Communication

G Protein Subunit, α3-3, Activates a Pertussis Toxin-sensitive Na+ Channel from the Epithelial Cell Line, A6*

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In nonpolar excitable cells, guanine nucleotide regulatory (G) proteins have been shown to modulate ion channel activity in response to hormone receptor activation. In polarized epithelia, hormone receptor-G protein coupling involved in the generation of cAMP occurs on the basolateral membrane, while the physiological response to this messenger is a stimulation of ion channel activity at the apical membrane. In the present study we have utilized the patch-clamp technique to assess if the polarized renal epithelium, A6, have topologically distinct G proteins at their apical membrane capable of modulating Na+ channel activity. In excised inside-out patches of apical membranes, spontaneous Na+ channel activity (conductance 8-9 picoamperes) was inhibited by the addition of 0.1 mM guanosine 5'-O-(2-thio)diphosphate to the cytosolic membrane surface without an effect on single channel conductance. In contrast, the percent open time of spontaneous Na+ channels increased from 6 to 50% following the addition of 0.1 mM GTP. The addition of preactivated pertussis toxin (100 ng/ml) to the cytosolic bathing solution of the excised patch inhibited spontaneous Na+ channel activity within a minute by 85% from approximately 47 to 7% open time and reduced the percent open time for Na+ channel activity to zero after approximately 3 min. The addition of 0.1 mM guanosine 5'- (3-O-thio)triphosphate or the addition of 20 pm purified human α3-3 subunit to pertussis toxin-treated membrane patches restored Na+ channel activity from zero to 35% open time. As little as 0.2 pm α3-3 subunit was capable of restoring Na+ channel activity. These data provide evidence for a role of pertussis toxin-sensitive G proteins in the apical plasma membrane of renal epithelia distal to signal transduction pathways in the basolateral membrane of these cells. This raises the possibility of a topologically distinct signal transducing pathway co-localized with the Na+ channel.

MATERIALS AND METHODS

A6 Cell Culture—A6 cells (ATCC CCL102) derived from Xenopus laevis renal tubular cells were purchased from the American Type Culture Collection (Rockville, MD) and used between passages 20 and 36. Cells were cultured on glass cover slips in tissue culture media as previously described (17). Briefly, cells were grown in a Coon's modified Ham's F-12 and Leibovitz's F-13 media modified to contain 108 mM NaCl and 25 mM NaHCO3. The mixture was supplemented with 10% fetal bovine serum (GIBCO). Cells were maintained at 37 °C in an atmosphere of humidified air plus 5% CO2. Cells were passaged and used between days 3 and 4 when cells were 60-70% confluent. Cells grown under these conditions express high conductance Na+ channels (17).

Patch-Clamp Measurement of Na+ Channel Activity—Patch-clamp pipettes were made of KG-33 glass capillaries (Garin Glass Co., Claremont, CA), pulled on a PB-7 pipette puller and heat-polished on an MF-9 pipette polisher (Narashige, Tokyo, Japan). All pipette shanks were coated with Sylgard 184 prior to polishing. Patch pipettes were prepared with an internal diameter of 0.1-0.5 μm and a resistance between 10 and 40 megohms in symmetric NaCl solutions. Actual currents and command voltages were obtained and driven with a PC-501 patch-clamp amplifier (Warner Instruments, Hamden, CT) using a head stage with a feedback resistance of 10 gigaohms and mounted onto a MN-2 micromanipulator (Narashige, Tokyo, Japan).

1 The abbreviations used are: G protein, signal-transducing nucleotide-binding protein of subunit structure αβγ; Gα, stimulatory G protein of adenylyl cyclase; Gβγ, the family of pertussis toxin-sensitive G proteins responsible for inhibition of adenylyl cyclase and modulation of ion channels; Gαi, stimulatory G protein of Ca2+ channels; GTPγS, guanosine 5'- O-(3-thio)triphosphate; GDPβS, guanosine 5'- O-(2-thio)diphosphate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH, piocisemien.

