Phosphorylation by Protein Kinase A of RCK1 K^+ Channels Expressed in *Xenopus* Oocytes†

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ABSTRACT: Phosphorylation-mediated regulation of voltage-gated K^+ channels has been implicated in numerous electrophysiological studies; however, complementary biochemical studies have so far been hampered by the failure to isolate and characterize any K^+ channel proteins of distinct molecular identity. We used the *Xenopus* oocyte expression system to study the biosynthesis and phosphorylation by protein kinase A (PKA) of rat brain RCK1 (Kv1.1) K^+ channel protein. RCK1 protein was isolated by immunoprecipitation from oocytes injected with RCK1 cRNA and analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The channel protein was expressed in the form of several polypeptides. The 57-kDa polypeptide, usually the major constituent, resided both in the cytosol and in the plasma membrane. Its levels were correlated with RCK1 current amplitudes (I_{RCK1}) and upon incubation of the cRNA-injected oocytes with tunicamycin, its molecular weight was decreased and at the same time I_{RCK1} was reduced. These results suggest that the membranal 57-kDa polypeptides represent functional channels that are N-glycosylated. Furthermore, a study of the phosphorylation of the RCK1 polypeptides revealed that the 57-kDa polypeptide was specifically targeted for phosphorylation by PKA. It could be phosphorylated *in vitro* by the catalytic subunit of PKA (PKA-CS). In its native state in intact oocytes, the 57-kDa polypeptide was partially phosphorylated and could be further phosphorylated *in vivo* by addition of a membrane-permeant cAMP analog. Site-directed mutagenesis demonstrated that phosphorylation of a single site on the C-terminus of the channel molecule fully accounts for these phosphorylations. This work thus characterizes biochemically what appears to be a functional K^+ channel polypeptide, demonstrates its phosphorylation by PKA *in vitro* and in intact cells, and localizes the phosphorylation site on the molecule.

The activity of voltage-gated K^+ channels is important in neuronal excitability and neurotransmission (Hille, 1992). The members that constitute this diverse group are encoded by distinct but related multigene subfamilies (Jan & Jan, 1990, Stuhmer, 1991) and differ in their conductance, gating mechanisms, pharmacological properties, and kinetics (Rudy, 1988). Integration of the techniques of electrophysiology and molecular biology and the use of expression systems such as *Xenopus* oocytes have provided considerable insight into K^+ channel structure–function relationships [for reviews, see Jan and Jan (1990), Stuhmer (1991), and Pongs (1992)]. However, the biosynthesis of these channels has not been extensively studied either in native tissues or in expression systems. In contrast to Na^+ and Ca^{2+} channels, the isolation and biochemical characterization of K^+ channels have been hampered both by their diversity and by their low density in the membrane. Also, there are no specific high affinity ligands or toxins that interact only with a unique type of channel [for a review, see Rehm (1991)]. A number of high-affinity peptide toxins have been used to characterize and partially purify toxin-binding components from numerous tissues (Moczydlowski et al., 1988; Castle et al., 1989). The only neuronal voltage-dependent K^+ channels that have been purified in this way are the brain DMB proteins (Black et al., 1988; Rehm & Lazdunski, 1988), which appear to be multimeric glycoproteins, with a broad α band of mass 75–90 kDa and two sharp bands of mass 42 and 38 kDa on sodium dodecyl sulfate (SDS)[1] gels. However, these purified proteins do not represent a unique K^+ channel type but rather a mixture of related but distinct K^+ channel molecules, probably belonging to the Shaker subfamily (Rehm, 1991). When reconstituted in lipid bilayers, these proteins produce functional K^+ channels, whose probability of opening is increased by protein kinase A (PKA) phosphorylation (Rehm et al., 1989).

Modulation by phosphorylation of the activity of different K^+ channels has been implicated in numerous electrophysiological studies carried out in different cell types, including neurons, muscle, and cardiac cells (Kaczmarek & Levitan, 1987; Levitan, 1988; Walsh & Kass, 1988, 1991; Benz et al., 1991; Bastin et al., 1990) and also *Xenopus* oocytes (Lotan et al., 1990; Moran et al., 1991; Busch et al., 1992; Blumenthal & Kaczmarek, 1992). The oocyte system has enhanced these studies because it allows expression of a single molecular species of an ion channel, which can then be studied by electrophysiological and molecular biology techniques. However, oocytes

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[1] Abbreviations: PKA, protein kinase A (cAMP-dependent protein kinase); PKA-CS, catalytic subunit of protein kinase A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; I_{RCK1}, current via RCK1 channels; Ab-1, antisera against RCK1; p-1, synthetic peptide against which Ab-1 was generated; Sp-cAMPS, adenosine cyclic 3′,5′-monophosphorothioate; PAS, protein A–sepharose; PMSF, phenylmethanesulfonyl fluoride; PP2A, protein phosphatase 2A; PP2B, calcineurin.

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have not yet been used to monitor the direct phosphorylation of expressed ion channel proteins. In this work, we used biochemical techniques to study phosphorylation, in *Xenopus* oocytes, of rat brain RCK1 (Kv1.1; Baumann et al., 1988), a voltage-gated K^+ channel of the Shaker subfamily which, when expressed in oocytes, functions as a delayed rectifier type (Stuhmer et al., 1989). The RCK1 protein is part of the rat brain K^+ channel population; its RNA is localized in several brain areas (Drewe et al., 1992). Using SDS–polyacrylamide gel electrophoresis (SDS–PAGE), we resolved the channel polypeptides, determined their cellular localization, and demonstrated their phosphorylation *in vitro* and *in vivo* by PKA. The biochemical study was complemented by a molecular biology study in which site-directed mutagenesis was used to identify the amino acid sequence forming the target site of the phosphorylation.

