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Phosphorylation Regulates an Inwardly Rectifying ATP-sensitive K^+- Conductance in Proximal Tubule Cells of Frog Kidney

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Abstract. K^+ channels in the renal proximal tubule play an important role in salt reabsorption. Cells of the frog proximal tubule demonstrate an inwardly rectifying, ATP-sensitive K^+ conductance that is inhibited by Ba^{2+}, G_{Ba}. In this paper we have investigated the importance of phosphorylation state on the activity of G_{Ba} in whole-cell patches. In the absence of ATP, G_{Ba} decreased over time; this fall in G_{Ba} involved phosphorylation, as rundown was inhibited by alkaline phosphatase and was accelerated by the phosphatase inhibitor F^-(10 mM). Activation of PKC using the phorbol ester PMA accelerated rundown via a mechanism that was dependent on phosphorylation. In contrast, the inactive phorbol ester PDC slowed rundown. Inclusion of the PKC inhibitor PKC-ps in the pipette inhibited rundown. These data indicate that PKC-mediated phosphorylation promotes channel rundown. Rundown was prevented by the inclusion of PIP-2 in the pipette. PIP-2 also abrogated the PMA-mediated increase in rundown, suggesting that regulation of G_{Ba} by PIP-2 occurred downstream of PKC-mediated phosphorylation. G-protein activation inhibited G_{Ba}, with initial currents markedly reduced in the presence of GTPγs. These properties are consistent with G_{Ba} being a member of the ATP-sensitive K^+ channel family.

Key words: Inwardly-rectifying K^+ channel — PIP-2 — Phosphorylation

Introduction

The mechanism by which epithelial cells absorb Na^+ involves the co-ordinated activity of membrane proteins located in the apical and basolateral membranes [17]. Salt reabsorption ultimately depends on the turnover of the Na^+ / K^+ ATPase located on the basolateral membrane of the cell, which maintains a low intracellular Na^+ concentration. Together with the negative membrane potential, this Na^+ concentration gradient provides the driving force for the apical uptake of Na^+ via a number of specialized proteins, such as Na^+ channels, Na^+-coupled co-transporters and Na^+-coupled antiporters. The Na^+ that enters the cell is removed via the basolateral Na^+/K^+ ATPase in exchange for K^+, which exits the cell via a basolateral K^+ conductance. The net result is the transepithelial movement of Na^+, while K^+ recycles across the basolateral membrane. This general mechanism for salt absorption was first proposed by Koefoed-Johnson and Ussing in the 1960s, and subsequent work has supported its validity in the renal proximal tubule of both amphibians and mammals.

A number of different K^+ channels have been identified in the renal proximal tubule that may play a role in salt reabsorption. On the basolateral membrane these channels fall into two general categories, those sensitive to intracellular ATP and those sensitive to membrane stretch. The ATP-sensitive K^+ channels have been observed in both rabbit and *Ambystoma* [2, 12, 18]. They are inwardly rectifying channels that show inhibition with millimolar levels of intracellular ATP. This sensitivity to ATP is thought to confer a link to the activity of the Na^+/K^+ ATPase, so-called pump-leak coupling [1]. The stretch-sensitive K^+ channels have been observed in *Necturus* and *Xenopus* [13, 30]. These channels are activated by either the application of pressure across the cell membrane or, physiologically, by increases in cell volume. Although less well studied, apical K^+ channels are also involved in electrolyte homeostasis. In primary cultures of rabbit proximal tubule cells large-conductance, Ca^{2+}- activated K^+ channels are

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observed that are thought to play a role in cell volume regulation [5]. The K^+ channel regulator KCNE1, together with the voltage-dependent channel KCNQ1, is expressed in the apical membrane of the mouse proximal tubule [37]. KCNE1 knockout mice have increased excretion of Na^+ and glucose, suggesting that proximal tubule transport is compromised in its absence [37]. In addition, proximal tubules isolated from these mice are unable to maintain the resting membrane potential in response to the stimulation of Na^+ glucose co-transport. It is apparent that both apical and basolateral K^+ channels play an important role in the reabsorption of Na^+ and solute by the renal proximal tubule.

