Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 8077–8081, August 1994
Physiology

Regulation of ROMK1 K^+ channel activity involves phosphorylation processes

(oocyte expression/kidney/patch clamp/ATP-regulated channel/phosphatase)

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Contributed by Gerhard Giebisch, March 7, 1994

ABSTRACT **An inwardly rectifying, ATP-regulated K^+ channel with a distinctive molecular architecture, ROMK1, was recently cloned from rat kidney. Using patch clamp techniques, we have investigated the regulation of ROMK1 with particular emphasis on phosphorylation/dephosphorylation processes. Spontaneous channel rundown occurred after excision of membrane patches into ATP-free bath solutions in the presence of Mg^{2+}. Channel rundown was almost completely abolished after excision of patches into either Mg^{2+}-free bathing solutions or after preincubation with the broad-spectrum phosphatase inhibitor, orthovanadate, in the presence of Mg^{2+}. MgATP preincubation also inhibited channel rundown in a dose-dependent manner. In addition, the effect of the specific phosphatase inhibitors okadaic acid (1 μM) and calyculin A (1 μM) was also investigated. The presence of either okadaic acid or calyculin A failed to inhibit channel rundown. Taken together, these data suggest that rundown of ROMK1 involves a Mg^{2+}-dependent dephosphorylation process. Channel activity was also partially restored after the addition of MgATP to the bath solution. Addition of exogenous cAMP-dependent protein kinase A (PKA) catalytic subunit led to a further increase in channel open probability. Addition of Na_2ATP, in the absence of Mg^{2+}, was ineffective, suggesting that restoration of channel activity is a Mg^{2+}-dependent process. Addition of the specific PKA inhibitor, PKI, to the bath solution led to a partial, reversible inhibition in channel activity. Thus, PKA-dependent phosphorylation processes are involved in the modulation of channel activity. This observation is consistent with the presence of potential PKA phosphorylation sites on ROMK1.**

Phosphorylation and dephosphorylation of specific amino acid residues is recognized as one of the most important mechanisms for regulating intracellular processes. Indeed, the role of protein phosphorylation in the regulation of channel activity is no exception. At any given time, the level of phosphorylation is determined by the relative specific activities of protein kinases and protein phosphatases.

Potassium channels in the distal nephron play a major role in the regulation of K^+ homeostasis through K^+ secretion (1, 2). A distinct population of ATP-sensitive, cAMP-dependent protein kinase A (PKA)- and protein kinase C (PKC)-regulated, inwardly rectifying low-conductance K^+ channels are primarily responsible for this function (3, 4)—a channel that belongs to a family of ATP-regulated K^+ channels, characterized by their dual sensitivity to ATP: high concentrations are inhibitory, whereas low concentrations are stimulatory. In addition, a hallmark of this particular class of channels is that channel activity rapidly declines, a process known as channel "rundown," when patches of membrane are excised in the inside-out configuration in the absence of ATP in the bathing medium (for review, see ref. 5).

Recently, the structure of a distinctive ATP-regulated K^+ channel (ROMK1) was reported (6). Several potential phosphorylation sites for both PKA and PKC are present on the predicted ROMK1 channel protein. Injection of ROMK1 cRNA into *Xenopus laevis* oocytes gives rise to a Ba^{2+}-sensitive, inwardly rectifying, ATP-regulated 39-pS K^+ channel. Studies to date indicate that ROMK1 shares striking functional characteristics with the native low-conductance channel of the distal nephron.