MATERIALS AND METHODS

Materials. Chemicals were from Sigma (Rishon Le Zion, Israel) unless stated otherwise. Vanadate (sodium orthovanadate) and okadaic acid were from Alomone Labs (Jerusalem). Adenosine cyclic 3′,5′-monophosphorothioate (Sp-cAMPS, sodium salt) was from Biolog (Bremen, FRG). [^{32}P]Orthophosphate and [γ-^{32}P]ATP were from Amersham. Tunicamycin was from Boehringer Manheim. Calcineurin (PP2B) and protein phosphatase 2A (PP2A) were from Upstate Biotechnology, Inc. (New York, NY).

Generation of the Antiserum. A 23 amino acid peptide that corresponds to the N-terminus of RCK1 (SGENADEASAAPGHPQDGSYPRQ) was synthesized and purified, and its amino acid composition was verified by standard methods in the Mount Sinai Medical School Protein Core Facility. This peptide (p-1) was coupled to KLH through a cysteine residue that was added to the C-terminus of the peptide. The antiserum generated against p-1 (Ab-1) was raised in rabbits by standard methods (Harlow & Lane, 1988).

Construction of cRNA of Wild-Type and Mutant RCK1 Channels. For electrophysiological studies, the RCK1 cRNA was generated from recombinant Bluescript plasmids, containing the cDNA insert, by linearization with *Not*I, followed by *in vitro* transcription with T3 RNA polymerase, as described (Moran et al., 1991). For biochemical studies, the RCK1 cDNA was subcloned into a vector, constructed by Dr. S. A. N. Goldstein (Brandeis University), which is a modified version of pGEM-9zf(−) with a 180 bp sequence at the 5′UTR and a 3′-polyadenylation sequence that confers high levels of expression (Swanson et al., 1990). An *Nco*I site was inserted by site-directed mutagenesis into the RCK1 cDNA at its initial ATG codon, and the fragment *Nco*I–*Hin*dIII (partial digest, spanning the whole coding region) was inserted into the vector, which had been digested with the same enzymes. This yielded the SupEx-RCK1 construct, from which cRNA was synthesized by *Not*I linearization and T7 RNA polymerase. This cRNA induced a higher level of expression of the RCK1 polypeptides in oocytes and correspondingly larger current amplitudes. In several experiments, the RCK1 cRNA was prepared from the construct RCK1-pSP64T (Liman et al., 1992), which yielded even higher levels of RCK1 polypeptides and current amplitudes; we refer to this construct as SSupEx-RCK1.

The substitution mutant of RCK1, R443C;S446A, was generated by the oligonucleotide-directed *in vitro* mutagenesis method using the Amersham mutagenesis kit. Oligonucleotide encoding the desired mutations (5′-GTGACCTCAGCTGCCGCAGCGCCTCTACTATCAGC-3′; underlined nucleotides encode the mutated residues) was annealed to the single-stranded template of SupEx-RCK1 and served as primer for the synthesis of the mutant channel sequence. The nucleotide sequence of the mutant was confirmed by DNA sequencing.

Oocytes and Electrophysiological Recordings. Frogs were maintained and dissected, and oocytes were prepared as described (Dascal & Lotan, 1992). For electrophysiological studies, oocytes were injected with 2–10 or 0.2–0.4 ng of cRNA prepared from the Bluescript construct or from SupEx-RCK1, respectively. For biochemical studies, the cRNA concentrations were up to 100-fold higher. Injected oocytes were incubated at 22 °C for 1–4 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.5) supplemented with 1.8 mM $CaCl_2$, 2.5 mM sodium pyruvate, 100 μg/mL streptomycin, and 100 units/mL penicillin (NDE solution; Dascal & Lotan, 1992) and then assayed electrophysiologically as follows: an oocyte was placed in a 1 mL bath constantly perfused at a slow rate with ND96 solution supplemented with 1 mM $CaCl_2$. Currents were recorded by the two-electrode voltage clamp technique as described (Lotan et al., 1988). To record large currents (>3 μA), we used a series resistance compensation circuit and electrodes (0.2–0.5 MΩ) that were regular glass electrodes with the tips slightly broken, filled with a KCl/agar cushion at the tip to prevent leakage of KCl (Schreibmayer et al., 1993). Currents were recorded by stepping the membrane potential from −80 to 0 mV. Net currents were estimated by arithmetical subtraction of the leak current calculated from the current elicited by a voltage step to −60 mV, where the K^+ current was not yet evoked. Digital subtraction of the capacity current was not performed.