In proximal tubule cells of *Rana temporaria* two K^+ conductances have been identified at the whole-cell level [27, 28]. One conductance is inhibited by Ba^{2+} and quinidine (G_{Ba}), while the second conductance is only inhibited by quinidine (G_{Quin}). Both channels are sensitive to millimolar levels of intracellular ATP. For G_{Ba} 2 mM ATP activates the conductance, via mechanisms that involve both hydrolytic and non-hydrolytic processes. G_{Quin} is also maintained by the presence of 2 mM ATP, but has an absolute requirement for ATP hydrolysis. A notable difference between these conductances concerns their conduction properties; G_{Ba} is an inward rectifier, while G_{Quin}, is an outward rectifier [27]. G_{Ba} has been identified at the single-channel level in patches of the basolateral membrane [11, 27], but as it has an ATP-sensitivity different from proximal tubule ATP-sensitive K^+ channels in other species, its physiological role remains conjectural [28]. The aim of the following study was to examine further the properties of G_{Ba}, in particular with respect to its regulation by protein kinase C.

Materials and Methods

Cell Isolation

Single proximal tubule cells were isolated by enzyme digestion from kidneys of *Rana temporaria* as described previously [10]. Frogs were killed by decapitation and the brain and spinal cord destroyed prior to removal of the kidneys.

Patch Experiments

A suspension of single cells was placed in a Perspex bath on the stage of an inverted microscope (Nikon Diaphot or Olympus 1X70) and standard patch-clamp techniques employed to investigate whole-cell currents [7]. Voltage protocols were driven from an IBM-compatible computer equipped with either a Labmaster TL-1 or Digidata interface (Axon Instruments, Foster City, CA), using the pClamp software, Clampex (Axon Instruments). Recordings were made using a List EPC-7 amplifier. To reduce stray capacitance and associated noise, patch pipettes were coated with Sylgard (Dow Coming). Patch pipette resistance was between 2 and 5 MΩ. Whole-cell clamp was obtained via the basolateral aspect of the cells and currents were saved directly onto the hard disk of the computer following low-pass filtering at 5 kHz. The series resistance of cells following patch rupture was 13.0 ± 0.75 MΩ (n = 100). Average steady-state currents at each potential were derived using Excel 2000. Cell area was calculated from the capacity transients seen in response to a 20 mV potential step, with membrane capacitance assumed to be 1 μF per cm^2. The mean cell capacitance was 45 ± 3 pF (n = 40). In all experiments 100 μM gadolinium chloride (Gd^{3+}) was added to the bathing solution to inhibit a barium- and Gd^{3+} -sensitive non-selective cation conductance also present in half of the cells [26].

Table 1. Experimental solutions

	Extracellular solution			Intracellular solutions
Conc (mM)	Isolation	20 KCl	100KCl_{bath}	100KCl_{pip}
NaCl	101			
KCl	5	20	100	100
$MgCl_2$		1	1	2
$CaCl_2$		2	2	
HEPES	10*	10**	10**	10**
Mannitol		150	10	20
EGTA				0.5

* Titrated to pH 7.4 with NaOH.
** Titrated to pH 7.4 with KOH.

Chemicals and Solutions

The composition of solutions is given in Table 1 and variations are given at the relevant position in the Results section. The osmolality of all solutions was measured (Roebling osmometer) and adjusted to within 1 mosmol per kg water of 215 mosmol per kg water, with water or mannitol, as appropriate. Chemicals were obtained from Sigma, and were of analytical grade. Alkaline phosphatase, protein kinase C ps (PKC-ps) and phosphatidylinositol-4,5-bisphosphate (PIP-2) were obtained from Calbiochem, UK.

Statistics

Results are given as means ± 1 SEM, with the number of experiments in parentheses (n). Significance was tested using ANOVAS and significance assumed at the 5 % level. The F value calculated from ANOVAS was presented as $F_{x,y}$, where x equals the between-group degrees of freedom and y equals the within-group degrees of freedom. Significance was determined using the F table. Statistical analysis of fractional data was carried out after these data had been normalized by logarithmic transformation.

