Phosphorylation of the ATP-sensitive, Inwardly Rectifying K⁺ Channel, ROMK, by Cyclic AMP-dependent Protein Kinase*

(Received for publication, May 22, 1995, and in revised form, December 20, 1995)

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Activity of the recently cloned ATP-sensitive epithelial K⁺ channel, ROMK (Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M. V., and Hebert, S. C. (1993) Nature 362, 31-38), is regulated by phosphorylation-dephosphorylation processes with cAMP-dependent protein kinase (PKA)-dependent phosphorylation events being required for maintenance of channel activity in excised membrane patches (McNicholas, C. M., Wang, W., Ho, K., Hebert, S. C., and Giebisch, G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8077-8081; Kubokawa, M., McNicholas, C. M., Higgins, M. A., Wang, W., and Giebisch, G. (1995) Am. J. Physiol. 269, F355-F362). To determine whether this channel is a substrate for PKA, ROMK tagged with the hemagglutinin epitope was transiently transfected into HEK293 cells. In vitro labeling of immunoprecipitated proteins from transfected cells showed that ROMK could be phosphorylated by PKA. Metabolic labeling of ROMK resulted in significantly increased phosphorylation upon pretreatment of the cells with forskolin, consistent with an action of cAMP-dependent protein kinase. Phosphoamino acid analyses of the ROMK phosphoproteins revealed that phosphate was attached exclusively to serine residues. Three putative PKA phosphorylation sites containing serine residues in the predicted ROMK proteins are shown directly to be substrates for PKA. Site-directed mutagenesis of each of these sites or double mutation of any two sites showed that ROMK proteins retained the ability to be phosphorylated by PKA both in vivo and in vitro to a variable extent, while triple mutation of all three PKA sites abolished the phosphorylation induced by cAMP agonists in transfected cells. Two-electrode voltage clamp experiments showed that PKA-dependent phosphorylation was required for ROMK channel activity and that at least two of the three sites were required for channel function when expressed in X. laevis oocytes. Taken together, these results provide strong evidence that direct phosphorylation of the channel polypeptide by PKA is involved in channel regulation and PKA-dependent phosphorylation is essential for ROMK channel activity.

ATP-sensitive (K⁺<sub>ATP</sub>) potassium channels have been identified in apical membranes of several renal epithelial cells, where they are in a position to play critical roles in mediating and regulating K⁺ secretion (Misler and Giebisch, 1992). These epithelial secretory K⁺<sub>ATP</sub> channels are characterized by a high open probability, inward rectification, exquisite pH sensitivity, and inhibition by cytosolic ATP (Misler and Giebisch, 1992; Wang et al., 1992). The low conductance (25–35 pS)<sup>1</sup> K⁺<sub>ATP</sub> channel in apical membranes of thick ascending limbs of Henle is critical to NaCl absorption, as it ensures that adequate luminal potassium is provided for efficient function of the Na⁺:K⁺Cl⁻ cotransporter (Hebert and Andreoli, 1984; Wang, 1994b). A similar apical K⁺<sub>ATP</sub> channel has been identified in principal cells in the cortical collecting duct, where it facilitates potassium secretion (Frindt and Palmer, 1987; Wang et al., 1990; Misler and Giebisch, 1992).

An inwardly rectifying, ATP-regulated K⁺ channel, ROMK<sup>1</sup>, was recently cloned from the outer medulla of rat kidney (Ho et al., 1993). ROMK, along with other subsequently identified K⁺ channel genes (Dascal et al., 1993; Kubo et al., 1993a, 1993b; Ashford et al., 1994; Suzuki et al., 1994; Zhou et al., 1994; Takumi et al., 1995) define a new family of inward rectifying K⁺ channels. The inward rectifying K⁺ channel protein contains an H5-like "pore-region" region related to the voltage-gated K⁺ channels and exhibits a characteristic topology featuring only two potential membrane-spanning segments. ROMK<sup>1</sup> channels expressed in Xenopus laevis oocytes display properties similar to those of the low conductance K⁺<sub>ATP</sub> channels identified in renal epithelia (Ho et al., 1993; Nichols et al., 1994; McNicholas et al., 1994; Kubokawa et al., 1995). Recently, splice variants of ROMK<sup>1</sup> denoted ROMK<sup>1</sup>-3 have been identified (Zhou et al., 1994; Boim et al., 1995), which display alternative splicing at the 5' end and give rise to channel proteins differing in their amino-terminal amino acid sequences. These isoforms are differentially expressed along the loop of Henle and distal nephron in the kidney; functional expression in X. laevis oocytes showed that they all form functional Ba<sup>2+</sup>-sensitive K⁺ channel (Boim et al., 1995).