Although the predicted ROMK1 channel protein shares no sequence homology with the ATP-binding cassette members (which includes cystic fibrosis transmembrane regulator), it has been suggested that ATP-dependent phosphorylation of a putative regulatory domain may alter channel gating (see ref. 6). In an attempt to elucidate the mechanisms involved in the regulation of ROMK1 K^+ channel activity, we have investigated the effects of ATP and PKA-dependent phosphorylation on restoration of ROMK1 K^+ channel activity. In addition, we have studied the modulation of channel rundown by dephosphorylation processes by exploring the effects of a variety of phosphatase inhibitors and the interaction of Mg^{2+} and ATP. Further, we have confirmed the data presented by Ho *et al.* (6), inasmuch as a Ba^{2+}-sensitive, inwardly rectifying, 42.6 ± 2.6 pS (150 mM KCl bath and pipette), ATP-regulated K^+ channel is expressed after injection of ROMK1 cRNA in *Xenopus* oocytes. A representative current–voltage relationship is shown in Fig. 1. A preliminary report of this data has been published (7).

METHODS

***Xenopus* Oocyte Preparation.** Stage V–VI oocytes were isolated, defolliculated by treatment with collagenase (2 mg/ml; Boehringer Mannheim) in zero Ca^{2+} hypotonic solution (82.5 mM NaCl/2.0 mM KCl/1.8 mM $MgCl_2$/5.0 mM Hepes, pH 7.4) combined with gentle agitation for 1 hr, and then washed in ND96 medium (96 mM NaCl/2.0 mM KCl/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/5.0 mM Hepes, pH 7.4). Thereafter, oocytes were maintained in supplemented ND96 medium (ND96 containing gentamycin at 50 μg/ml and 2.5 mM sodium pyruvate). Twenty-four hours later oocytes were injected with 50 nl (5 ng) of ROMK1 cRNA. Experiments were performed on days 3–8 after injection.

Oocytes were placed in a hypertonic solution (220 mM *N*-methylglucamine/220 mM aspartic acid/2 mM $MgCl_2$/10 mM EGTA/10 mM Hepes, pH 7.4 at room temperature) and

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Abbreviations: PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; PKI, PKA inhibitor; CA, calyculin A; OA, okadaic acid; PP, protein phosphatase; CCD, cortical collecting tubule.

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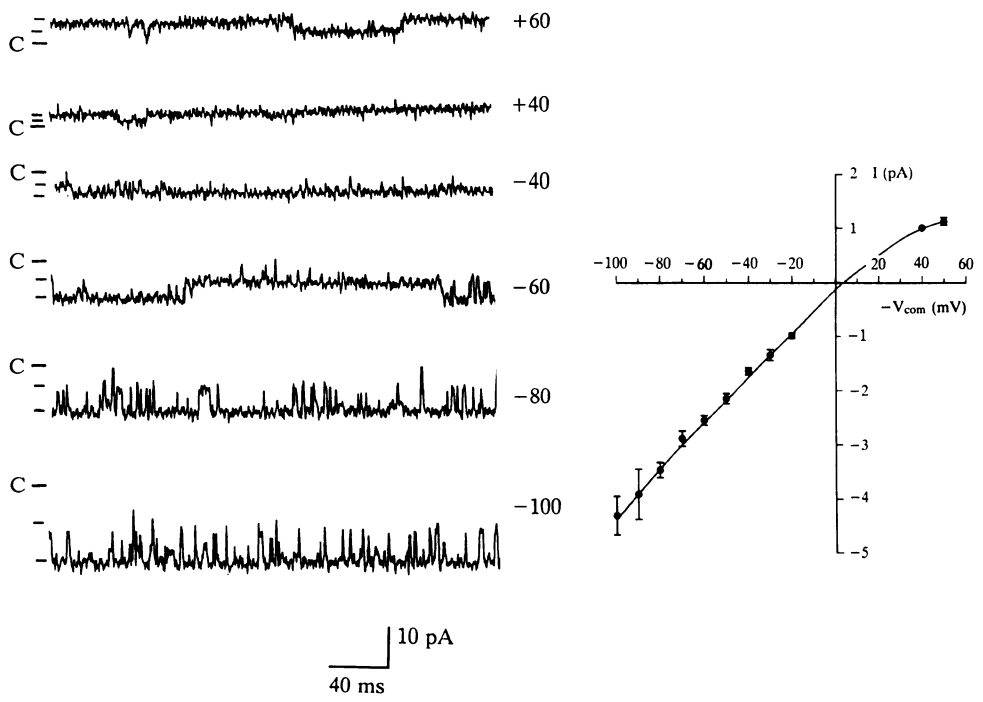

