Guanine Nucleotide-Binding Protein, α_{i-3} , Directly Activates a Cation Channel in Rat Renal Inner Medullary Collecting Duct Cells

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Abstract

We examined whether GTP binding proteins (G proteins) regulate sodium conducting channels in the apical membrane of renal inner medullary collecting duct (IMCD) cells and thereby modulate sodium absorption. Patch clamp studies were conducted on inside-out patches of the apical membrane of IMCD cells grown in primary culture. Guanosine 5'-triphosphate (GTP) and the nonhydrolyzable GTP analogue, GTP γ S, which activate G proteins, increased the open probability of the cation channel. In contrast, the nonhydrolyzable GDP analogue, GDP β S, which decreases G protein activity, inhibited the channel. Pertussis toxin also reduced the open probability of the channel. Addition of the α_{i-3} subunit of G_i to the solution bathing the cytoplasmic surface of the membrane increased the open probability in a dose-dependent manner (2–200 pM). The threshold concentration for activation by α_{i-3} was 2 pM. Activation of the cation channel by α_{i-3} was not mediated via a protein kinase. The IMCD is the first polarized epithelium in which an ion channel has been shown to be directly regulated by a G protein. Thus, G proteins are important elements in regulating sodium absorption by the IMCD.

Introduction

Guanine nucleotide-binding proteins (G proteins)¹ are a family of proteins composed of three subunits (α , β , γ) which couple plasma membrane receptors to a variety of enzymes and ion channels (1). According to the generally accepted mode of action of G proteins, agonist binding to an external receptor induces a conformational change in the G protein

that leads to the displacement of guanosine 5'-diphosphate (GDP) by guanosine 5'-triphosphate (GTP) on the α subunit. The α -GTP complex dissociates from the $\beta\gamma$ dimer, and in most cases, interacts with the effector and elicits a response. In some instances, however, the $\beta\gamma$ subunit may mediate a response (2–5). The intrinsic GTPase activity of the α subunit hydrolyzes GTP to GDP, and the α -GDP complex reassociates with the $\beta\gamma$ dimer ending the response (1).

Several ion channels are regulated by G proteins (1, 6–16). G_i activates K^+ channels in isolated atrial cells (1, 6–9) and the GH₃ pituitary cell line (15, 16), G_s stimulates Ca^{2+} channels in skeletal muscle and cardiac cells (1, 11, 12), and G_o activates Ca^{2+} channels in neuronal cells (13) and K^+ channels in hippocampal neurons (14). The $\beta\gamma$ subunits activate K^+ channels in atrial cells, probably via phospholipase A_2 (2–5). Our previous studies showed that electrogenic sodium absorption by LLC-PK₁ cells, a renal epithelial cell line, may also be regulated by a G protein (17, 18). Pertussis toxin (PTX), a compound that prevents receptor-dependent activation of G_i and G_o (1, 7, 13, 15, 19, 20), reduced sodium absorption across this epithelium (17, 18). Although PTX inhibition of electrogenic sodium absorption indicates that a G protein regulates an epithelial Na^+ channel, direct evidence is lacking.

The objective of the present study was to investigate whether G proteins regulate sodium conductive channels in the apical membrane of inner medullary collecting duct (IMCD) cells. Intracellular microelectrode studies on isolated and perfused IMCD show that electrogenic sodium absorption is a two-step process (21). Sodium diffuses across the apical membrane, from the urine into the cell, through an amiloride-sensitive conductive pathway and is pumped across the basolateral membrane into the blood by the Na^+ - K^+ -dependent adenosine triphosphatase (21). Patch clamp studies on freshly isolated IMCDs and on IMCD cells in primary culture have shown that sodium uptake across the apical membrane is mediated by an amiloride-sensitive cation channel (22). The channel is highly selective for sodium versus chloride, does not discriminate between sodium and potassium, and has a single channel conductance of 28 pS (22). Atrial natriuretic peptide, via its second messenger guanosine 3',5'-cyclic monophosphate, inhibits the cation channel and sodium uptake across the apical membrane (23).