Both the secretory K⁺<sub>ATP</sub> channel in renal epithelia (Wang and Giebisch, 1991a, 1991b) and ROMK channels expressed in X. laevis oocytes are regulated by phosphorylation and dephosphorylation processes, with activation of channel activity by cAMP-dependent protein kinase (PKA) (McNicholas et al., 1994). The predicted ROMK channel protein contains three PKA consensus phosphorylation sites, suggesting that ROMK may be a substrate for PKA and that direct phosphorylation of the channel polypeptide may play a role in channel regulation by this serine-threonine kinase. Phosphorylation of specific amino acid residues on other ion channels is one mechanism of regulating channel properties (for review, see Levitan (1988, 1994)). Thus, in the present report we investigated whether the modulation of ROMK channel activity by PKA is associated with...

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*This work was supported by National Institutes of Health Research Grant DK 37605 (to S. C. H.) and a postdoctoral fellowship from the Medical Research Council of Canada (to Z. C. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: pS, picosiemens(s); PKA, cAMP-dependent protein kinase; HA, hemagglutinin; PCR, polymerase chain reaction; IBMX, 3-isobutyl-1-methylxanthine.
with direct phosphorylation of the ROMK channel polypeptide. A functional HA-tagged ROMK1 channel cDNA construct was transiently expressed in HEK293 cells and expression confirmed biochemically. We observed phosphorylation of ROMK1 protein both in vivo and in vitro, as indicated by PKK-induced \(^{32}\)P phosphate incorporation. We also found that phosphate is attached exclusively to serine residues and the extent of \(^{32}\)P incorporation is increased by preincubation of cells with forskolin. Site-directed mutagenesis, coupled with phosphopeptide mapping, identified three serine phosphorylation sites in ROMK2. Expression of these serine mutants of ROMK2 channels in X. laevis oocytes showed that at least two sites were required for channel function. Mutation of all three PKK phosphorylation sites rendered the channel inactive and abolished the phosphorylation of channel protein induced by cAMP agonists in transfected HEK293 cells.

**MATERIALS AND METHODS**

Construction of HA-tagged ROMK—The HA epitope of influenza virus hemagglutinin (Wilson et al., 1984; Meloche et al., 1992) was introduced into the 3’ end of the ROMK coding region by polymerase chain reaction (PCR) using ROMK1pSPORT and ROMK2pSPORT as templates with primers P-1 (TGACCGTGCTACAGG) and P-HA (TCAGCTAAGCATAATCAGGAACATCATAAGGATACATCTGGTGTCTGC). Amplification was performed on a PTC-100™ programmable controller (M. Research, Inc.) using the cycling parameters as described previously (Boim et al., 1995). The PCR products were digested with Msd and Nhel and subcloned into Msdl and Nhel digested wild-type ROMK to generate the HA-tagged ROMK (ROMK-HA/pSPORT). The Msd-Nhel fragment containing HA was also subcloned into both ROMK1pSVL and ROMK2pSVL for transient transfection.

Site-directed Mutagenesis of ROMK2—Site-directed mutagenesis of ROMK2 pKA Site—Site-directed mutagenesis of ROMK2 pKA sites was performed in ROMK2pSPORT according to method of Kunkel (1985). For single mutations, which modified ROMK2 by mutating individually the three potential PKA phosphorylation sites, three primers were used to change the indicated serine to alanine: S25A, TCTTCCTTCTTTGGCCACCAGCCTTG; S200A, CTGCCAATCAGTAAGGCCTTCCTAAGAT; and S294A, CCTCTGGGACATATGCCGTGCGGACC. Three silent restriction sites modified ROMK2 by mutating individually the three potential PKA phosphorylation sites, three primers were used to change the indicated serine to alanine: S25A, TCTTCCTTCTTTGGCCACCAGCCTTG; S200A, CTGCCAATCAGTAAGGCCTTCCTAAGAT; and S294A, CCTCTGGGACATATGCCGTGCGGACC. Three silent restriction sites (S25A, Msd; S200A, Stul; S294A, NdeI) generated by these primers were used to confirm subcloning. Mutants containing double or triple changes were accomplished by PCR using the relevant single mutant ROMK2 as template and subcloned into the single or double mutant ROMK2. For expression in HEK293 cells, these constructs were transferred into the eukaryotic expression vector ROMK2/pSPORT and tagged with the HA epitope. All mutants were verified by DNA sequencing.