FIG. 1. Current–voltage relationship for ROMK1 K^+ channel activity in the cell-attached configuration (n = 10) with symmetrical KCl solutions. A representative trace is shown; closed channel level is indicated by "C". Downward deflections represent inward currents.

allowed to shrink for 2–5 min; the vitelline membrane was removed and then transferred into a chamber mounted on an IMT-2 Olympus microscope.

Experimental Media. Standard bath solution was 150 mM KCl/1.0 mM $MgCl_2$/5.0 mM EGTA/5.0 mM Hepes, pH 7.4; Mg^{2+}-free bath solution was 150 mM KCl/5.0 mM EDTA/5.0 mM Hepes, pH 7.4. Standard pipette solution was 150 mM KCl/1.0 mM $CaCl_2$/1.0 mM $MgCl_2$/5.0 mM Hepes, pH 7.4. Stock solutions of 10 mM MgATP (Sigma) were made in standard bath solution. PKA (Sigma) was dissolved in ultrapure water containing dithiothreitol at 6 mg/ml, and appropriate volumes of stock solutions were added to the bath solution to yield a final concentration of 40 units/ml. PKA inhibitor (PKI; Sigma, no. P0300), was dissolved in standard bath solution prior to use. Stock solutions (100 mM) of sodium orthovanadate (Na_3VO_4) were made. Solutions were maintained at 4°C. Stock solutions were diluted by adding appropriate aliquots directly to the bath chamber. Experiments were performed at room temperature (20–22°C). Calyculin A (CA) and okadaic acid (OA) were purchased from LC Laboratories (Woburn, MA).

Patch Clamp Technique. Single channel recordings were obtained from membrane patches in cell-attached and inside-out configurations (8). Recording pipettes were constructed from borosilicate glass capillaries (Dagan Instruments, Minneapolis).

Single channel currents were recorded with a patch clamp amplifier (List Electronics, Darmstadt, Germany), filtered at 1 kHz using an 8-pole Bessel filter, and stored on videotape after pulse code modulation (Sony model PCM-501). For analysis, data were redigitized and transferred into an IBM-compatible microcomputer and analyzed using PCLAMP