Plasma Membrane Preparation and Electron Microscopy. Plasma membranes were separated mechanically, essentially as described (Sadler & Maller, 1981). Defolliculated oocytes were incubated for 10 min in ice-cold hypotonic solution [5 mM NaCl, 5 mM Hepes, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM pepstatin, and 1 mM 1,10-phenanthroline], and plasma membranes together with the vitelline membranes (extracellular collagen-like matrix; Dumont, 1972) were removed manually by use of watchmaker forceps. The plasma membrane together with the vitelline membrane appeared as a transparent sheet which appeared either whitish or brownish; the brownish color usually turned whitish upon further trituration with a Pasteur pipet. The remainder of the cell, consisting of cytoplasm and intracellular organelles (here collectively termed cytosol), was usually left as an almost intact sphere. The two fractions were collected separately by a Pasteur pipet and pelleted by centrifugation for 10 min in a microfuge. The transparent sheets were examined morphologically by electron microscopy (see below). The whitish sheet was verified as plasma membrane attached to vitelline membrane; it was devoid of other cellular components, whereas the brownish sheet also contained cortical granules [data not shown; see also Wall and Patel, (1989)]. For electron microscopy, transparent sheets from 30 oocytes were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixated in 1% osmium tetroxide in the same buffer. After dehydration, the preparation was embedded in Epon Epoxy resin. Sections were obtained with an LKB Ultratom III, stained with uranyl acetate and lead citrate, and examined in a JEM-100B electron microscope.

Metabolic Labeling with [^{35}S]Methionine and Immunoprecipitation. This was done essentially as described (Thornhill, 1987). Oocytes were injected with RCK1 cRNA and

incubated for 4 h in NDE solution, and then in NDE containing 2 mCi/mL [^{35}S]methionine for 2–3 days at 22 °C. Ten oocytes, 30 plasma membranes, or 15 cytosolic fractions were homogenized in 100 μL of solubilization buffer [4% SDS, 10 mM EDTA, 50 mM Tris (pH 7.5), 1 mM PMSF, 1 μM pepstatin, and 1 mM 1,10-phenanthroline] and heated to 100 °C for 2 min. Following the addition of 100 μL of H_2O and 800 μL of immunoprecipitation buffer [190 mM NaCl, 6 mM EDTA, 50 mM Tris (pH 7.5), and 2.5% Triton X-100], homogenates were centrifuged for 10 min at 1000*g* and 4 °C, to remove the insoluble yolk and pigment particles. The supernatant was precleaned by the addition of protein A–sepharose (PAS), followed by shaking for 3 h at 4 °C and pelleting. The supernatant was then incubated for 16 h with Ab-1, in the presence or absence of 20 μM p-1 peptide. The antibody–antigen complex was incubated for 3 h at 4 °C with PAS and then pelleted by centrifugation for 1 min at 8000*g*. Immunoprecipitates were washed 4 times with immunowash buffer [150 mM NaCl, 6 mM EDTA, 50 mM Tris (pH 7.5), 0.1% Triton X-100, and 0.02% SDS]; the final wash contained no Triton. Samples were boiled in SDS–gel loading buffer and electrophoresed on 8% polyacrylamide–SDS gel together with standard molecular mass markers (205–45 kDa).

Tunicamycin Treatment. Oocytes were preincubated for 24 h in NDE medium containing 2 μg/mL tunicamycin, and then RCK1 cRNA was injected with 0.2 ng of tunicamycin dissolved in water. Tunicamycin was present in the medium for the entire period of oocyte incubation.

In Vitro Phosphorylation. For phosphorylation, 15–30 oocytes, 15 cytosolic fractions or 30 plasma membranes were homogenized in 150–300 μL of medium composed of 20 mM Tris (pH 7.4), 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 50 μg/mL PMSF, 1 mM iodoacetamide, 1 μM pepstatin, and 1 mM 1,10–phenanthroline. Debris was removed by centrifugation at 1000*g* for 10 min at 4 °C. After addition of Triton X-100 to a final concentration of 4%, followed by centrifugation for 15 min at 4 °C, the supernatant was incubated for 16 h at 4 °C with Ab-1. The antigen–antibody complex was precipitated by mixing with PAS at 4 °C for 45 min and sedimenting at 8000*g* for 1 min. The pellet was washed twice with phosphorylation buffer (25 mM Hepes, 5 mM $MgCl_2$, 5 mM EGTA, and 0.2% Triton X-100, pH 7.4). Phosphorylation was performed by incubation of the pellet with 1 μg of the catalytic subunit of PKA (PKA-CS; reconstituted in 5 mM dithiothreitol) and 10 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol), in phosphorylation buffer for 5 min at 30 °C. The reaction was stopped by washing twice with 1 mL of RIA buffer [50 mM sodium phosphate buffer (pH 7.4), 50 mM KF, 75 mM NaCl, 2.5 mM EDTA, 0.01% NaN_3, and 25 mM Tris (pH 7.4)]. In some experiments, an additional washing [0.08% SDS, 1 mM EDTA, and 50 mM Tris (pH 6.8)] was performed; this resulted in a reduction of background and 57-kDa polypeptide signals. Samples were boiled in SDS–gel loading buffer and analyzed on 8% polyacrylamide gels. To determine the specificity of binding, Ab-1 was incubated with 20 μM p-1 prior to incubation with the oocyte homogenate.

Dephosphorylation. Dephosphorylation was performed essentially as described (Rossie & Catterall, 1987). The immunoprecipitate was washed with a solution containing 40 mM Hepes/Tris (pH 7.6), 40 mM NaCl, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$, 0.1% Triton X-100, and 1 mg/mL BSA. PP2B (2.4 μg), calmodulin (1 μM), and PP2A (0.1 unit), dissolved in total volume of 50 μL of the wash solution, were added, followed by incubation at 36 °C for 10 min. Dephosphorylation was stopped by dilution with wash solution at 0 °C, and the immunoprecipitate was washed twice in this solution.