**RESULTS**

**Epitope Tagging of ROMK Protein**—Since our antibodies to the wild-type ROMK channel protein are not immunoprecipitating, we tagged ROMK1 and ROMK2 channels with the influenza virus hemagglutinin epitope, HA, at the carboxyl termini (Fig. 1A). This allowed for immunoprecipitation of ROMK protein with anti-HA monoclonal antibody (Meloche et al., 1992). Fig. 1B shows that in vitro translation of equal amounts of cDNA from the untagged (lane 1) and HA-tagged (lane 2) constructs resulted in equal amounts of HA-tagged ROMK protein.
Heterologous Expression of the ROMK1 Protein in HEK293 Cells—Although the physiological studies shown in Fig. 2 demonstrate that the HA-tagged ROMK functions as a K⁺ channel in X. laevis oocytes, we wished to study the phosphorylation of the HA-tagged ROMK protein in a mammalian cell line. Thus, we also assessed whether the HA-tagged channel protein could be expressed at the plasma membrane of HEK293 cells. First, we were able to detect the 45-kDa ROMK1-HA protein in crude membrane preparations by Western blot (Fig. 3A). Furthermore, we used biotin, which specifically labels plasma membrane proteins, to assess the cell-surface expression of ROMK1-HA. Fig. 3B shows that the 45-kDa ROMK1-HA protein was detected by cell-surface labeling with biotin in transiently transfected, but not untransfected, HEK293 cells. These experiments demonstrate that the ROMK1-HA channel protein was both synthesized and transported to the plasma membrane of these mammalian cells.
Phosphorylation of ROMK by PKA

Phosphorylation of the ROMK1 Protein by PKA—We next determined whether the expressed ROMK1 protein could be phosphorylated either in vitro or in vivo. For in vitro phosphorylation, HA-tagged ROMK proteins expressed in transfected HEK293 cells were immunoprecipitated by anti-HA antibody, and phosphorylated in vitro by PKA in the presence of [γ-32P]ATP. B, phosphoamino acid analysis of the in vitro phosphorylated ROMK1 phosphoprotein by electrophoresis at pH 3.5. Molecular mass is given in kDa. The position of the ROMK1 bands is indicated by arrows. Phosphorylation sites are labeled by P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; FSK, forskolin.

Phosphorylation of ROMK by PKA

Site-directed Mutagenesis of Putative PKA Sites—Three potential PKA phosphorylation sites with serine residues can be identified in ROMK1 or ROMK2 (Ser-44, Ser-219, and Ser-313 or Ser-25, Ser-200, and Ser-294, respectively). We prepared seven ROMK2 mutant constructs, including three single mutants, two double mutants, and one triple mutant form, in which all three serine residues were mutated to alanine residues (see “Materials and Methods”). Mutant forms of ROMK2 in p5V7 vector were HA-tagged in order to immunoprecipitate the mutant ROMK proteins expressed in transfected HEK293 cells. First, we assessed whether the PKA phosphorylation site ROMK2 mutants affect the translation of these cDNAs both in vitro and in HEK293 cells. Fig. 5 shows that in vitro translation of equal amounts of cDNAs (1 μg) from the wild-type and mutant ROMK2 constructs yielded the −42-kDa bands (the apparent molecular mass of the ROMK2) of similar intensity. We also transiently transfected the wild-type or mutant HA-tagged ROMK2 cDNA constructs into HEK293 cells and measured protein expression by cell-surface labeling with biotin. The results shown in Fig. 5 indicate that all four mutants were expressed at the plasma membrane of HEK293 cells at a level indistinguishable from that of wild-type HA-tagged ROMK2 (similar results were observed in the other two single mutants and one double mutant form; data not shown). These experiments demonstrate that the mutation of PKA phosphorylation sites does not produce structural alterations that prevent the synthesis and transport of the HA-tagged mutant ROMK proteins to the plasma membrane of HEK293 cells.

We next examined the ability of each mutant to be phosphorylated following exposure to forskolin and IBMX. Fig. 7A shows that the single mutant ROMK2 channels, R2-S25A and R2-S200A, exhibited −3-fold increases in [32P] incorporation in...
In vitro translation of wild-type and PKA phosphorylation site mutant ROMK2. The wild-type and mutant ROMK2 cDNA constructs were in vitro translated using TNT™-coupled reticulocyte lysate and [35S]methionine. Produced proteins were resolved on 8% SDS-polyacrylamide gel. Control, in vitro translation in the absence of cDNA. Molecular mass is given in kDa. The position of the ROMK2 bands are indicated by arrow.