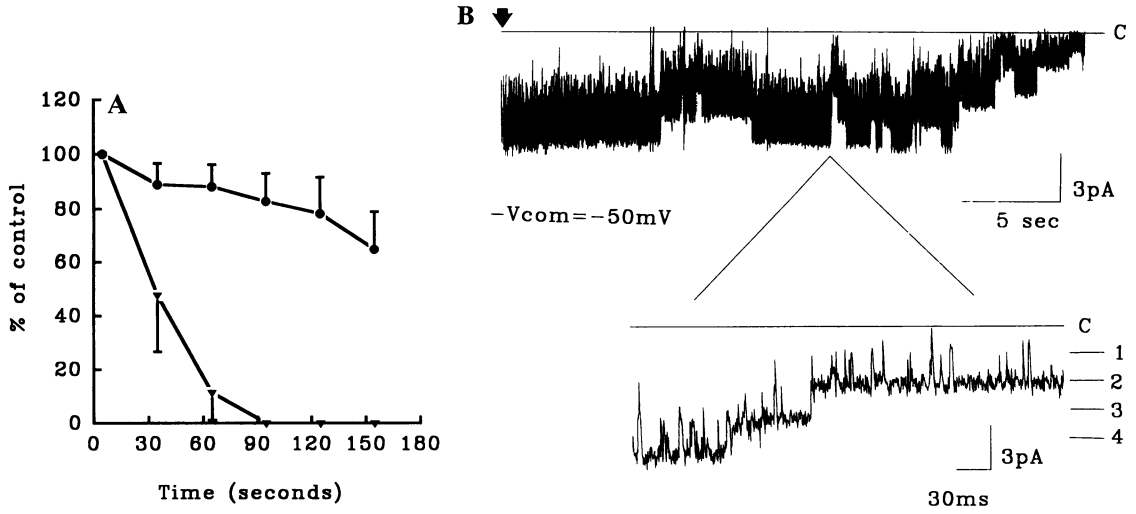

FIG. 2. (*A*) Effect of Mg^{2+} on channel rundown. In the absence of Mg^{2+} (●) in the bath solution, channel rundown is partially inhibited (n = 9). In the presence of Mg^{2+} (▼), channel activity rapidly declines (n = 6). (*B*) Channel recording of ROMK1 K^+ channel activity made in an inside-out patch (point of excision = arrow) with Mg^{2+}/ATP-free solutions. Channel activity rapidly declines under these conditions. C, closed level. $-V_{com}$ = −50 mV.

(Axon Instruments, Foster City, CA) and SCAP (M. Hunter, Leeds University, Leeds, U.K.) software systems. Since in some experiments many channel levels were present, manual analysis was carried out to assess channel open probability. Data are presented as the mean ± SEM. Significance was assessed using Student's *t* test. In time course experiments, unless otherwise stated, data are shown where "control" levels were taken over the first 10 sec immediately postexcision. Thereafter data points, again measured over 10-sec periods, were compared with this value and expressed as a percentage of control values.

RESULTS

Channel Rundown Is Mg^{2+} Dependent. Fig. 2*A* illustrates the dependence of channel rundown on the presence of cytosolic Mg^{2+}. In the presence of 1 mM Mg^{2+} (−ATP), channel activity rapidly declines and rundown is generally complete within 1–2 min. A representative trace is shown in Fig. 2*B*. On average, channel activity declines by ≈50% at 30 sec after excision. In contrast, in the absence of Mg^{2+}, channel activity is maintained and decreases only by 10% at 20 sec and only by 37% after 3 min.

The Effect of Phosphatase Inhibitors on Channel Rundown. Since Mg^{2+} is known to be an essential cofactor for certain phosphatases, this Mg^{2+}-dependent channel rundown could result from the stimulatory effect of Mg^{2+} on these enzymes. We therefore employed a variety of phosphatase inhibitors to test this hypothesis. Orthovanadate is a broad-spectrum phosphatase inhibitor, and when membrane patches were excised in the presence of 3 mM orthovanadate (+ Mg^{2+}), channel activity was partially maintained—decreasing by only 43% 3 min after excision. Importantly, as shown in Fig. 3, immediately after washout, channel activity rapidly declined and disappeared completely within 1 min. Lower

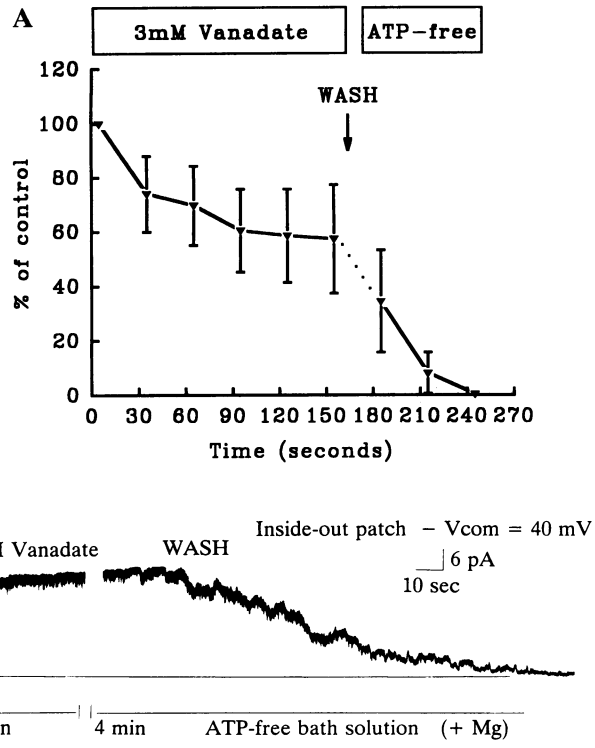

FIG. 3. Effect of 3 mM orthovanadate on channel rundown. (*A*) In the presence of 3 mM orthovanadate, channel activity is partially maintained and then immediately declines to zero following washout (+ Mg^{2+}, −ATP) (n = 9). (*B*) A representative trace is shown. c, Closed level.