Results

Regulation of G_{Ba}

To examine the regulatory mechanisms underlying the run-down of G_{Ba}, experiments were conducted with no ATP in the pipette solution, together with a number of modulators of protein kinases, protein phosphatases and G-proteins. Whole-cell patches were obtained with 20 KCl in the bath (Table 1) and 100 KCl_{pip} in the pipette (Table 1). Whole-cell

potential was held at 0 mV and then transiently stepped to +33 mV followed by a second step to −33 mV for 300 ms duration. This protocol was repeated at 5-second intervals over a 10-minute recording period. The measured V_{rev} of the K^+- current was −33 mV, while that of the Cl^- current was +33 mV [25]; by recording the current flow at +33 and −33 mV, respectively, predominantly K^+-or Cl^--selective currents could be recorded, although this study only shows data concerned with the K^+-selective current. As the K^+-selective current comprises two different conductances, one that is blocked by Ba^{2+} and one that is not, the current passed by G_{Ba} was defined as the current sensitive to 5 mM Ba^{2+} (I_{Ba}, a maximal inhibitory concentration) at +33 mV [27]. Changes in I_{Ba} were taken as the difference between the Ba^{2+}-sensitive current measured initially on achieving the whole-cell configuration and that recorded after ten minutes (Fig. 1).

Under the control circumstance (0 ATP in the pipette) the initial I_{Ba} was 1.46 ± 0.17 pA/pF (n = 34). As observed previously, I_{Ba} decreased significantly over ten minutes to 0.66 ± 0.09 pA/pF (n = 34) (Fig. 1). Statistical analysis of the initial currents under all experimental conditions gave an F value of $F_{14,171}$ = 2.49. Statistical analysis of the currents at ten minutes expressed relative to the initial current gave an F value of $F_{14,171}$ = 7.79.

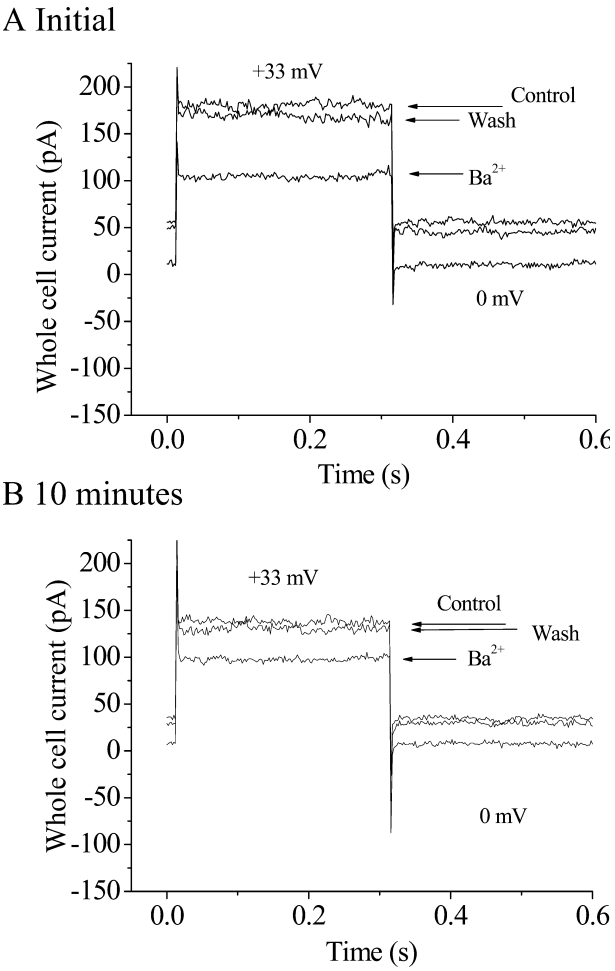

Fig. 1. Typical whole-cell traces of K^+ currents obtained from the same cell in the absence of pipette ATP initially on achieving the whole-cell configuration (*A*) and after 10 minutes (*B*). Each figure shows the current recorded under the control circumstance, on addition of 5 mM Ba^{2+} to the bath, and on wash-out of Ba^{2+}.

Role of Phosphatases

Protein phosphatase activity was examined by inclusion of either 25 units/ml of exogenous alkaline phosphatase (AP), 10 mM F^- [31] or 1 μM calyculin A (calA, to inhibit protein phosphatases 1 and 2A) in the pipette. Initial currents were unchanged between 0 ATP and the test pipette solutions. However, the fall in I_{Ba} in the absence of intracellular ATP was reduced by alkaline phosphatase (Fig. 2), whereas inhibition of endogenous phophatases by 10 mM F^- had the opposite effect and increased the rate of rundown (Fig. 2). Omitting Mg^{2+} from the pipette solution (osmolality maintained with mannitol) increased the magnitude of initial currents, but was without effect on the current decrease over time. Calyculin A had no effect on either the initial current or the degree of current decrease with time.