In the present study, we found that the α subunit of G_i directly activates the cation channel in IMCD cells and thereby regulates sodium uptake across the apical membrane.

Methods

We conducted patch clamp studies on a homogeneous population of rat IMCD cells in primary culture. Cells were isolated, grown in culture

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1. *Abbreviations used in this paper:* ATP γ S, adenosine 5'-O-(3-thiophosphate); GDP β S, guanosine 5'-O-(2-thiophosphate); G protein, guanine nucleotide-binding protein; GTP γ S, guanosine 5'-O-(3-thiophosphate); IMCD, inner medullary collecting duct; β -NAD, nicotinamide adenine nucleotide; PTX, pertussis toxin.

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and characterized as described (22). Over 90% of the cells in culture bound a lectin from *Dolichos biflorus*, a selective marker for the collecting duct cell type within the inner medulla (22, 24). We did not study the contaminating cells. As previously shown (22), cation channels were seen in the apical membrane of freshly isolated IMCDs, and in cells in confluent monolayers grown on filter bottom cups (Millipore CM; Millipore Corp., Bedford, MA) and collagen-coated (Matrigel; Collaborative Research, Bedford, MA) glass coverslips. Because the properties of the channel were independent of the substratum, cells were grown exclusively on Matrigel-coated glass coverslips for this study.

Single channel ion currents were measured with a current to voltage converter (Yale Mark V design), low-pass filtered at 100 or 300 Hz, and digitized at 1 KHz with an IBM AT computer. Data were recorded for at least two 20-s periods, and usually for three to four periods, (with a 30-s delay between each period) during the control and experimental periods. The channel amplitude or unitary current level (i) was determined by constructing amplitude histograms of the currents. For patches containing multiple channels, the number of channels (n) was calculated as described by Frindt and Palmer (25). We assumed n number of channels were open when the current was between $(n - 1/2)i$ and $(n + 1/2)i$. The probability of a single channel being open (P_o) was calculated as described (22). In brief, channels were considered open when the current was larger than $i/2$. P_o was defined as the total time spent in the open state divided by the total time of the record (22). In patches with multichannel events, P_o was estimated from $(\sum_{n=1}^N P_n)/N$, where P_n is the open probability for each unitary current level n , and N is the total number of channels in the patch (see reference 22 for details). As shown previously, the channels in multichannel patches were identical and independent (22, 23). All experiments were conducted in a paired fashion (i.e., each patch served as its own control) using excised, inside-out patches of the apical membrane. Data are reported as means $\pm$ SE. The statistical significance of an experimental maneuver was determined by the paired Student's t test. $P < 0.05$ was considered significant.

The patch pipette contained: NaCl, 140 mM; KCl, 5 mM; CaCl₂, 1 mM; MgCl₂, 1 mM; and Hepes, 10 mM; pH 7.4. The bath solution was: NaCl, 5 mM; KCl, 140 mM; CaCl₂, 1 mM; MgCl₂, 1 mM; and Hepes, 10 mM; pH 7.4. Unless stated otherwise, the bath solution flowed continuously (10 ml/min) through a 0.5-ml chamber. The composition of the bath solutions could be changed rapidly by a six-way valve.

The α_{i-3} subunit of G_i, a gift from Dr. Lutz Birnbaumer and his co-workers, was purified from human erythrocytes and activated with GTP γ S as described (6, 9). The effects on the channel of the activating buffer solution and heat-inactivated α_{i-3} were also examined as described (6). Activated α_{i-3} , denoted as α_{i-3}^* , was added to a static bath solution, which corresponds to the cytoplasmic side of the membrane patch.