In Vivo Phosphorylation. Thirty oocytes were incubated for 4.5 h in NDE solution containing 0.5 mCi of [^{32}P]-orthophosphate. During the last 30 min, 0.5 mM Sp-cAMPS, an analog of cAMP which is membrane-permeant and relatively resistant to phosphodiesterases, was added to the test group. To preserve the state of phosphorylation of the channels, separation of the plasma membrane and all subsequent steps were performed in the presence of the following protein phosphatase inhibitors: okadaic acid, 50 nM; vanadate, 0.5 mM; and KF, 50 mM. The homogenization medium contained 100 mM sodium phosphate buffer (pH 5.8), 10 mM EDTA, 5 mM β-glycerophosphate, 5 mg/mL BSA, 50 μg/mL PMSF, 1 mM iodoacetamide, 1 μM pepstatin, 1 mM 1,10-phenanthroline, and protein phosphatase inhibitors as above. Immunoprecipitation was as in the *in vitro* experiments.

Quantification of Labeling Intensities. Relative intensities of the labeled bands were estimated quantitatively by use of the software ImageQuant on PhosphorImager (Molecular Dynamics).

Statistical Analysis. Data are presented as means ± SEM. The two-tailed *t*-test was used to calculate the statistical significance of differences between two populations.

RESULTS

Biosynthesis of RCK1 Polypeptides. Antiserum (Ab-1) was generated against a 23 amino acid peptide (p-1) that corresponds to the N-terminus of the RCK1 K^+ channel. Ab-1 was characterized initially by its ability to immunoprecipitate the RCK1 core polypeptide synthesized in a cell-free translation system programmed with RCK1 cRNA. Ab-1 specifically immunoprecipitated the core polypeptide since in the presence of the p-1 peptide the RCK1 core polypeptide was not precipitated (data not shown).

Oocytes were injected with RCK1 cRNA and metabolically labeled by incubation for different time periods in medium enriched with [^{35}S]methionine. The channels were isolated by detergent solubilization of the oocyte proteins, followed by their immunoprecipitation with Ab-1 and analysis by SDS–PAGE and autoradiography. As illustrated in Figure 1A, after cRNA injection and incubation for 2 days with [^{35}S]-methionine, Ab-1 precipitated (from whole oocyte homogenates) radiolabeled products that migrated as ~57- and ~54-kDa polypeptides, and occasionally also a weakly labeled ~50-kDa polypeptide (Figure 1A, lane 5). After additional days of incubation, the 57-kDa band thickened, and a band corresponding to polypeptides of larger molecular mass, weakly labeled and very diffuse, appeared (Figure 1A, lane 2). The center of this band varied (depending on oocyte batch and duration of incubation) between 70 and 80 kDa, and we will refer to it as 80 kDa. These polypeptides, probably representing various stages of channel biosynthesis and glycosylation (see below), were not observed in native (control) oocytes, that were not injected with cRNA, or in oocytes that were injected with cRNA but immunoprecipitated in the presence of 20 μM p-1 peptide which competed with RCK1 polypeptides for binding to Ab-1 (Figure 1A).

Prior to biochemical processing, voltage-dependent K^+ currents via the RCK1 channels (I_{RCK1}) were tested by the two-electrode voltage clamp technique. The currents characteristics were of a delayed rectifier type, as described by Stuhmer et al. (1989) (Figure 1C shows traces of currents