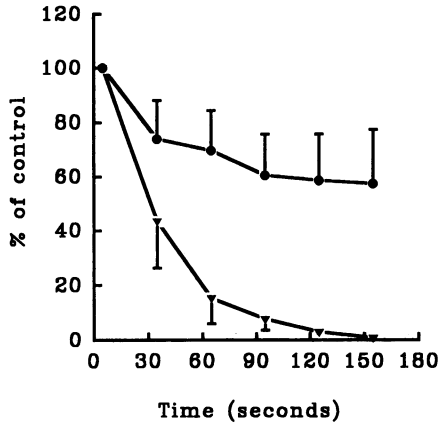

FIG. 4. Effect of 1.0 mM (▼; n = 7) and 3.0 mM (●; n = 9) orthovanadate on K^+ channel activity.

concentrations [0.1 mM (data not shown) and 1 mM (Fig. 4)] of orthovanadate were less effective in inhibiting channel activity. To elucidate which phosphatase is present, more specific phosphatase inhibitors were used. OA and CA are both potent inhibitors of protein phosphatases (PPs) 1 and 2A. Both OA and CA, at 1 μM (+ Mg^{2+}), were clearly less effective than 3 mM orthovanadate at inhibiting rundown; only a modest (9%) level of channel activity remained 2–3 min after excision (Fig. 5). These results strongly suggest that a Mg^{2+}-dependent phosphatase plays an important role in channel rundown, with additive, yet small, contribution from PP1 and/or PP2A.

MgATP Can Inhibit Channel Rundown in a Dose-Dependent Manner. Although Mg^{2+} accelerates the channel rundown process, Mg^{2+} is also required for maintaining channel activity in the presence of ATP. Fig. 6 demonstrates that MgATP slowed channel rundown in a dose-dependent manner. Experiments were performed in which membrane patches were excised into bath solutions containing either 300 μM or 500 μM MgATP (−PKA). Complete inhibition of channel rundown was not observed. In the presence of 0.3 mM MgATP, 20% of the channel activity remained 3 min postexcision; with 0.5 mM MgATP, 46% of the channel activity remained intact. Channel activity remaining 3 min postexcision for 0.3 and 0.5 mM MgATP was, however, not significantly different.

Restoration of Channel Activity Is Dependent on PKA and ATP. The observation that MgATP can maintain ROMK1 channel activity suggests that phosphorylation plays a regulatory role. As PKA is known to stimulate the apical low-

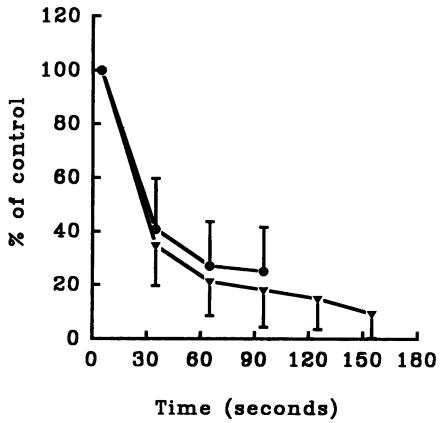

FIG. 5. Effect of 1 μM OA (●; n = 4) and 1 μM CA (▼; n = 7), potent inhibitors of PP-1 and PP-2A, respectively, on channel rundown.