2 H. Cantiello and D. Ausiello, unpublished observations.
The resultant signal was filtered at 500 Hz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). Data were acquired, digitized, and stored in a hard disc of a personal computer (Unitech, Boston, MA) through an 8-bit interface (Tecmar, Solon, OH) until further analysis with FCamp 5.08 (Axon Instruments, Burlingame, CA). Patch pipette solution was in mM: 115 NaCl, 5 KCl, 0.8 MgSO4, 1.2 CaCl2, and 10 Hepes, pH 7.4. The perfusion solution was a modification of the patch pipette solution where Na+ was replaced equimolarly with K+ as to contain 115 mM KCl and 5 mM NaCl, all other solutions being the same. Both solutions were filtered (Milipore, 0.22-μm pore size) before each experiment. Data were obtained solely from excised inside-out patches and are presented as percent of total open time, i.e., 100 × p (p = open probability). Only single-channel tracings were analyzed. Data are expressed as mean ± S.E. where p = number of channels analyzed. Percent open time in spontaneous channels ranged between 7.1 and 75% of total time analyzed (>20 s). Open probability did not statistically change at a given holding potential for up to 15 min. Channels for different experimental protocols were chosen depending on the spontaneous activity, i.e., channels that displayed lower percent open time were chosen for GTP or α3-3 activation and channels with higher activity (>40%) were used for either GDP/β/S or pertussis toxin.

Pertussis Toxin Addition—Pertussis toxin was obtained from Peninsula Laboratories, Belmont, CA and stored at −20°C. The toxin was activated by a stock solution containing 100 mM NaCl, 20 mM Na2HPO4, pH 7.0, 10 mM dithiothreitol, and 1 mM NAD+ as previously described (2). Stock solutions were kept at 0°C until the time of the experiment. Activated pertussis toxin (2 μl) was added to the chamber (0.4 ml) flushing the cytosolic side of the excised membrane patches to a final concentration of 100 ng/ml.

GTP and Analog Additions—GTP, GTPβ/S, and GDPβ/S were added to the perfusion chamber bathing the cytosolic side of the membrane patch from a stock solution containing 10 mM Hepes, pH 7.6, at 0°C to a final concentration of nucleotide of 0.1 mM.

Activated α3-3 Addition—Human α3-3 activated with GTP/β/S and purified by ion-exchange chromatography (2) was the kind gift of Dr. Lutz Birnbaumer. Activated α3-3 was stored in a solution containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 7.5 mM MgCl2, and 20 mM KCl in bovine serum albumin-coated tubes at −70°C. 2 μl of the activated α3-3 stock solution at 20°C was added to the perfusion chamber at the cytosolic surface of the patch with an albumin-coated pipette dispenser to a final concentration of α3-3 of 20 PM in most experiments.

RESULTS

Basal Na+ Channel Activity—We used the patch-clamp technique to obtain single-channel currents from the apical surface of A6 epithelial cells grown to partial confluence. Spontaneous channel activity was present in approximately 60% of successful patches. The Na+ channel displayed no detectable rectifying properties between ±100 mV, was selective for Na+–K+, 5:1, and had a conductance of 8.87 ± 0.34 pS (n = 78) in symmetrical Na+ solutions (Na+ = 115 mM). Single-channel activity was amiloride-sensitive with an apparent K<sub>i</sub> < 10<sup>-7</sup> M. These data are in agreement with those previously reported by Hamilton and Eaton (17).