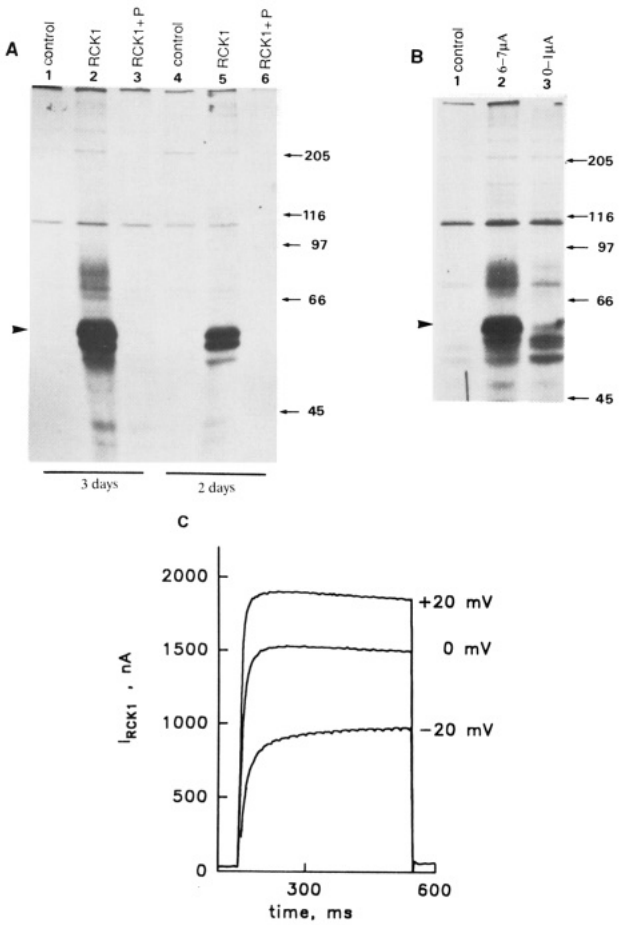

FIGURE 1: SDS–PAGE analysis of immunoprecipitated RCK1 polypeptides expressed in cRNA-injected oocytes metabolically labeled with [^{35}S]methionine. (A) Correlation of RCK1 polypeptide expression with length of incubation period. Lanes 2 and 5, cRNA-injected oocytes that were labeled with [^{35}S]methionine for 48 and 24 h, respectively, starting 16 h after injection. Lanes 1 and 4, native (control) oocytes labeled as in lanes 2 and 5. Lanes 3 and 6 are the same as lanes 2 and 5, respectively, but immunoprecipitation was carried out in the presence of the synthetic peptide p-1. All oocytes were from the same donor and were injected on the same day. (B) Correlation of RCK1 polypeptide expression with current (I_{RCK1}) amplitudes in the same oocytes. Lanes 2 and 3, cRNA-injected oocytes that expressed currents of 6–7 and 0–1 μA, respectively, when the membrane potential was stepped to 0 mV; lane 1, native (control) oocytes. Arrowheads denote the 57-kDa polypeptide. In each lane, the immunoprecipitate of 10 oocytes was loaded. (C) RCK1 currents (I_{RCK1}) evoked by depolarizing voltage steps from a holding potential of −80 mV to the indicated values in normal ND96 solution (2 mM [K^+]). Currents were recorded by the two-electrode voltage clamp technique.

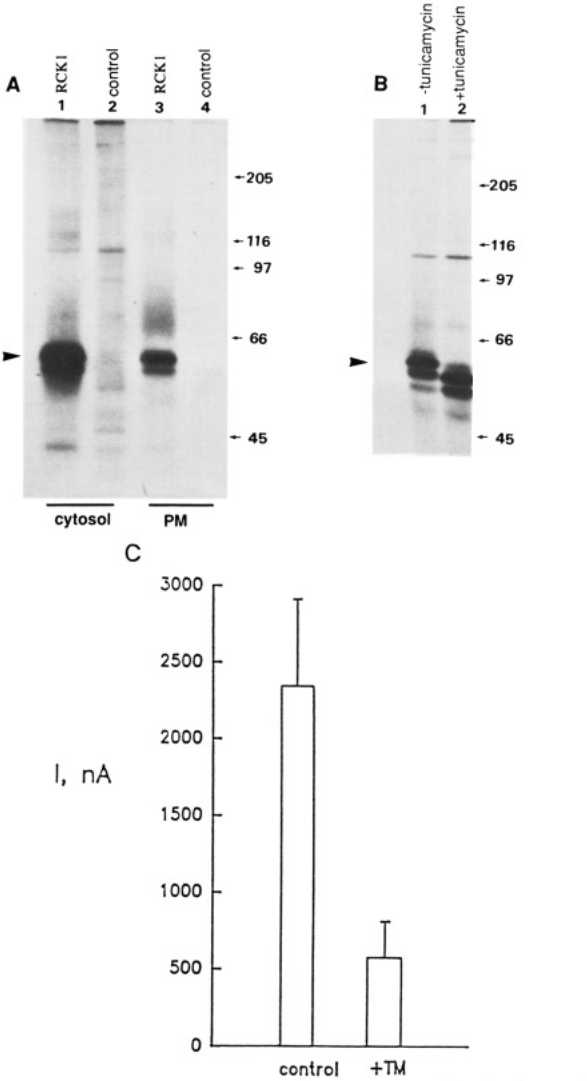

FIGURE 2: SDS–PAGE analysis of subcellular distribution and N-glycosylation of immunoprecipitated RCK1 polypeptides expressed in [^{35}S]methionine-labeled oocytes. (A) RCK1 polypeptides reside both in the plasma membrane (PM) and in the cytosol. Lanes 1 and 3, 15 cytosolic fractions and 30 plasma membranes of cRNA-injected oocytes, respectively. Lanes 2 and 4, 15 cytosols and 30 plasma membranes of native (control) oocytes, respectively. (B) Some RCK1 polypeptides are N-glycosylated. Lanes 1 and 2, cRNA-injected oocytes incubated in the absence and in the presence of tunicamycin, respectively (15 oocytes per treatment). Arrowheads denote the 57-kDa polypeptide. (C) I_{RCK1} amplitudes are reduced in the presence of tunicamycin. Assayed oocytes were incubated with (16 oocytes) or without (9 oocytes) tunicamycin (TM); they exhibited current amplitudes of up to 3 μA. Results are means ± SEM; $P < 0.002$.

evoked by stepping the membrane potential to different values). In three experiments, one of which is shown in Figure 1B, oocytes with low and high levels of macroscopic current expression were divided into separate groups; higher levels of expression of functional channels (larger macroscopic currents) were correlated with stronger labeling of the 57- and 80-kDa polypeptides.

To determine which of the RCK1 polypeptides reside in the oocyte's plasma membrane, experiments were carried out in which the plasma membrane was separated manually from the rest of the cell. The plasma membrane (together with the vitelline membrane, a fibrous noncellular membrane) appeared as a transparent sheet. The rest of the cell containing cytoplasm, nucleus, endoplasmic reticulum, Golgi apparatus, etc. (collectively termed the cytosol) usually appeared as an almost intact sphere. SDS-PAGE analysis of the two fractions revealed that the polypeptides of 54, 57, and 80 kDa were present in both plasma membrane and cytosol (Figure 2A).