Role of PKCl

To activate protein kinase C (PKC) cells were preincubated with 100 nM phorbol-12-myristate-13-acetate (PMA) for 10 minutes prior to whole-cell clamp, and PMA was subsequently included in the bath and pipette solutions. PMA had no effect on the initial current, but enhanced rundown (Fig. 3). To confirm that the effect of PMA was via a specific effect on PKC, experiments were also carried out with 100 nM 4α-phorbol 12,13-didecanoate (PDC), an inactive phorbol ester that does not stimulate PKC activity. Again cells were incubated for 10 minutes prior to obtaining the whole-cell configuration and PDC was also present in both the bath and pipette solutions throughout the experiment. PDC had no effect on the initial current but, in contrast to PMA, decreased the rate of rundown (Fig. 3). PMA exerted its effects via phosphorylation since alkaline phosphatase (25 units/ml added to the pipette) completely abrogated the effects of PMA. Finally, to inhibit PKC, whole-cell recordings were obtained with 10 μM PKC-ps added to the pipette solution, which abolished rundown (Fig. 3). Previous studies have suggested that the inhibitory effect of PKC on Kir channels could be due to depletion of cellular PIP-2 [39]. PIP-2 [50 μM in the pipette) had no effect on the initial currents but abolished the decrease in current typically seen in the absence of ATP (Fig. 3). In addition, PIP-2 also negated the effect of PMA (Fig. 3).

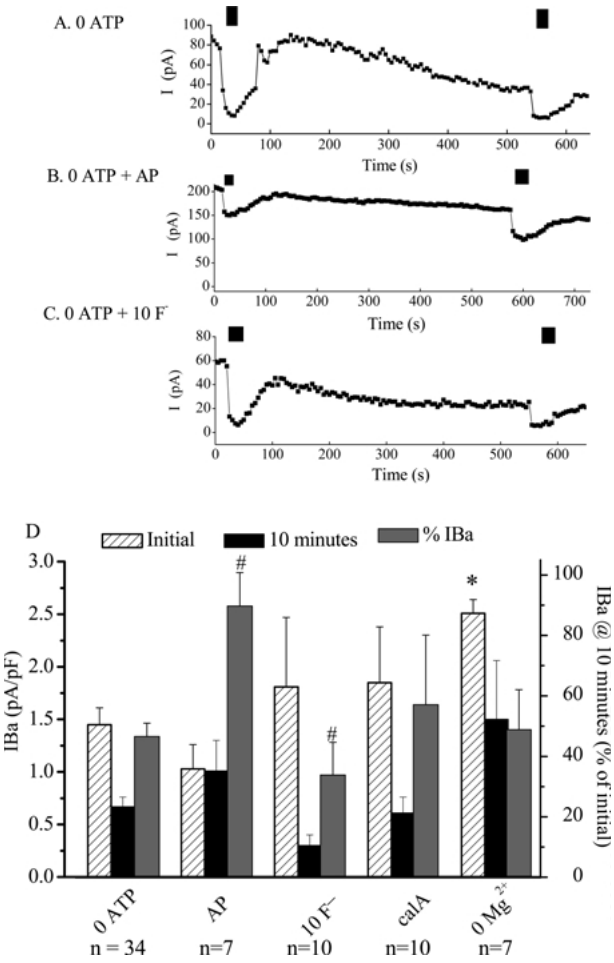

Fig. 2. Role of phosphorylation in rundown of G_{Ba}. Whole-cell currents recorded at +33 mV with 20 mM KCl (bath) and 100 mM KCl_{pip} (pipette) in the absence of ATP (*A*), in the presence of alkaline phosphatase (AP) (*B*) and in the presence of 10 mM F^- (*C*). The black bars indicate when 5 mM Ba^{2+} was present in the bathing solution. (*D*) Mean I_{Ba} recorded initially and after 10 minutes, plus % I_{Ba} remaining at 10 minutes. Statistical analysis was carried out on the absolute initial currents (*) or on the fraction of the initial current remaining after 10 minutes (#). * and # indicate a significant difference to the 0 mM ATP circumstance.