Results

Cation channels were active in 30% of excised, inside-out patches of the apical membrane. The open probability (P_o) of the channel remained constant for long periods of time during control experiments (~ 30 min). Guanosine 5'-O-(3-thio)phosphate (GTP γ S, 10^{-4} M) in the bath solution increased the P_o in every experiment (Fig. 1 and Table I); the effect was evident within 30 to 90 s, and took 2 to 5 min to develop fully. In contrast, in independent experiments, guanosine 5'-O-(2-thio)phosphate (GDP β S, 10^{-4} M) decreased the P_o (Fig. 2 and Table I). Activation by GTP γ S and inhibition by GDP β S could not be reversed by washing the nucleotides from the bath. In addition, GTP (10^{-4} M) increased the P_o in five of eight experiments, however, it was less effective than GTP γ S (Table I). These results indicate that a G protein regulates the cation channel.

Pertussis toxin ADP-ribosylates G_i and G_o and slows the rate of receptor-dependent activation of G proteins by GTP and its analogues (1, 7, 13, 15, 19, 20). To determine whether the G protein regulating the cation channel was sensitive to PTX, we added the activated subunit of PTX (A protomer), with β -nicotinamide adenine nucleotide (NAD, 1 mM), adenosine 5'-triphosphate (1 mM) and DL-dithiothreitol (DTT, 2×10^{-5} M), to the bath solution of inside-out patches. PTX inhibited the channel in every experiment (Fig. 3, A and B, and Table II). Inhibition was first observed after a variable delay of 30 s to 5 min; maximum inhibition was seen after a delay of 2–10 min. The buffer solution, without PTX, in the bath had no appreciable effect on P_o ($n = 5$). The activity of the channel never increased when PTX was washed out of the bath. Although GTP could not reverse PTX-inhibition (Fig. 3 C and Table II), the channel was reactivated by GTP γ S (Fig. 3 D and Table II).

In the next experiment, we added the activated α_{i-3} subunit of G_i, α_{i-3}^* , to the bath solution of untreated membrane patches. In five experiments, α_{i-3}^* in the bath (2–200 pM) increased the P_o in every experiment (Table I). α_{i-3}^* also stimulated the channel in inside-out patches after endogenous G

Table I. Effect of G Protein Activation and Inactivation on the Cation Channel in Excised Inside-out Patches of the Apical Membrane of IMCD Cells

Compound	Concentration	Open probability	
		Control	Experimental
GTP γ S (9)	10^{-4} M	0.17 ± 0.05	$0.47 \pm 0.05^*$
GTP (8)	10^{-4} M	0.30 ± 0.10	$0.49 \pm 0.11^\dagger$
GDP β S (7)	10^{-4} M	0.57 ± 0.06	$0.25 \pm 0.10^\dagger$
α_{i-3}^* (5)	2–200 pM	0.28 ± 0.10	$0.44 \pm 0.11^\dagger$

The numbers in parentheses after each compound is the number of patches analyzed. All compounds were added to the bath solution in paired experiments. The P_o ranged between 0.01 and 0.99 in different membrane patches (control); however, in individual patches P_o was constant for long periods (~ 30 min). In general, we added activators when P_o was low and inhibitors when P_o was high. Our results, however, were independent of the P_o during the control period. Data are reported as mean $\pm$ SE. * $P < 0.01$. $^\dagger P < 0.05$.

Figure 2. GDPβS inhibition of the cation channel in an inside-out patch of the apical membrane. (A) Control. Two channels were present in the patch; the single channel P_o was 0.38. (B) 1 min after the addition of GDPβS (10^{-4} M) to the bath. Although there were two channels in the patch, simultaneous openings of both channels were not observed. In this experiment P_o decreased to 0.01. The voltage across the patch was -60 mV.

protein was inactivated by PTX (Fig. 4, and Table II). In two membrane patches, 2 pM α_{i-3}^* activated the channel (Fig. 4 and Table II). Although this concentration of α_{i-3}^* was ineffective in four of six PTX-treated membrane patches, higher concentrations of α_{i-3}^* (20 and 200 pM) in the bath of the same

Figure 3. Pertussis toxin inhibition and GTPγS activation of the cation channel in an inside-out patch. (A) Control. One channel was present in the patch and the P_o was 0.30. (B) 2 min after the addition of preactivated PTX (100 ng/ml), with β -NAD (10^{-3} M), ATP (10^{-3} M) and DTT (2×10^{-5} M) to the bath. P_o decreased to 0. (C) 3 min after the addition of GTP (10^{-4} M) to the bath. No change in channel activity was observed. (D) 5 min after the addition of GTPγS to the bath. P_o increased to 0.23. The voltage across the patch was -60 mV.