Glycosylation of RCK1 Polypeptides. In three experiments with different oocyte donors, treatment with tunicamycin (an inhibitor of N-linked glycosylation) led to the almost complete disappearance of the 80- and 57-kDa bands and an apparent thickening of the 54- and 50-kDa bands (Figure 2B), indicating that the 80- and 57-kDa polypeptides were N-glycosylated. Accordingly, in oocytes treated with tunicamycin and later subjected to phosphorylation by PKA-CS (see next paragraph), the 57-kDa band disappeared, and a band was seen at about 54 kDa (Figure 3B). In these experiments, electrophysiological assays revealed that tunicamycin treatment resulted in a 35% reduction in I_{RCK1} amplitudes, from 13.2 ± 0.6 μA ($n = 20$) in control oocytes to 8.5 ± 0.6 μA ($n = 26$) in tunicamycin-treated oocytes ($P < 0.001$). However, measurement of I_{RCK1}

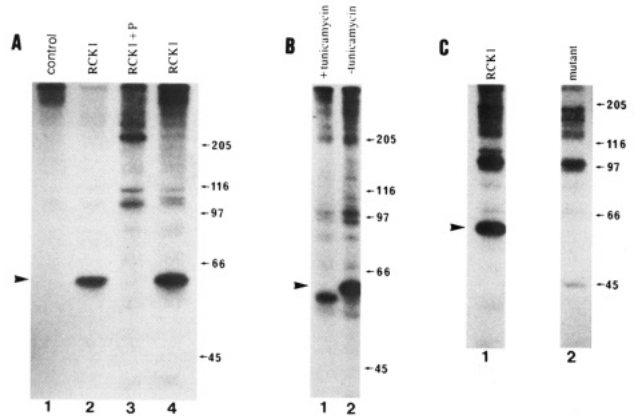

FIGURE 3: RCK1 57-kDa polypeptide is phosphorylated *in vitro* by PKA-CS in the presence of [γ-^{32}P]ATP; the double mutation R443C;S446A abolishes this phosphorylation. (A) PKA-CS-catalyzed phosphorylation of RCK1 polypeptides immunoprecipitated from whole oocytes. Lane 1, native (control) oocytes. Lanes 2 and 4, RCK1-expressing oocytes 3 and 6 days after cRNA injection, respectively. Lane 3, same as lane 2, but immunoprecipitation was carried out in the presence of the p-1 peptide (P). All oocytes were from the same donor and were injected on the same day. (B) Effect of tunicamycin treatment on PKA-catalyzed phosphorylation in whole oocytes. Lanes 1 and 2, RNA-injected oocytes, incubated in the presence and in the absence of tunicamycin, respectively. (C) Lane 1, RCK1-expressing oocytes. Lane 2, oocytes expressing the mutant R443;S446A . On each lane, immunoprecipitates from 15 whole oocytes were loaded. Arrowheads denote the 57-kDa polypeptide.

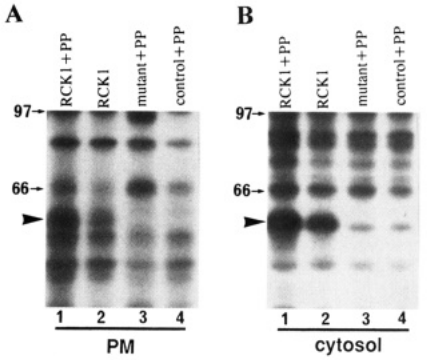

FIGURE 4: PKA-CS-catalyzed phosphorylation of the RCK1 57-kDa polypeptide, in the presence of [γ-^{32}P]ATP, is increased after prior dephosphorylation by PP2B and PP2A (PP); the double mutation R443C;S446A abolishes this phosphorylation. RCK1 polypeptides were immunoprecipitated from plasma membrane (A) and cytosolic (B) fractions of oocytes from the same donor. (A, B) Lanes 1 and 2, RCK1-expressing oocytes with and without prior dephosphorylation, respectively. Lanes 3 and 4, oocytes expressing mutant R443C;S446A and native oocytes (control) subjected to prior dephosphorylation, respectively. Arrowheads denote the 57-kDa polypeptide; the arrows denote migration of molecular mass markers. (A) and (B) are two parts of the same gel, but the autoradiogram shown in (A) was exposed for about twice as long as the autoradiogram shown in (B). The intensity of labeling of the 57-kDa band without prior dephosphorylation was 40% (plasma membrane; PM) and 46% (cytosol) of the intensity observed with dephosphorylation.

amplitudes higher than 3 μA suffers from problems of series resistance, leading to inaccuracies, mainly underestimation of the current amplitudes (Schreibmayer et al., 1993) and thus possible underestimation of the tunicamycin effect. To avoid these errors, we conducted a separate electrophysiological experiment on oocytes injected with cRNA at concentrations about 25–50 times lower (see Materials and Methods), which resulted in relatively low levels of expression of RCK1 channels ($I_{RCK1} < 3\ \mu A$). Treatment of these oocytes with tunicamycin caused a 76% reduction of the current amplitude (Figure 2C).

In Vitro Phosphorylation of RCK1 Polypeptides by PKA-CS. In seven experiments, incubation of channels immunoprecipitated with Ab-1 from whole oocytes in the presence of [γ-^{32}P]ATP and PKA-CS, followed by SDS–PAGE analysis, resulted in all cases in a phosphorylated band that migrated as a polypeptide of 57 kDa. No such immunoprecipitation occurred in the presence of 20 μM p-1 peptide which competed with RCK1 polypeptides for binding to Ab-1, or when native (control) oocytes were used (Figure 3A). Native oocytes of some donors yielded a faint band which migrated as a 57-kDa polypeptide and might correspond to an endogenous K^+ channel of the native oocyte (Parker & Miledi, 1988).