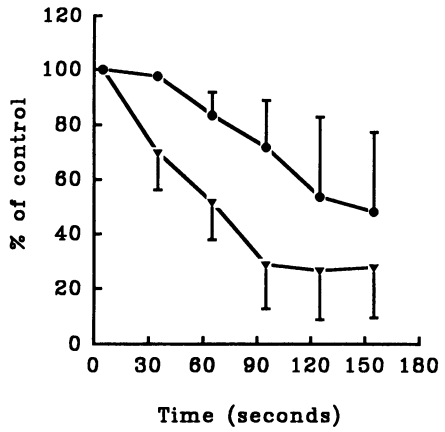

FIG. 6. MgATP in the bath solution slows channel rundown in a dose-dependent manner: 0.5 mM (●; $n = 4$) and 0.3 mM MgATP (▼; $n = 9$) are shown.

conductance K^+ channel in the principal cells of the cortical collecting tubule (CCD), we examined the effect of ATP on channel restoration in the presence of exogenous PKA. After complete rundown, MgATP (0.1–1.0 mM) restored modest levels of channel activity in 37% (22 out of 60) of the patches (Fig. 7). Restoration of channel activity requires the presence of Mg^{2+}, because in the absence of Mg^{2+}, addition of Na_2ATP was ineffective. Addition of the catalytic subunit of PKA (20 units) in the presence of MgATP led to a further increase in channel activity in 49% (25 out of 51) of the patches (Fig. 7).

Additional evidence for the involvement of PKA in the regulation of ROMK1 channel activity is provided by the reversible inhibition of channel activity upon addition of PKI (20 μM) (Fig. 8). In the absence of exogenous PKA, addition of 20 μM PKI to the bath solution led to a maximal decrease in channel activity of 39% (Fig. 9). In the presence of exogenous PKA, PKI was ineffective (Fig. 9).

DISCUSSION

One mechanism put forward to explain channel rundown is protein dephosphorylation (9), not only in rundown of ATP-sensitive K^+ channels but also cardiac Cl^- channels (10) and smooth muscle cell Ca^{2+}-activated K^+ channels (11). Dephosphorylation was suggested because rundown channels could be "refreshed" by the application of MgATP (4, 12).

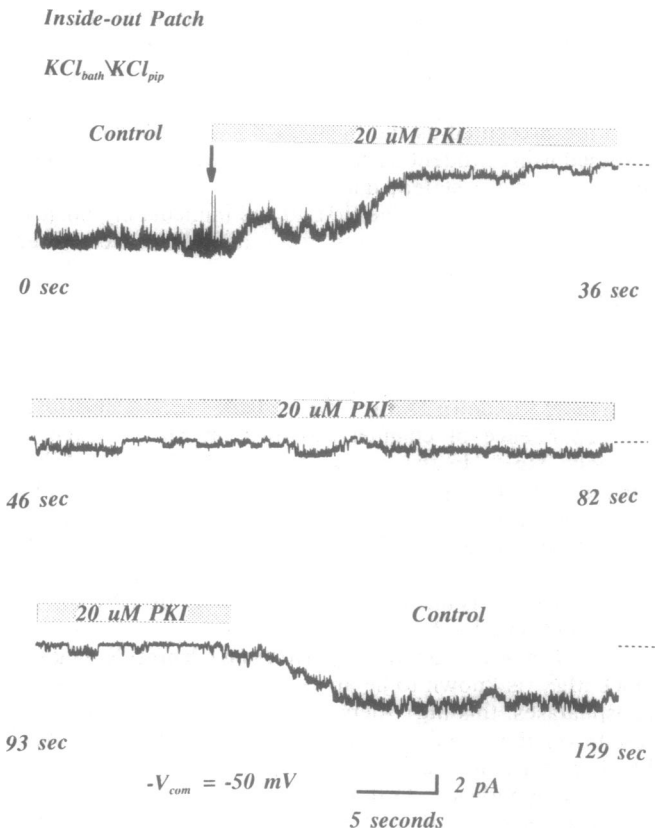

FIG. 8. Effect of PKI. This shows a continuous (time course indicated) channel recording made in an inside-out patch in the presence of MgATP bath solutions. PKI is added at the point indicated by the arrow; the stippled bar indicates exposure time. Note that channel activity was restored immediately after washout of PKI. The dashed line indicates the closed level.