Expression of PKA phosphorylation site mutant ROMK2 at the plasma membrane of HEK293 cells. The wild-type and mutant ROMK2 cDNA constructs were transiently transfected into HEK293 cells and cell-surface labeled with biotin. Biotinylated cells were immunoprecipitated by anti-HA antibody and analyzed on 10% SDS-polyacrylamide gel. Molecular mass is given in kDa. The position of the ROMK2 bands are indicated by arrow.

Metabolic labeling of wild-type and mutant forms of ROMK2 in transfected HEK293 cells with 32Pi. The wild-type and mutant ROMK2 cDNA constructs were transiently transfected into HEK293 cells, metabolically labeled with 32Pi, and treated with or without forskolin and IBMX. The ROMK2 proteins were immunoprecipitated with anti-HA antibody and protein A-Sepharose and then analyzed on 10% SDS-polyacrylamide gels. Molecular mass is given in kDa. The position of ROMK1 bands are indicated by arrow. FSK, forskolin.

Response to cAMP agonists in vivo (a similar result was observed with the R2-S294A mutant; data not shown). Double mutation of the potential PKA phosphorylation sites (R2/S25A/S200A, R2/S25A/S294A, and R2/S200A/S294A; Fig. 7B) resulted in approximately a 50% decrease in the intensity of phosphorylation with forskolin and IBMX compared with the wild-type ROMK2 protein. The triple mutant (R2-S25A/S200A/S294A), which lacks all putative PKA phosphorylation sites, had only a basal level of phosphorylation and failed to show enhanced phosphorylation intensity after forskolin and IBMX treatment (Fig. 7B, lanes 9 and 10). These results indicate that in the absence of all three putative phosphorylation sites, the HA-tagged ROMK2 protein is not a substrate for PKA. Phosphorylation of the wild-type and mutant HA-tagged ROMK2 proteins are summarized in Table I.

Tryptic Phosphopeptide Mapping of ROMK Labeled in Vitro—In order to verify that all three serine residues are substrates for PKA-mediated phosphorylation, we compared two-dimensional maps of tryptic phosphopeptides from wild-type ROMK2 and from individual mutant and triple mutant forms of ROMK2 (Fig. 8). Serine mutant forms of the HA-tagged ROMK2 cDNA were transfected into HEK293 cells and in vitro labeled using PKA and [γ-32P]ATP. The resultant ROMK2 phosphopeptides were excised from SDS-polyacrylamide gel, digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, and fingerprinted by horizontal electrophoresis and ascending chromatography. By comparison of the wild-type and mutant HA-tagged ROMK2 fingerprints, specific spots on autoradiograms were assigned to each serine residue. As shown in Fig. 8, we were able to identify three phosphopeptides that were clearly associated with the three PKA consensus phosphorylation sites in the HA-tagged ROMK2 protein. These findings suggest that, at least in vitro, all three serine residues can be phosphorylated by PKA.

Functional Expression of the Wild-type and Mutant ROMK2 cRNA in X. laevis Oocytes—We determined the effects of the PKA phosphorylation site mutations on ROMK channel activity in X. laevis oocytes by two-electrode voltage clamp analysis. Oocytes were injected with equal amounts of channel cRNA and examined on the same day after injection. Endogenous cAMP levels in our oocytes are sufficient to give maximal or near-maximal levels of ROMK activity (e.g. forskolin does not enhance ROMK current). Fig. 9 shows that the Ba2+-sensitive K+ current from single-site mutant HA-tagged ROMK2 was ~40% lower than currents observed in oocytes expressing wild-type ROMK2. On the other hand, oocytes injected with cRNA from double- or triple-site mutant ROMK2 showed no detectable Ba2+-sensitive K+ currents. These results indicate that phosphorylation of ROMK2 is absolutely required for channel activity and that at least two of the three serine residues must