Table II. Effect of Pertussis Toxin and Exogenous G Protein on the Cation Channel in Excised Inside-out Patches of the Apical Membrane of IMCD Cells

Compound	Concentration	Open probability	Open probability
		Control	Experimental
PTX (9)	100 ng/ml	0.57 ± 0.11	0*
PTX (15)	200 ng/ml	0.55 ± 0.06	$0.10 \pm 0.05^*$
GTP (3)	10^{-4} M	0	0
GTPγS (2)	10^{-4} M	0	0.34 ± 0.21
α_{i-3}^* (2)	2 pM	0.15 ± 0.04	0.26 ± 0.02
α_{i-3}^* (3)	20 pM	0.04 ± 0.04	0.56 ± 0.26
α_{i-3}^* (1)	200 pM	0	0.52
α_{i-3}^* (6)	2–200 pM	0.05 ± 0.03	$0.46 \pm 0.14^\dagger$

The numbers in parentheses after each compound is the number of patches analyzed in paired experiments. All compounds were added to the bath solution. Guanine nucleotides and G protein was added after PTX was washed from the bath solution. The last row of data are pooled data from all experiments conducted with α_{i-3}^* after PTX inactivation of the channel. PTX reduced P_o gradually; complete inhibition was usually observed after 2–10 min. In some experiments P_o did not decrease to 0: in those experiments either the patch broke before complete inhibition was achieved or PTX was washed from the bath before complete inhibition was produced. Data are reported as mean $\pm$ SE. * $P < 0.01$, $^\dagger P < 0.05$.

patch always increased channel activity (Table II). Furthermore, α_{i-3}^* activated the cation channel in a dose-dependent manner (Table II). Neither the activating buffer solution ($n = 5$) nor heat-inactivated α_{i-3}^* (100°C for 30 min; $n = 3$) had an appreciable effect on the channel in control patches or in PTX-treated patches.

The channels activated by exogenous α_{i-3}^* in PTX-treated patches had properties similar to the channels observed in control solutions. In particular, the single channel conductance (28 pS) and ion selectivity ($P_{\text{Na}}:P_{\text{K}} = 1:1$; $P_{\text{Na}}:P_{\text{Cl}} = 13:1$) were similar ($n = 2$). In addition, cGMP (10^{-4} M) inhibited the channel (23) both before inactivation by PTX and after reactivation by exogenous α_{i-3}^* ($n = 2$).

Discussion

Our data show that the GTP-binding protein, G_i , regulates the cation channel in the apical membrane of renal epithelial (IMCD) cells, and thus regulates Na^+ influx across the apical membrane. The cation channel is the first ion channel which is shown to be gated directly by a G protein in a polarized epithelium. Our results are consistent with the immunocytochemical study of Ercolani et al. (17) who localized the sodium channel and G_i to the apical membrane of LLC-PK₁ cells and to observations that PTX inhibits electrogenic sodium absorption by LLC-PK₁ cells (18).

The α subunit of G proteins also activates ion channels in excitable cells and in hormone-secreting cells. In atrial cells α_i couples the muscarinic receptor directly to a K^+ channel (1, 7–9) and in GH₃ cells α_i couples somatostatin receptors to a K^+ channel (16). In these cell types the α subunit has been shown to activate channels directly as opposed to an indirect

Figure 4. Pertussis toxin inhibition and α_{i-3}^* activation of the cation channel in an inside-out patch. (A) Control. Three channels were present in the patch: P_o was 0.64. (B) 4 min after the addition of preactivated PTX (200 ng/ml) to the bath. Occasional brief open events were observed; simultaneous openings of more than one channel were never seen. P_o decreased from 0.64 to 0.10. (C) 2 min after the addition of α_{i-3}^* (2 pM) to a static bath. (PTX was washed out of the flowing bath solution). Simultaneous channel openings were frequently observed. P_o increased from 0.10 to 0.24. The voltage across the patch in all three current records was -60 mV.