Effect of Guanine Nucleotides and Pertussis Toxin on Single Na+ Channel Activity—The effect of GTP, its analogs, and pertussis toxin was assessed on control channel activity as well as patches that did not show any spontaneous channel activity. The percent open time of spontaneous channels in 4/4 patches increased from 6 to 50% (Fig. 1, A and B) following addition of 0.1 mM GTP. Na+ channel activity was also observed on addition of 0.1 mM GTP to the bathing solution in 3/5 patches that had not displayed spontaneous Na+ channel activity (data not shown). In contrast, GDP/β/S inhibited Na+ channel activity from spontaneously active channels (Fig. 1, C and D, Table I) by approximately 84% without an effect on the single-channel conductance. Addition of preactivated pertussis toxin (100 ng/ml) to the cytosolic bathing solution inhibited spontaneous Na+ channel activity within 1 min by 85.2% from 46.5 ± 9.3 to 6.87 ± 2.49 (p < 0.01) percent open time (see Fig. 2) and reduced the percent open time to zero in all 11 patches treated after approximately 3 min. Pertussis toxin was also without effect on the single-channel conductance, 9.35 ± 2.35, versus 8.84 ± 2.19 pS. Removal of pertussis toxin did not reverse its effect. In contrast, addition of 0.1 mM GTP/γ/S restored Na+ channel activity in all four patches previously exposed to pertussis toxin where percent open time increased from 0 to 55.4 ± 10.6 (p < 0.01) (Fig. 2). The addition of 0.1 mM GTP was not able to reverse pertussis toxin inhibition of Na+ channel activity (data not shown). The addition to the perfusion buffer of ATP (0.1 mM) alone or with cAMP (0.1 mM) or phorbol esters (0.1 μM) did not stimulate Na+ channel activity in the presence or absence of pertussis toxin.

Effect of Purified α3-3 Subunit on Single Na+ Channel Activity—To demonstrate that the effect of pertussis toxin was mediated through the action of a G-like protein, purified activated human α3-3 subunit (Fig. 3 and Table II) was added to the bathing solution of the cytosolic side of the patch. α3-3 (20 pm) reversed the effect of pertussis toxin on Na+ channel activity in 100% of the patches studied. The effect of α3-3 was dose-dependent, with as little as 0.2 pm purified subunit activating the Na+ channel (data not shown). The addition of GTP/γ/S alone in a 5000-fold excess of its equimolar concentration with the chromatographically purified α3-3 (2) was without effect, making it unlikely that stimulation of the Na+ channel by activated α3-3 was due to contaminating GTP/γ/S. To rule out this possibility, GDP/β/S (100 μM) was added to several patches prior to the addition of activated α3-3 (20 pm). The α3-3 subunit stimulated Na+ channel activity to the same extent as α3-3 in the absence of GDP/β/S (data not shown).

| Table I |
|-------|
| Effect of GTP/β/S on Spontaneous Single Na+ Channel Activity from A6 Cells |
| Control | GDP/β/S |
| % open time | 43.8 ± 13.6 (3) | 6.95 ± 4.38 (3) | p < 0.025 |
| γ (pS) | 10.5 ± 1.69 (3) | 8.90 ± 1.35 (3) | NS* |

* Not significant.

Fig. 1. Guanine nucleotide regulation of single epithelial Na+ channels. Single channel currents are shown from excised inside-out patches from the apical membrane of partially confluent A6 cells. Upward deflection indicates open state, i.e., an inward Na+ current. Holding potential was +60 mV. Tracing A, patch with minimal spontaneous Na+ channel activity (% open time = 2.5, 10 events). Tracing B, same patch as A, 1 min after addition of 0.1 mM GTP to the perfusion chamber (% open time = 30.6, 96 events). Tracing C, patch with highest Na+ channel activity (% open time = 60.5, 75 events). Tracing D, same patch as C, 2 min after addition of 0.1 mM GDP/β/S (% open time = 0, 0 events).
by pertussis toxin-sensitive G proteins, including been proposed as a consequence of the ability of the activity was +80 mV.

perfusion chamber.

channels.