\[2\] Z.-C. Xu, Y. Yang, and S. C. Hebert, unpublished studies.
tubules have shown that the low conductance, ATP-sensitive K⁺ channel (KATP) present in apical membranes of rat cortical collecting duct and MTAL cells is activated by PKA (Wang and Giebisch, 1991a; Kubokawa et al., 1995a; Wrana et al., 1994), the recent patch clamp study of ROMK2 expressed in X. laevis oocytes (Boim et al., 1995) and both isoforms require PKA-mediated phosphorylation processes for maintenance of channel activity. Although the HA epitope tag has been used frequently for verifying the expression of membrane proteins (Attisano et al., 1993; Wranas et al., 1994), the caveat with epitope tagging of proteins is that the added epitope may disrupt protein sorting and function. We have shown, however, that the HA-tagged ROMK1 and ROMK2 channels can be functionally expressed in X. laevis oocytes (Fig. 2 (ROMK1) and Fig. 9 (ROMK2)) and that these epitope-tagged constructs are detected by cell-surface biotinylation in HEK293 cells (Fig. 3B (ROMK1) and Fig. 6 (ROMK2)), indicating that the ROMK-HA proteins are functional and are expressed at plasma membranes.

The observations that the immunoprecipitated ROMK1 protein from transiently transfected HEK293 cells could be phosphorylated in vitro by PKA (Fig. 4A) and that forskolin with IBMX increased γ-[32P]ATP incorporation into the ROMK1 (Fig. 4C) and ROMK2 (Fig. 7) proteins isolated from transfected X. laevis oocytes demonstrates that addition of the catalytic subunit of PKA and MgATP was required to restore channel activity following phosphatase-induced channel run-down (McNicholas et al., 1996). Thus, these physiological studies suggest that, similar to the native low conductance K⁺ channel, ROMK channels are regulated by PKA-mediated phosphorylation and dephosphorylation processes, and that PKA-dependent phosphorylation is required for maintaining channel activity. Phosphorylation of K⁺ channels in non-renal cells is also thought to be an important mechanism for modulation of these metabolically regulated channels (Ashcroft, 1988; Misler and Giebisch, 1992). However, it is not known whether ROMK, or indeed any of the other inwardly rectifying K⁺ channels, are substrates for protein kinases. In the present study, we describe the direct phosphorylation of ROMK K⁺ channel protein in transiently expressed HEK293 cell by PKA.

We epitope-tagged ROMK1 and ROMK2 at the carboxyl terminus in order to immunoprecipitate ROMK with anti-HA antibody. It should be noted that although ROMK1 and another isoform, ROMK2, differ at the amino terminus due to alternatively splicing, they both form similar functional K⁺ channels when expressed in X. laevis oocytes (Ashcroft, 1988; Misler and Giebisch, 1992). However, it is not known whether ROMK, or indeed any of the others, is involved in inwardly rectifying K⁺ channels, are substrates for protein kinases. In the present study, we describe the direct phosphorylation of ROMK K⁺ channel protein in transiently expressed HEK293 cell by PKA.

It is well established that a variety of voltage-gated and ligand-gated ion channels are substrates for protein kinases, and phosphorylation of ion channel proteins on serine, threonine, or tyrosine residues is considered a ubiquitous mechanism of regulating ion channel activity (for review, see Catterall and Levitan, 1988). Studies on renal tubules have shown that the low conductance, ATP-sensitive K⁺ channel (KATP) present in apical membranes of rat cortical collecting duct and MTAL cells is activated by PKA (Wang and Giebisch, 1991a; Kubokawa et al., 1995a; Wang, 1994a). A recent patch clamp study of ROMK2 expressed in X. laevis oocytes demonstrated that addition of the catalytic subunit of PKA and MgATP was required to restore channel activity.

FIG. 8. Two-dimensional tryptic phosphopeptide mapping of ROMK2 labeled in vitro. The wild-type or mutant ROMK2 cDNA constructs were transiently transfected into HEK293 cells, immunoprecipitated by anti-HA antibody, and phosphorylated in vitro by the catalytic subunit of PKA in the presence of [γ-32P]ATP. The ROMK phosphoproteins were gel-purified and digested with trypsin. The tryptic peptides were then resolved by electrophoresis and chromatography in two dimensions. The origins are marked with a circle.

be phosphorylated in order for the channel to be active. This is consistent with the previous patch clamp study by McNicholas et al. (1994) showing that PKA-dependent phosphorylation is essential for ROMK channel function. Moreover, the significant difference in magnitude of current between the wild-type and single-site mutant channels suggests that maximal channel activity requires phosphorylation of all three serine residues. Finally and interestingly, these results suggest that none of the sites is more or less important for channel function, i.e. any combination of two sites gives rise to the same level of channel activity. These functional effects of PKA phosphorylation site mutants are summarized in Table I.