mechanism involving protein kinases (1, 6, 12, 18, 26). The $\beta\gamma$ subunit of G_i also activates the muscarinic K^+ channel in cardiac myocytes, but indirectly via phospholipase A_2 (2, 3). Our studies on the cation channel in IMCD cells are also consistent with a direct stimulation of the channel by α_{i-3} as opposed to an indirect affect via activation of a protein kinase. In preliminary experiments we found that adenosine 5'-*O*-(3-thio)phosphate (ATP γ S), a nonspecific activator of protein kinases, PMA, an activator of protein kinase C, and exogenous cGMP-dependent protein kinase inhibited the cation channel. Furthermore, adenosine 3',5'-cyclic monophosphate (cAMP), which stimulates protein kinase A, had no effect on the channel in cell-attached patches (22). These observations are consistent with previous studies, which show that protein kinase C inhibits electrogenic sodium absorption by LLC-PK $_1$ cells (18) and A6 cells (27, 28). Because kinases inhibit the cation channel, our data show that the activation of the channel by the α_{i-3}^* subunit of G_i is not mediated by a kinase. Thus, α_{i-3} activates the cation channel directly.

Our observation that PTX completely inhibited the cation channel in IMCD cells indicates that G protein-receptor coupling is required for channel activity. Hormone receptors in epithelial cells, however, are localized primarily in the basolateral membrane (29). In our experiments, channels in inside-out patches of the apical membrane remained active for over 30 min in the absence of exogenous agonist and GTP. Given these observations, we suggest at least two disparate modes by which G proteins regulate ion channels in the apical membrane of polarized epithelial cells.

First, there may be a classical receptor-G protein complex in the apical membrane. The receptor G protein complex may either have a high basal activity, sufficiently high to account for active channels in inside-out patches in the absence of known agonist and exogenous GTP, or that locally produced autacoids, such as lipoxygenase products, bind to the receptor (2, 3, 30, 31). In this scenario, autacoids derived from the plasma membrane would bind to the receptor and activate the G protein. This concept requires that the membrane patch contains a pool of GTP. PTX would inhibit receptor-dependent G protein activation and inactivate the channel. At the present time, however, a receptor has not been identified in the apical membrane of IMCD cells, and an autacoid-hormone dependent mechanism has not been demonstrated. Regulation of the cation channel by autacoids that bind to apical receptors, such as lipoxygenase products, or other hormones such as bradykinin, could be tested with specific analogues and inhibitors (3, 30), and will be examined in future studies.

A second possible mode of G protein regulation of ion channels in polarized epithelia involves a unique mechanism not requiring a traditional membrane receptor to interact with the G protein. In epithelia, including the IMCD, the G protein-ion channel complex in the apical membrane is geographically separated from G protein-receptor complexes in the basolateral membrane (29). Because hormones bind to receptors in the basolateral membrane and activate ion channels located in the apical membrane of epithelia, it is possible that an effector activated by the G protein complex in the basolateral membrane may serve as an intracellular agonist, and either bind to and directly activate the G protein-cation channel complex in the apical membrane or activate the complex by phosphorylation (32, 33). PTX would block the interaction between the apical G protein and its effector, the cation channel. Although this action of G proteins is potentially novel it may have evolved in epithelial cells because of the geographical separation of basolateral membrane receptors and apical membrane transport mechanisms. Such a separation would require an intracellular messenger to couple events at the basolateral membrane to transport mechanisms in the apical membrane.

In summary, our data show that the cation channel in the apical membrane of IMCD cells is activated directly by the α_{i-3}^* subunit of G_i . Accordingly, sodium absorption by the renal IMCD, a polarized epithelium, is directly regulated by a G protein. It has now been shown in a variety of cell types including electrically excitable cells, hormone-secreting cells, and epithelial cells that G proteins regulate ion channels.

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