouse mandibular duct cells are studied in the whole-cell patch-clamp configuration with a Na^+-rich bath solution and a Na^+-free, low-Cl^- pipette solution (containing NMDG-glutamate at 150 mmol/liter), the predominant conductance seen is an amiloride-sensitive Na^+ conductance that

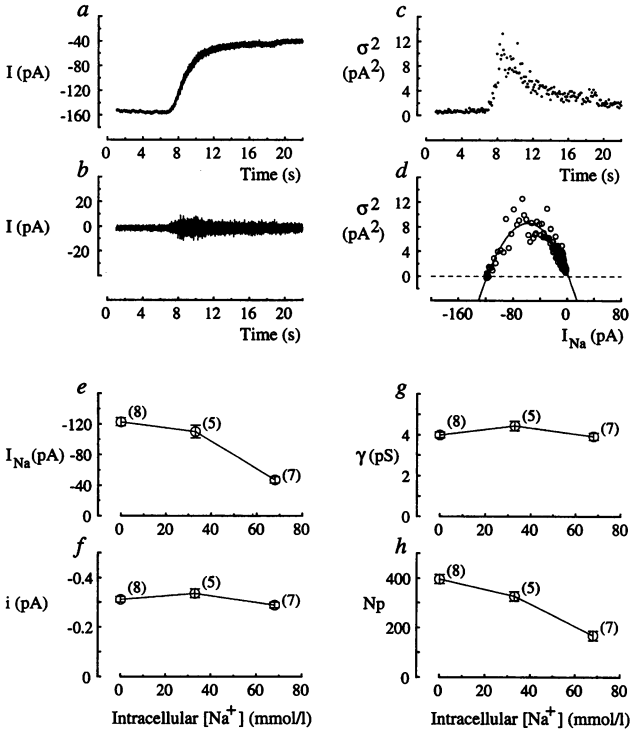

FIG. 2. Single-channel conductance (γ) and activity (N_Tp) of Na^+ channels, measured by applying fluctuation analysis (12, 28) to the whole-cell current-noise generated by the weak, electroneutral, epithelial Na^+ channel blocker, CDPC (27). (*a*) Time-course of the effect of CDPC at 200 μmol/liter on the whole-cell current of a duct cell bathed in a solution containing Na-glutamate at 157 mmol/liter, with an NMDG-glutamate pipette solution. The holding potential was −80 mV. (*b*) The record in *a* after high-pass filtering. (*c*) Time-course of the current variance during the addition of CDPC at 200 μmol/liter. (*d*) The relation between the current variance and the mean whole-cell Na^+ current. The unbroken line is a least-squares fit of Eq. **2** to the data. (*e*) Dependence of the whole-cell Na^+ current (I_{Na}) on pipette Na^+. (*f*) Dependence of the single-channel current (*i*) on pipette Na^+. (*g*) Dependence of single-channel conductance (γ) on pipette Na^+. (*h*) Dependence of the channel activity (N_Tp) on pipette Na^+.

is not voltage-activated and is permeable to Li^+ but not to K^+. The channel type underlying this conductance appears to be the epithelial Na^+ channel (ENaC), which is known to be expressed in these cells (1). In the present experiments, we observed that the magnitude of the amiloride-sensitive Na^+ current (and therefore the Na^+ conductance) declined with increasing Na^+ concentration in the pipette solution (Fig. 1), the half-maximum reduction in Na^+ conductance being observed at a pipette Na^+ concentration of 33 mmol/liter, a value within the physiological range for cytosolic Na^+ concentration in exocrine tissues (24–26).

The inhibitory effect was specific to Na^+, as shown by studies in which we replaced all the $NMDG^+$ in the pipette solution with K^+, which did not significantly inhibit the amiloride-sensitive Na^+ conductance [NMDG-glutamate pipette solution: 1.094 ± 0.12 nS (n = 5) versus K^+-glutamate pipette solution: 0.845 ± 0.16 nS (n = 6), P = 0.252; see also refs. 3 and 19]. Furthermore, because the pipette solutions used in our experiments were buffered at pH 7.2 with Hepes and had a constant, low Cl^- concentration and because the extracellular and pipette solutions both contained EGTA (1 mmol/liter and 5 mmol/liter, respectively) with no added Ca^{2+}, the decline in Na^+ conductance cannot have been due to a change in intracellular pH or in the cytosolic concentrations of Cl^- or free Ca^{2+}. Because inhibition of epithelial Na^+ channels due to increases in intracellular free Ca^{2+} has been extensively