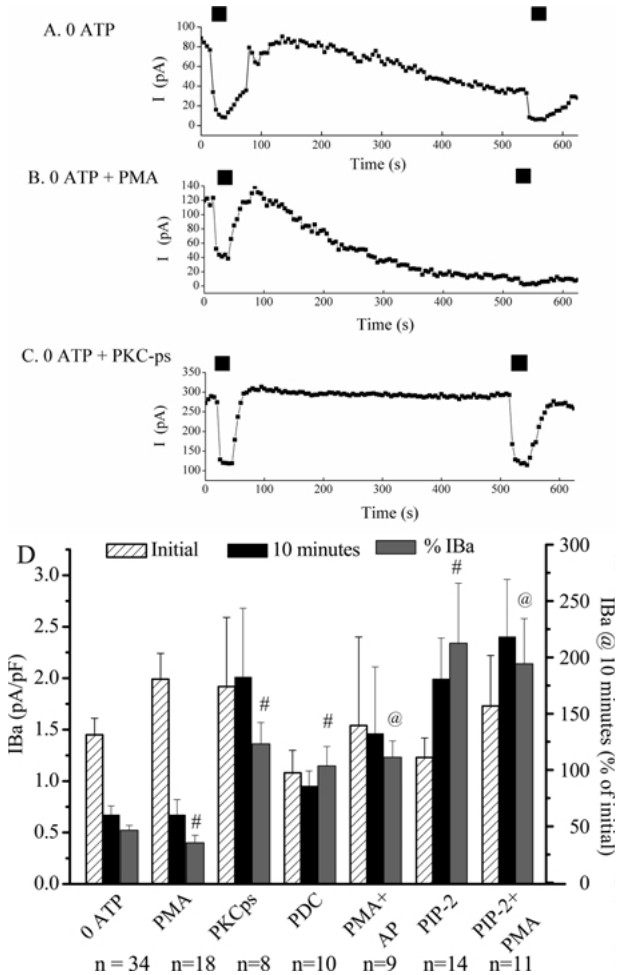

Fig. 3. Role of PKC in rundown of G_{Ba}. Whole-cell currents recorded at +33 mV with 20 mM KCl (bath) and 100 mM KCl_{pip} (pipette) in the absence of ATP (*A*), in the presence of PMA (*B*) and in the presence of PKCps (*C*). The black bars indicate when 5 mM Ba^{2+} was present to the bath. (*D*) Mean I_{Ba} measured initially, at 10 minutes and at 10 minutes expressed as a % of the initial level. # indicates a significant difference to the 0 ATP circumstance. @ indicates a significant difference to the PMA circumstance.

Role of G-proteins

G protein activity was investigated by inclusion of either 10 μM GTPγS, 1 mM F^- (for activation, [4]) or 10 μM guanosine 5′-O-(2-thiodiphosphate) (GDPβS, for inhibition) in the pipette solution. To investigate whether G protein regulation depended upon activation of protein phosphatases 1 and 2A [3, 29], the phosphatase inhibitor calyculin A was added to the pipette solution together with GTPγS. With GTPγS alone or GTPγS together with calyculin A, the initial I_{Ba} was reduced compared to control (Fig. 4). In addition, rundown of I_{Ba} was apparently markedly reduced by both GTPγS and 1 mM F (Fig. 4). On the other hand, rundown was unaffected by GDPβs, indicating that G proteins were not tonically active (*see* Discussion). When calyculin A was added to the pipette solution together with GTPγS there was no additional effect on rundown (Fig. 4).

Discussion

G_{Ba} decreased over time in the whole-cell configuration, with I_{Ba} falling by half within ten minutes of achieving the whole-cell configuration. Rundown is a property demonstrated by many K^+ channels, of renal and non-renal origin [8, 15, 16, 18, 24, 38] and may be affected by the phosphorylation state. Rundown of KIR 1.1 is prevented in the absence of intracellular Mg^{2+} or by exposure to the phosphatase inhibitor orthovanadate, suggesting that dephosphorylation is the key event mediating rundown [19].