to activate phospholipases (19). In the case of non-

subunits of the heterotrimeric G proteins (2, 4-7, 12, 18). There is precedent for apical hormone receptors which can alter CAMP production and increase apical Na+ channel activity (1, 20). These data expand the role of G proteins in modulating epithelial Na+ transport. Epithelia may use G proteins both at proximal and distal steps of signal transduction, such that G proteins mediate changes in second messengers which in turn activate intracellular metabolic pathways (i.e. protein kinases), which modify the activity of more distal G proteins coupled to ion channels, and epithelia may also use G proteins localized in apical membranes as distinct signal transduction pathways capable of directly modifying ion channels. The inability of activators of protein kinases (ATP, cAMP, or phorbol esters) to stimulate Na+ transport in our present study lends further support for a distinct apical membrane signal transduction pathway.

Although no apically located receptor has yet been discovered that couples to an amiloride-sensitive Na+ channel, other ion channels are modulated by a receptor ligand-G protein interaction (2-8). Our present data lend support to such a G protein-receptor interaction in the apical membrane. The following are consistent with this hypothesis: 1) all spontaneous Na+ channel activity appears to be modulated by an activated G protein since it is completely suppressible with the addition of GDPβS; 2) pertussis toxin inhibits spontaneous Na+ channel activity in isolated perfused membrane patches which can be overcome by GTPγS, consistent with the well established mechanism of action of the toxin as an uncoupler of receptor-dependent G protein action (21); and 3) GTP is effective in the stimulation of Na+ channel activity, unusual in the absence of agonist receptor stimulation of G proteins (21). There is precedent for apical hormone receptors coupled to Na+ transport in renal epithelia; for example, angiotensin II stimulates Na+ uptake at the apical membrane of renal proximal tubules (22). Should such a receptor exist in the A6 cell, it probably binds a product that is endogenously produced by the apical membrane, such as a phospholipid.

**DISCUSSION**

A family of ion channels has been demonstrated to be gated by pertussis toxin-sensitive G proteins, including K+ (2-7), Ca2+ (8-12), and cation-selective channels (18). In each case, channel activity has been shown to increase in response to the exogenous addition of guanine nucleotide-activated α subunits of the heterotrimeric G proteins (2, 4-7, 12, 18). An additional role for βγ subunits as channel activators has also been proposed as a consequence of the ability of the βγ subunits to activate phospholipases (19). In the case of non-polar cells, hormone receptor modulation of ion channels is G protein-linked which implies the co-localization of receptor, G protein, and "effector," i.e. ion channel. Our present data suggest that in the polarized renal epithelia, A6, a pertussis toxin-sensitive G protein is present as a modulator of Na+ channels in the apical membrane and is topographically dis-

**TABLE II**

| Effect of activated human purified α-3 subunit on single Na+ channel activity from A6 cells |
|-----------------------------------------------|
| Control | Pertussis toxin | α-3 (20 pm) |
| % open time | 46.5 ± 9.29 (4) | 0 (4) | 32.4 ± 13.1 (4) |
| γ (pS) | 9.6 ± 2.1 (3) | 12.1 ± 4.4 (3) | NS* |

* Not significant.
The spontaneous Na\(^+\) channel activity in an excised membrane patch vigorously perfused for greater than 20 min, which is always pertussis toxin-sensitive, makes it unlikely that a residual ligand from intact cells or media remains present in isolated membranes. Preliminary data in our laboratory\(^3\) shows that arachidonic acid metabolites can activate the same G protein-sensitive Na\(^+\) channel in pertussis toxin-treated membranes described in the present study. Recent data have shown that arachidonic acid or its metabolites are capable of activating both receptor-coupled (19) and non-coupled (23) K\(^+\) channels. It remains to be demonstrated whether the observed \(a_3\) stimulation of Na\(^+\) channels is the consequence of G protein modulation of phospholipid metabolism in apical membranes. Our data demonstrate, however, that the regulation of Na\(^+\) channel activity in renal epithelia must now include a role for apically localized pertussis toxin-sensitive G proteins.

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