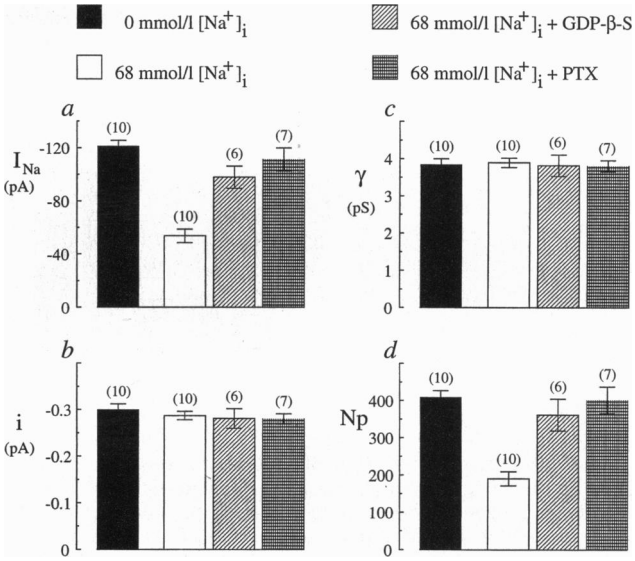

FIG. 3. Effects of G protein modulators on the whole-cell Na^+ current in duct cells. (*a–d*) The whole-cell Na^+ current (*a*), the single-channel current (*b*), the single-channel conductance (*c*) and the channel activity (*d*), measured using CDPC fluctuation analysis (see Fig. 2) at a pipette potential of −80 mV. Experiments were conducted using the following pipette solutions: (*i*) a Na^+-free NMDG-glutamate solution; (*ii*) a solution containing Na^+ at 68 mmol/liter; (*iii*) a solution containing Na^+ at 68 mmol/liter plus GDP-β-S at 100 μmol/liter; and (*iv*) a solution containing Na^+ at 68 mmol/liter plus pertussis toxin (PTX) at 500 ng/ml. In solutions *ii–iv*, Na^+ was supplemented with $NMDG^+$ to maintain a summed cation concentration of 157 mmol/liter. The Goldman equation was used to estimate the single-channel conductance in symmetrical 157 mmol/liter Na^+ solutions from the single-channel current at −80 mV.

reported in the literature (6, 14, 15), we further investigated whether a change in free Ca^{2+} concentration could be mediating the effects we observed. We found that the reduction in Na^+ conductance seen when cytosolic Na^+ concentration is increased persists even when the pipette Ca^{2+} concentration is buffered with BAPTA, a more powerful Ca^{2+} chelator than EGTA, in concentrations as high as 20 mmol/liter and the bath solution contains 1 mmol/liter EGTA with no Ca^{2+} added to either solution (data not shown).

In principle, a decline in Na^+ conductance induced by increasing intracellular Na^+ could be the result either of a decrease in channel activity (N_Tp) or a decrease in single-channel conductance (γ). To investigate which of these was the actual cause, we applied fluctuation analysis to the noise generated during the onset of inhibition of the Na^+ channels by CDPC, a weak electroneutral channel blocker (12, 27), a technique that permits us to determine the single-channel current (*i*) and the activity (N_Tp) of the Na^+ channels from whole-cell recordings (Fig. 2). We found that the decline in Na^+ conductance with increasing intracellular Na^+ was attributable to a decline in channel activity (Fig. 2*h*) rather than a change in the single-channel conductance, which did not alter with increasing intracellular Na^+ (Fig. 2*g*).

The Role of G Proteins. We then investigated the mechanism by which intracellular Na^+ controlled the activity of the Na^+ channels. We found that inclusion of GDP-β-S at 100 μmol/liter [which competitively inhibits the binding of GTP by G proteins (29)] in a pipette solution containing Na^+ at 68 mmol/liter reversed the inhibitory effect of Na^+ on the whole-cell Na^+ current and increased Na^+ channel activity to a level not significantly different from that observed when the pipette solution contained no Na^+ at all (Fig. 3). Similarly, we found that pertussis toxin [which ADP ribosylates G proteins of the G_i and G_o classes so as to prevent their activation by