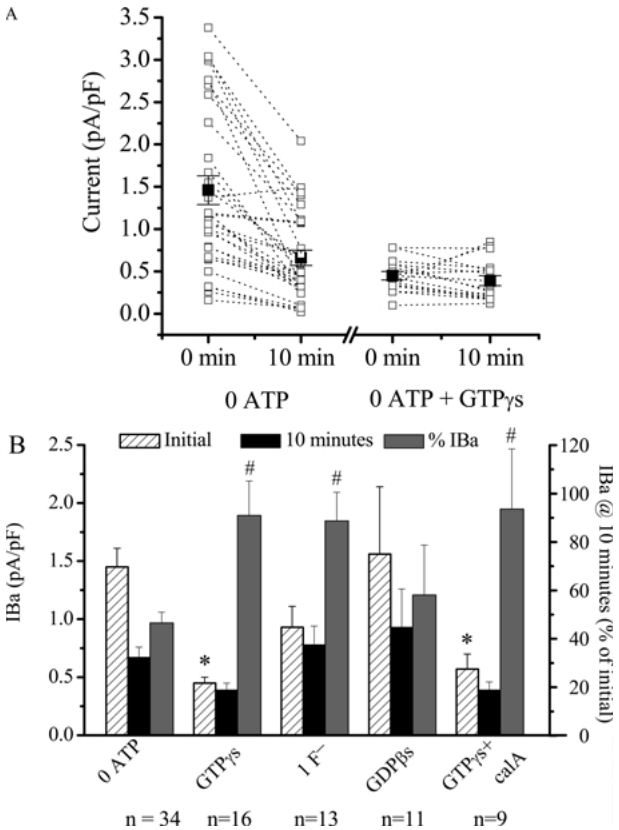

Fig. 4. Role of G-proteins in regulation of G_{Ba}. (*A*) I_{Ba} initially on achieving the whole-cell configuration and at 10 minutes, with 0 mM ATP or 0 mM ATP plus GTPγs. Each empty square represents an individual whole-cell recording. The filled squares are the mean data for each dataset. (*B*) Mean I_{Ba} recorded initially and at 10 minutes. Statistical analysis was carried out on the absolute initial currents (*) or on the fraction of the initial current remaining after 10 minutes (#). Both * and # indicate a significant difference to the 0 mM ATP circumstance.

Rundown of the native apical K^+ channel in rat principal cells is also prevented by inhibition of phosphatase activity or by removal of Mg^{2+} [14]. A decrease in rundown with the phosphatase inhibitor okadaic acid has also been observed with a number of other K^+ channels [3, 21]. Conversely, calyculin A induces inhibition of the basolateral K^+ channel in rat distal colon, suggesting that this channel is maintained by dephosphorylation [33].

G_{Ba} also seems to be regulated by phosphorylation. The fall in G_{Ba} was prevented by exogenous phosphatase and was accelerated by the non-specific serine / threonine phosphatase inhibitor F^-. The effect of F^- suggests that, under the experimental conditions, a serine / threonine phosphatase is active in the cell, although a contribution from G-protein activation, which inhibits G_{Ba}, cannot be excluded. The lack of an effect of calyculin A rules out a role for phosphatases 1 or 2A in regulating G_{Ba}. Finally, in the nominal absence of intracellular Mg^{2+}, the decrease in G_{Ba} was unaffected, suggesting the involvement of a Mg^{2+}- independent phosphatase. It should be noted, however, that when the nominally, Mg^{2+}-free pipette solution also contained EDTA, the rundown of G_{Ba} was accelerated to such an extent that its rate could not be determined (*data not shown*). This suggests that even small levels of intracellular Mg^{2+} could be important in regulation, and therefore a role for a Mg^{2+}-dependent phosphatase cannot be ruled out. This involvement of phosphatases in maintaining G_{Ba} is in contrast to the majority of previous studies, but is similar to a K^+ channel in rat distal colon, where inhibition of a phosphatase inhibits channel activity [33].