The amino acid sequence of the RCK1 channel contains a consensus site for cAMP-dependent phosphorylation in the cytosolic C-terminus (Baumann et al., 1988). The amino acid sequence of this site is ArgArgSerSerSer, and the middle Ser^{446} is the most likely candidate for phosphorylation (Kennelly & Krebs, 1991). However, since the other two serine residues are also candidates (albeit with lower probability), we decided to generate two substitution mutations in this site. Thus, Arg^{443} was replaced with cysteine and Ser^{446} with alanine, rendering the double mutant R443C;S446A. To compare mutant and wild-type channels, both types of cRNA were injected in adequate concentrations so that the two types of channels expressed currents of similar amplitudes. Currents through the mutant channel resembled the delayed rectifier type of current through the wild-type channel, with respect to both kinetics and voltage dependence (not shown). The mutation abolished the phosphorylation by PKA-CS of the 57-kDa polypeptide in whole oocytes (Figure 3C).

The 57-kDa polypeptide was phosphorylated by PKA-CS in both the cytosolic and the plasma membrane fractions in six experiments with different donors (Figure 4A, lane 2; Figure 4B, lane 2). Figure 4 shows that the double mutation R443C;S446A abolished phosphorylation in both fractions (Figure 4A, lane 3; Figure 4B, lane 3).

To determine whether the channels in their native state in intact oocytes are phosphorylated to some extent by endogenous protein kinases, the immunoprecipitated channels were dephosphorylated, prior to phosphorylation by PKA-CS, by the protein phosphatases PP2B and PP2A (Figure 4A, lane 1; Figure 4B, lane 1). Dephosphorylation increased the intensity of the subsequent PKA-CS-induced ^{32}P-labeling of the 57-kDa polypeptide (Figure 4A,B; compare lanes 1 and 2 in each panel). Comparison of labeling intensities with and without prior dephosphorylation yielded an estimate of the extent of the basal phosphorylation (assuming that the phosphatases dephosphorylated the channels completely) in every experiment. The extent of basal phosphorylation varied among donors. Values obtained for basal phosphorylation were 55% in whole oocytes (one donor), 60%, 0%, and 73% in the plasma membrane (three donors), and 54%, 40%, and 25% in the cytosol (three donors).

cAMP-Induced Phosphorylation of RCK1 Polypeptides in Intact Oocytes. To study the phosphorylation of RCK1 polypeptides *in vivo* (in intact oocytes), native oocytes and oocytes injected with RCK1 cRNA were incubated with [^{32}P]-orthophosphate for 4.5 h, and during the last 30 min, a membrane-permeant cAMP analog, Sp-cAMPS, was added to the incubation solution. RCK1 polypeptides were then immunoprecipitated with Ab-1 in the presence of protein phosphatase inhibitors and analyzed by SDS–PAGE. In both the cytosolic and plasma membrane fractions of oocytes injected with RCK1 cRNA and treated with Sp-cAMPS, the 57-kDa polypeptide was phosphorylated (Figure 5A, lanes 2 and 3; Figure 5B, lanes 2 and 4), and migrated at the same

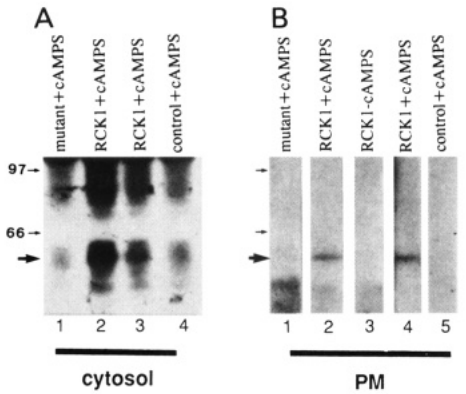

FIGURE 5: Sp-cAMPS-induced phosphorylation of RCK1 polypeptides in intact oocytes, metabolically labeled with [^{32}P]orthophosphate; the double mutation R443C;S446A abolishes this phosphorylation. Phosphorylation was induced by incubation of oocytes for 30 min with 0.5 mM Sp-cAMPS. RCK1 polypeptides were immunoprecipitated from cytosolic (A) and plasma membrane (B) fractions. (A) Lanes 2 and 3, RCK1 cRNA-injected oocytes; the cRNA was derived from SSupEX-RCK1 or SupEx-RCK1 constructs, respectively. Lane 4, native (control) oocytes. Lane 1, oocytes injected with the mutant R443C;S446A cRNA. (B) Lanes 4 and 2, RCK1 cRNA-injected oocytes; the cRNA was derived from SSupEx-RCK1 or SupEx-RCK1 constructs, respectively. Lane 5, native (control) oocytes. Lane 1, oocytes injected with the mutant cRNA. Lane 3, RCK1 cRNA-injected oocytes not treated with Sp-cAMPS. Thick arrows denote the 57-kDa polypeptide as verified also by migration of [^{35}S]methionine-labeled RCK1 polypeptides on the same gel (not shown). Thin arrows denote migration of 97- and 66-kDa molecular mass markers.

molecular mass as the 57-kDa polypeptide labeled metabolically by [^{35}S]methionine (not shown). This 57-kDa phospholypeptide was detected in oocytes of five out of seven donors examined, in amounts that correlated with the current amplitudes expressed in those oocytes. Thus, in the experiment shown in Figure 5, two groups of oocytes injected with cRNAs derived from different expression constructs (see Materials and Methods), SupEx -RCK1 (Figure 5A, lane 3; Figure 5B, lane 2) and SSupEx-RCK1 (Figure 5A, lane 2; Figure 5B, lane 4), and therefore expressing currents of different amplitudes, 18 ± 5 ($n = 9$) and 30 ± 2 ($n = 6$) μA, respectively, exhibited different amounts of [^{32}P]phosphate labeling. In two experiments, the 57-kDa polypeptide was faintly labeled after 4.5 h incubation with [^{32}P]orthophosphate in oocytes that were not treated with Sp-cAMPS (not shown).