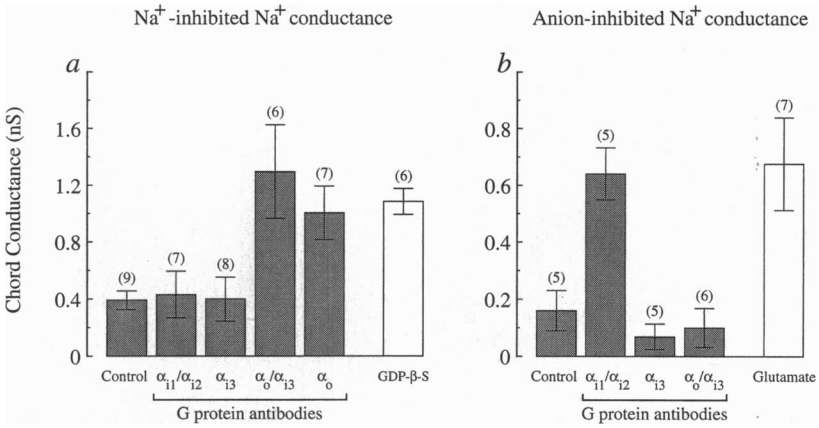

FIG. 4. Effects of the inclusion of antibodies against various G protein α-subunits on the amiloride-sensitive Na^+ conductance measured at -80 mV (*a*) with Na^+ at 68 mmol/liter in a glutamate-rich pipette solution and (*b*) with NMDG-NO_3 at 155 mmol/liter in the pipette solution. For ease of comparison, we have also included the Na^+ conductance observed with a pipette solution containing Na^+ at 68 mmol/liter and GDP-β-S at 100 μmol/liter (as shown in *a*, calculated from the data in Fig. 3) and the Na^+ conductance observed with a glutamate-rich pipette solution containing zero Na^+ (*b*). In the experiments in *a*, Na^+ current was measured by the addition of amiloride (cf. Fig. 1 of ref. 18) and, in the experiments in *b*, it was measured by substitution of bath Na^+ by $NMDG^+$ (cf. Fig. 2 of ref. 18); we have previously shown that these two methods give identical results. The experiments on the effects of anions on the Na^+ current used NO_3^- rather than Cl^- in the pipette solution to eliminate contamination by the hyperpolarization-activated ClC-2-type current found in these cells (18, 32).

receptors (30, 31)] also inhibited the effects of Na^+ at 68 mmol/liter in the pipette solution on Na^+ channel activity (Fig. 3). Given that we have previously reported that the Na^+ current was not affected by GDP-β-S or pertussis toxin when the pipette contained a Na^+-free NMDG-glutamate solution (18), the present results indicate that GDP-β-S and pertussis toxin act by preventing the inhibitory effects of intracellular Na^+, rather than by producing some kind of direct stimulatory effect on the Na^+ conductance.

Finally, we show that inclusion in the pipette solution of antibodies directed against the α-subunits of G_o proteins abolishes the inhibitory effect of Na^+ in the pipette solution on the Na^+ conductance, whereas inclusion of antibodies directed against the α subunits of G_{i1}, G_{i2}, and G_{i3} had no effect (Fig. 4*a*). We have previously reported that the Na^+ conductance in salivary duct cells is inhibited by the presence of anions such as Cl^-, Br^-, and NO_3^- in the cytosol (19) and have shown that this effect is mediated by a pertussis toxin-sensitive G protein (18). We thus examined whether the effect of anions on the Na^+ conductance is inhibited by antibodies directed against the α-subunit of G_o. We found that the effects of inclusion of NO_3^- in the pipette solution are inhibited by antibodies directed against the C-terminal peptides of the α-subunits of G_{i1}/G_{i2} but not of G_o proteins (Fig. 4*b*).

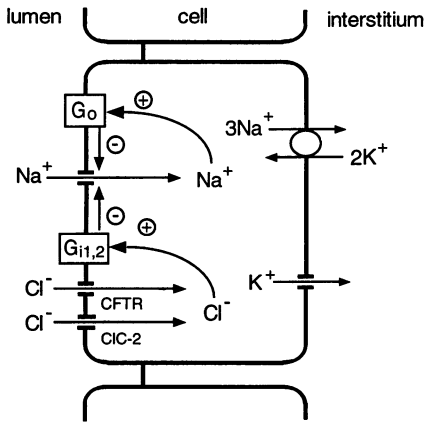

FIG. 5. Proposed model for feedback regulation of Na^+ channels in salivary duct cells by cytosolic Na^+ and Cl^- acting through G proteins.

This study demonstrates that intracellular Na^+ influences the activity of epithelial Na^+ channels independent of previously identified controlling agents such as changes in intracellular pH, Cl^- or Ca^{2+} concentration. We find that this effect of intracellular Na^+ is blocked by agents that inhibit G_o, a G protein expressed in high concentrations in salivary duct cells for which a physiological role has not previously been established (33). Since inclusion of the nonhydrolyzable analogue of GTP, GTP-γ-S, in a Na^+-free (NMDG-glutamate) pipette solution inhibits the epithelial Na^+ conductance in these cells (18), it would appear that active G_o actually mediates the effects of cytosolic Na^+ on the Na^+ channels, rather than simply producing a state in which the Na^+ channels are able to interact with and be blocked by cytosolic ions. The observation that pertussis toxin, which prevents G protein activation by receptors (30, 31), blocks the action of cytosolic Na^+ further suggests that a receptor of some type is involved. We do not know whether this hypothetical receptor is specific for cytosolic Na^+ or whether cytosolic Na^+ is acting by altering the efficacy of coupling between G_o and a receptor for an unknown extracellular ligand.

Interactions between epithelial Na^+ channels and G proteins, particularly G_{i3}, have been widely reported (34–37), and G_{i3} has been reported to form part of the purified Na^+ channel complex (35, 37). The physiological significance of these interactions has been unclear, however. By showing that Na^+ channels are modulated by G_o in response to changes in cytosolic Na^+ concentration and by a different G protein (either G_{i1} or G_{i2}) in response to changes in cytosolic concentrations of anions (18, 19) (Fig. 5), the present work indicates that G proteins play a critical role in the phenomenon of homocellular regulation.

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