Alternative suggestions for potential mechanisms of rundown are (*i*) loss of some intracellular factor (13); (*ii*) proteolytic degradation as in cardiac Ca^{2+} channels (14); and (*iii*) binding of Mg^{2+} ions to the channel or some modulatory protein, resulting in an allosteric change in channel protein structure and entry into a long-lived closed state (15).

We present evidence for inhibition of channel rundown in the absence of "cytosolic" Mg^{2+}, as demonstrated previously in CRI-G1 insulin-secreting cells (15). ROMK1 channel

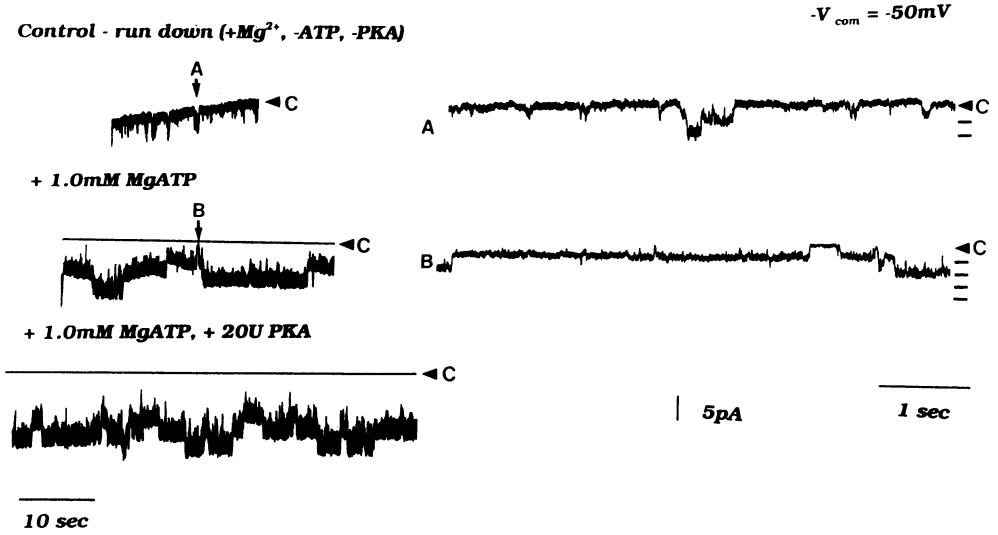

FIG. 7. Channel activity declines to zero (inside-out) in ATP-free solutions (+ Mg^{2+}). ◂ C, closed level, which is restored by addition of 0.5 mM MgATP and 20 units of PKA. Expanded traces are shown on the right side of the figure, corresponding to points A and B.

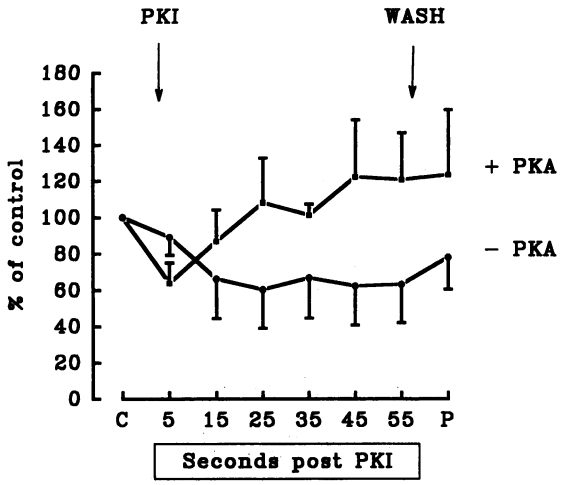

FIG. 9. Time course of the effect of PKI in the presence (+ PKA; $n = 4$) and absence (− PKA; $n = 4$) of exogenous PKA. "C" represents control conditions. Initial control activity was measured over 30 sec immediately prior to the addition of PKI and normalized to 100%. "PKI" data points were compared with this value. PKI washout is indicated by the arrow. The final data point was measured over the first 30 sec after washout (point P).

activity rapidly declines in the presence of cytosolic Mg^{2+}; however, in the presence of MgATP, rundown was partially inhibited. In contrast, others have demonstrated that rundown proceeds in Mg^{2+}-free solutions (4, 16), possibly reflecting cell-specific phosphatase activity.