In terms of phosphorylation, the data support the hypothesis that G_{Ba} is regulated by PKC. Stimulation of PKC with PMA accelerated rundown, while the inactive phorbol ester PDC decreased the rate of rundown, possibly by binding to endogenous PKC and thus reducing the activation by endogenous diacylglycerol. In addition, inhibition of PKC maintained G_{Ba}. All these results are consistent with a role for PKC in regulation. The mechanism by which PKC is acting involves phosphorylation, as alkaline phosphatase prevented the action of PMA. In addition, the data also suggest the involvement of PKC-mediated changes in PIP-2 levels, as PIP-2 was able to prevent rundown in the presence of PMA. This mechanism of inhibition by PKC is similar to ROMK, which also shows relief of inhibition by PKC on exposure to PIP-2 [39]. Interestingly, it has been shown that PMA decreases membrane PIP-2 levels in *Xenopus* oocytes, ventricular myocytes and a mouse collecting duct cell line [22, 39]. PIP-2 was also able to prevent the rundown of G_{Ba} typically observed in the absence of pipette ATP. Such regulation has been observed in a number of other Kir channels, including GIRK1/GIRK4, ROMK and Kir 6.2 [6, 36, 39]. It is also interesting to speculate that rundown subsequent to activation of PKC may occur via dynamin-mediated internalization [9, 35], but in the absence of suitable antibodies, such experiments are not feasible. Nonetheless, in the present study the initial current was unaffected by pre-treatrnent with PMA, indicating that activation of PKC alone is not sufficient to cause channel rundown. The effects of PKC and phosphatase modulation were observed in the absence of pipette ATP, suggesting that sufficient ATP was present at the cytosolic surface of the channel to allow phosphorylation. Such an "ATP pool" is thought to fuel the sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^+ / K^+ ATPase [20, 34].

G_{Ba} was also regulated by G-proteins. Both GTPγs and 1 mM F^- prevented the decrease in G_{Ba} with time, with the change in I_{Ba} under the two circumstances significantly reduced compared to zero ATP. However, in the presence of GTPγs the initial I_{Ba} was reduced compared to 0 ATP. Indeed, the initial I_{Ba} recorded with GTPγs was not significantly different from that

recorded at 10 minutes with 0 ATP. The GTPγs data suggest that, rather than maintaining G_{Ba}, activation of G-proteins inhibited the conductance so that G_{Ba} could decrease no further. Hence the apparent lack of rundown actually masks an initial strong inhibition. The effect of 1 mM F^- is not as clear-cut, as the initial I_{Ba} was not significantly different from control, although at 0.93 ± 0.18 pA/pF it was rather low. This deviation from the GTPγs data could be a consequence of a reduced level of G-protein activation with 1 mM F^-, and therefore a reduced level of inhibition on achieving the whole-cell configuration. The inhibition of G_{Ba} by G-protein activation is similar to a number of inwardly rectifying K^+ channels expressed in both epithelial and non-epithelial tissue, e.g., Kir 3.1, and K^+ channels from alveolar type II cells and rat nerve terminals [3, 21, 32]. The mechanism of G-protein mediated inhibition of G_{Ba} is not clear; as phosphatase activity maintains G_{Ba} we would not expect the inhibition by G-proteins to be mediated by activation of phosphatases 1 and 2A. However, as such regulation has been previously demonstrated in a rat neuronal K^+ channel and Kir2.1 [3, 29], it was investigated in this study. Calyculin A did not reverse the effect of GTPγs, indicating that the inhibition by G-proteins does not involve activation of phosphatases 1 and 2A. G_{Ba} is not tonically activated by G-proteins, since GDPβs was without effect. GDPβs binds to G- proteins and prevents binding of endogenous GTP, leading to G-protein inhibition. However, the data presented here show a clear inhibitory effect of G-protein activation on channel activity, suggesting that G-proteins may regulate the activity of G_{Ba} in vivo.

In conclusion, G_{Ba} shares several of the properties of basolateral K^+ channels thought to be involved in cellular homeostasis in rabbit and *Ambystoma* renal proximal tubule. It is regulated by PKC-mediated phosphorylation, leading to channel rundown. Rundown was prevented by the phospholipid PIP-2, suggesting that PIP-2 may act downstream of PKC-mediated phosphorylation. G_{Ba} was also inhibited by activation of G-proteins. Thus, like many other Kir channels, the regulation of G_{Ba} is multifactorial, involving PKC, PIP-2, and G-proteins. When considered together with the previously published data concerning its ATP-sensitivity [27], we propose that G_{Ba} is a member of the ATP-sensitive family of K^+ channels.

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