The R443C;S446A mutant channels did not undergo *in vivo* phosphorylation by Sp-cAMPS stimulation in either the cytosolic (Figure 5A, lane 1) or the plasma membrane fraction (Figure 5B, lane 1). Faint labeling of the 57-kDa band in the cytosol of native oocytes and oocytes injected with mutant cRNA (Figure 5A, lanes 4 and 1, respectively) may have resulted from phosphorylation of endogenous K^+ channels.

DISCUSSION

Biosynthesis of the RCK1 Channel. This study complements electrophysiological studies pointing to phosphorylation-mediated regulation of voltage-gated K^+ channels. As a first step in the biochemical characterization of a molecularly defined voltage-gated K^+ channel, a polyclonal antiserum has been generated that immunoprecipitates the rat brain RCK1 channel from *Xenopus* oocytes. SDS–PAGE analysis of immunoprecipitated RCK1 products metabolically labeled by [^{35}S]methionine shows that the RCK1 channels are expressed in oocytes in the form of several polypeptides, mainly of molecular mass ~54 and ~57 kDa, and a set of polypeptides of lower (~50 kDa) and higher (65–90 kDa) molecular mass. It should be noted that the estimation of the molecular mass of hydrophobic polypeptides from SDS–PAGE is imprecise, as they tend to run faster than typical standard proteins of similar molecular mass (Takagi, 1991). If the 54-kDa band, which is the main form of RCK1 when N-glycosylation is inhibited by tunicamycin, corresponds to a "net" channel molecule (molecular mass calculated from the predicted primary sequence is 56.4 kDa), the extent of molecular mass underestimation is about 2.5 kDa.

The 57-kDa and larger polypeptides are N-glycosylated, as shown by the experiments with tunicamycin (Figure 2B); those with molecular mass higher than 57 kDa may be extensively glycosylated channel proteins. Since the major subunit of the purified rat brain K^+ channel protein (DMB protein) is about 75–90 kDa (Rehm, 1991), it is possible that the glycosylation of RCK1 in the oocyte is less extensive than in the brain [see Soreq (1985)]; it is not known, however, whether the DMB protein corresponds to RCK1.

Our data suggest that the 57-kDa polypeptide corresponds to the functional RCK1 channel, because (i) its synthesis, resolved by SDS–PAGE, was correlated with functional channel activity (I_{RCK1} amplitudes) and (ii) treatment with tunicamycin, which eliminated the 57-kDa band almost completely, reduced the current amplitude by up to 76%.

RCK1 Channels as Substrates for in Vitro and in Vivo Phosphorylation by PKA. Our results demonstrate that the RCK1 channel serves as a substrate for phosphorylation by PKA. The product phosphorylated *in vitro*, both in the cytosolic and in the plasma membrane fractions, is the 57-kDa polypeptide. Recently, similar *in vitro* phosphorylation studies have shown that a related K^+ channel (Kv1.3) serves as a substrate for PKA phosphorylation in Jurkat cells (Cai & Douglass, 1993).

Also, the RCK1 channel, in its native state, is partially phosphorylated in the cytosol and in plasma membranes of intact oocytes. This conclusion is inferred from two observations: (i) RCK1 polypeptides immunoprecipitated from metabolically [^{32}P]phosphate-labeled oocytes were sometimes found to be weakly labeled even without stimulation by a cAMP analog; (ii) *in vitro* PKA-induced incorporation of [^{32}P]phosphate into the 57-kDa polypeptides was significantly higher after their *in vitro* dephosphorylation with protein phosphatases (PP2B and PP2A), indicating that the phosphatases removed phosphate already basally incorporated into the RCK1 polypeptide (the level of basal phosphorylation appeared to vary significantly among oocytes from different donors). Interestingly, basal phosphorylation has also been reported for Kv1.3 K^+ channel in intact Jurkat cells (Cai & Douglass, 1993) and for Na^+ channels in intact neurons (Rossie & Catterall, 1987).

We further provide evidence that in metabolically labeled intact oocytes, Sp-cAMPS (a membrane-permeant analog of cAMP) causes incorporation of [^{32}P]phosphate into the RCK1 polypeptide, presumably through activation of endogenous PKA. This finding provides the first evidence for cAMP-induced phosphorylation of K^+ channels in intact cells.

Site of Phosphorylation by PKA. The RCK1 sequence contains one consensus phosphorylation site at the polypeptide's intracellular C-terminus. Insertion of two mutations at this site abolished *in vitro* phosphorylation by PKA-CS and eliminated both basal and cAMP-stimulated phosphorylation in the intact oocytes, indicating that this (most probably Ser^{446}) is the only site phosphorylated by PKA.

Although some studies have demonstrated modulation of activity of several voltage-dependent K^+ channels by PKA,

it was not clear whether the target for phosphorylation is the channel itself. In this work, we have shown that a K^+ channel of known molecular identity serves as a substrate for PKA phosphorylation and can be phosphorylated *in vivo* by addition of a cAMP analog. Moreover, we have identified the site of phosphorylation on the channel molecule. These findings constitute an essential first step toward understanding of the molecular events underlying modulation by PKA phosphorylation of voltage-dependent K^+ channels.

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