A variety of phosphatase inhibitors were employed to elucidate whether the Mg^{2+} response reflects dephosphorylation processes. There are two major protein-serine/threonine phosphatase families, types 1 and 2. Orthovanadate is a relatively broad-spectrum phosphatase inhibitor known to act on PP-1, PP-2A, and PP-2C but not PP-2B (17). Interestingly, PP-2C is also dependent on Mg^{2+} (18). We observed that orthovanadate (3 mM) mimics Mg^{2+}-free conditions. Decavanadate has previously been shown to reactivate run-down ATP-sensitive channels from guinea pig ventricular myocytes (19). OA (18) and CA (20) are potent inhibitors of PP-1 and PP-2A. OA (1 μM) and CA (1 μM) were clearly less effective than either orthovanadate or Mg^{2+}-free incubation. These data suggest that the major phosphatase involved, at least in the oocyte expression system, could be PP-2C. It remains to be determined, however, whether the same phosphatase is involved in regulation of the native small conductance K^+ channel of the CCD.

Restoration of ROMK1 K^+ channel activity also involves phosphorylation processes. The predicted ROMK1 channel protein contains several potential phosphorylation sites for cAMP-dependent PKA (6). Addition of MgATP to the bath solution after channel rundown led to partial restoration of channel activity, suggesting membrane-associated PKA (21). Further restoration occurred after addition of exogenous PKA catalytic subunit. However, to date we have only seen partial restoration of channel activity after PKA and MgATP, which may reflect complete inactivation after proteolytic degradation. However, the situation may be more complex inasmuch as we have obtained preliminary data in which the run-down channel activity was only partially restored after application of ATP and PKA. Channel activity then increased upon insertion of the patch pipette into the oocyte ("patch-cramming") and then disappeared immediately after removal, suggesting that complete degradation of the channel protein had not occurred. This may reflect the presence of cytosolic phosphatase inhibitors or involvement of other kinases.

Addition of PKI (in the absence of exogenous PKA; + MgATP) to the bath solution led to a 39% decrease in channel activity. This is reminiscent of the decline in activity of native rat CCD principal cell ATP-sensitive K^+ channel, in which a 32–34% reduction in channel open probability was observed in similar experimental conditions (4). PKI is known to complex with PKA (22), and, in the presence of exogenous PKA catalytic subunit, PKI was ineffective. Additionally, there is a significant difference ($P = 0.008$) between the effects of PKI in the presence or absence of exogenous PKA (all data points were averaged from 30 to 60 sec after PKI addition).

In summary, we have demonstrated that the regulation of ROMK1 channel activity involves phosphorylation processes. Channel rundown is dependent on the presence of cytosolic Mg^{2+} ions and can be partially inhibited by phosphatase inhibitors. Our data suggests that the active phosphatase in the oocyte expression system is predominantly PP-2C. In addition, we have demonstrated that channel activity can be restored by the application of MgATP and the PKA catalytic subunit. Thus, ROMK1 ATP-regulated K^+ channels share common characteristics with the low-conductance K^+ channel found in the CCD (4) and with other members of the ATP-sensitive K^+ channel family.

We thank Mr. M. Lombardi and Dr. M. Boim for preparing ROMK1 cRNA and Dr. M. E. Egan for invaluable discussion. This study was supported by National Institutes of Health Grants DK-17433 (G.G.) and DK-37605 (S.C.H.).

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