DISCUSSION

It is well established that a variety of voltage-gated and ligand-gated ion channels are substrates for protein kinases, and phosphorylation of ion channel proteins on serine, threonine, or tyrosine residues is considered a ubiquitous mechanism of regulating ion channel activity (for review, see Catterall and Levitan, 1988). Studies on renal tubules have shown that the low conductance, ATP-sensitive K⁺ channel (KATP) present in apical membranes of rat cortical collecting duct and MTAL cells is activated by PKA (Wang and Giebisch, 1991a; Kubokawa et al., 1995a; Wang, 1994a). A recent patch clamp study of ROMK2 expressed in X. laevis oocytes demonstrated that addition of the catalytic subunit of PKA and MgATP was required to restore channel activity.

FIG. 9. Ba²⁺-sensitive K⁺ currents in X. laevis oocytes injected with the wild-type or mutant HA-tagged ROMK2 cRNA. X. laevis oocytes were injected with the wild-type and mutant ROMK2 cRNA and Ba²⁺-sensitive K⁺ currents (I_Ba) were recorded under two-electrode voltage clamp in solution of 5 mM K⁺. Data are expressed as mean ± S.E. K⁺ currents (µA) are as follows: wild-type, 4.29 ± 0.58 (n = 7); S25A, 2.61 ± 0.70 (n = 7, p < 0.02); S200A, 2.30 ± 0.42 (n = 7, p < 0.05); S294A, 2.85 ± 0.39 (n = 7, p < 0.05). p values were calculated by comparing wild-type with each mutant form.

McNicholas, C. M., Yang, Y., Giebisch, G., and Hebert, S. C. (1996) Am. J. Physiol., in press.
HEK293 cells in vivo demonstrate that ROMK channels are substrates for PKA-mediated phosphorylation and are consistent with the electrophysiological studies indicating that ROMK channels are regulated by PKA-dependent processes (McNicholas et al., 1994). Phosphoamino acid analyses of both in vitro and in vivo phosphorylated ROMK1 proteins demonstrated that phosphate is incorporated only at serine residues Ser-44, Ser-219, and Ser-313 by PKA (Fig. 4, B and D).

It is quite common that mutant forms of proteins fail to be transported to the correct cellular location (Cheng et al., 1990; Welsh and Smith, 1993). Thus, we tested for plasma membrane expression of ROMK2 in transiently transfected HEK293 cells by cell-surface biotinylation. The results in Fig. 6 clearly show that the serine-to-alanine mutations did not prevent the ROMK2 mutant proteins from sorting to the plasma membrane of HEK293 cells. Several lines of evidence from in vitro or in vivo phosphorylation strongly suggest that all three PKA sites are directly involved in phosphorylation of ROMK. First, the in vitro phosphorylated wild-type ROMK proteins examined by two-dimensional TLC analysis show three phosphopeptides, which represent three PKA sites that can be abolished by site-specific mutagenesis (Fig. 8A). Second, studies on the triple mutant ROMK2 clearly demonstrate that there is a direct correlation between the ROMK channel activity and no other sites are indicated phosphorylation residues mutated.

From analysis of these results, which are summarized in Table I, two major conclusions can be reached. First, like the CFTR Cl− channel and the insulin receptor (Cheng et al., 1991; Zhang et al., 1991), phosphorylation of ROMK by PKA is degenerate, meaning that no one individual site is essential, and yet more than one site is required for maintaining channel activity. Second, studies on the triple mutant ROMK2 clearly indicate that there is a direct correlation between the ROMK phosphorylation and channel activity, and no other sites are detectable upon phosphorylation of ROMK by PKA. At present, we have not yet examined the ROMK single-channel properties using patch clamp techniques. These latter studies may reveal other aspects of channel function, which are modulated by specific phosphorylated residues (e.g. the characteristics of MgATP- or pH-mediated channel inhibition).

In summary, the present study demonstrates that ROMK is a phosphoprotein, that the channel can be phosphorylated by PKA, and that three PKA sites containing serine residues are essential for ROMK channel activity. Given the critical importance of this channel for renal K+ secretion and recycling, these findings provide important insights into the functional regulation of ROMK and possibly other KATP channels.

Acknowledgments—We thank Drs. Jonathan Lytton, Gerhard Giebisch, Carmel McNicholas, Wenhui Wang, and David Mount for valuable discussion during the course of this work. We also thank Dr. Seng H. Cheng (Genzyme) for kindly providing the TCC electrophoresis apparatus and Dr. Mike Bienkowski (Upjohn) for supplying the ROMK apSVL construct.

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J. Biol. Chem. 1996, 271:9313-9319.
doi: 10.1074/jbc.271.16.9313

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