Inhibition of ROMK potassium channel by syntaxin 1A

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Inhibition of ROMK potassium channel by syntaxin 1A. Am J Physiol Renal Physiol 288: F284–F289, 2005. First published September 28, 2004; doi:10.1152/ajprenal.00320.2004.—ROMK potassium channels are present in the cortical collecting duct (CCD) of the kidney and serve as apical exit pathways for K⁺ secretion in this nephron segment. K⁺ secretion in the CCD is regulated by multiple factors. In this study, we show that syntaxin 1A, but not syntaxin 3 or 4, inhibited whole cell ROMK currents in Xenopus laevis oocytes. Syntaxin 1A, but not syntaxin 3 or 4, interacted with the COOH-terminal cytoplasmic domain of ROMK in vitro. Coexpression with synaptobrevin 2 reversed inhibition of whole cell ROMK currents by syntaxin 1A. In excised inside-out membranes of oocytes, application of fusion proteins containing the cytoplasmic region of syntaxin 1A to the cytoplasmic face caused a dose-dependent inhibition of ROMK. We further examined regulation of the K⁺ channels in the CCD by syntaxin 1A. Application of botulinum toxin C1 to the excised inside-out membranes of the CCD caused an increase in the activity of K⁺ channels. In contrast, application of toxin B had no effects. These results suggest that syntaxin 1A causes a tonic inhibition of the K⁺ channels in the apical membrane of the CCD. Binding of synaptobrevin 2 to syntaxin 1A during docking and fusion of transport vesicles to the plasma membranes of CCD may lead to activation of these channels.

As the final common pathway for K⁺ secretion in the CCD, ROMK is a target for regulation by multiple factors. These factors include peptide hormones such as vasopressin and bradykinin, intracellular pH, and changes in dietary K⁺ intake (9, 11, 13). Some of the factors (such as intracellular pH) regulate opening probability of the channel (6). Others (such as changes in dietary K⁺ intake) regulate the density of active channels (27). An increase in the density of active channels may be caused by an increase in channel expression, insertion of channels from an intracellular pool, and/or activation of preexisting silent channels.

Docking and fusion between transport vesicles and target acceptor membranes (such as fusion of the transport vesicles with plasma membranes in biosynthetic trafficking) involve the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (31). Every transport vesicle contains on its surface proteins that specify that vesicle’s target (v-SNAREs). Target membranes possess cognate partners (t-SNAREs) that interact with the v-SNAREs. The specific interaction between v-SNAREs and their cognate t-SNAREs ensures delivery of transport vesicles to their distinct and specific targets (31).

v-SNAREs include synaptobrevins, synaptotagmins, and synaptophysins. t-SNAREs include syntaxins and synaptosomal-associated protein of 25 kDa (SNAP-25) (31). Each of the SNAREs belong to a large gene family. For example, in mammalian cells at least 13 different syntaxins, 8 different synaptobrevins, and 3 SNAP-25 homologs have been identified (2, 4, 7, 28). Two factors may contribute to the multiplicity of isoforms for SNAREs. First, multiple isoforms of SNAREs are needed to allow specific targeting to distinct intracellular loci throughout the cell (28). For example, syntaxin 1, 2, 3, and 4 are present in the plasma membranes and involved in vesicle trafficking to this destination (2, 4). In contrast, syntaxin 5 is present in the Golgi complex and plays an important role in membrane trafficking between the endoplasmic reticulum and the Golgi (2, 7). Syntaxin 6, 7, and 8 are involved in endosomal trafficking (28). The other contributing factor for the multiplicity of SNAREs is the heterogeneity of SNAREs in different tissues. In the kidney, syntaxin 4 and 3 and synaptobrevin 2 have been localized to the apical membrane, basolateral membrane, and intracellular vesicles of collecting ducts, respectively, suggesting that they have roles in docking and fusion of vesicles in this nephron segment (15, 19, 20, 25).

Besides the role in vesicle docking and fusion, SNARE proteins also play important roles in directly regulating the function of the proteins in the target membranes. Syntaxin 1A, the neuronal t-SNARE, inhibits the activity of voltage-gated Ca²⁺ channels via direct protein-protein interaction (3, 30, 33, 34).
suggest that a pool of silent K+ and that synaptobrevin 2 reverses the inhibition. These results are consistent with the participation of synaptobrevin 2 in colonic and airway epithelial cells and the epithelial Na+ channel (ENaC) in renal epithelial cells through protein-protein interaction (23, 24, 29). In this study, we report that syntaxin 1A inhibits ROMK in Xenopus laevis oocytes and in the CCD and that synaptobrevin 2 reverses the inhibition. These results suggest that a pool of silent K+ channels are present in the CCD, which may be activated by synaptobrevin during fusion of transport vesicles to the plasma membranes.

MATERIALS AND METHODS

Molecular biology. Wild-type ROMK1 cDNA is in the pSPORT plasmid (12). Oocyte expression constructs (in pGEMM vector) of rat syntaxin 1A, 3, and 4 are gifts from Dr. I. Bezprozvanny (UT Southwestern Medical Center, Dallas, TX). Rat synaptobrevin 2 and synaptotagmin 2 (in pBlueScript) are gifts from Dr. T. Südhof and Dr. M. Cobb (UT Southwestern Medical Center). mCAP cRNAs for ROMK1, synaptotagmin 2, synaptobrevin 2, and syntaxin isoforms were transcribed in vitro (17). For construction of fusion proteins, nucleotide sequences corresponding to the cytoplasmic domains of syntaxin 1A (amino acid 1–265; GST-S1A-ΔC), syntaxin 3 (amino acid 1–263; GST-Syn3-ΔC), and syntaxin 4 (amino acid 1–263; GST-Syn4-ΔC) were amplified by PCR and subcloned into the pGEX-2T bacterial expression vector (Pharmacia). Production and purification of the respective glutathione S-transferase (GST) fusion proteins were performed as described (14, 18). Hexahistidine-tagged fusion proteins containing the COOH terminus of ROMK1 (amino acid 181–391; H6-RKC) were produced using the pRSET vector (Invitrogen) as described (18).

Two-electrode voltage-clamp recording. X. laevis oocytes were prepared as previously described (14, 17, 18, 35). Oocytes were injected with cRNA for ROMK1 and/or SNARE proteins as indicated. Current-voltage relationships (−100 to +100 mV, in 25-mV steps) were measured in oocytes at −23°C by two-electrode voltage clamp using an OC-725C oocyte clamp amplifier (Warner Instruments, Hamden, CT), pCLAMP7 software, and a Digidata 1200A digitizer (Axon Instrument). The resistance of current and voltage microelectrodes (filled with a 3 M KCl solution) was 1–2 MΩ. The bath solution contained (in mM) 96 KCl, 1 MgCl2, 1 CaCl2, and 5 HEPES (pH 7.5 by KOH).

Single-channel patch-clamp recording. Patch-clamp pipettes (pulled from borosilicate glass, Warner Instruments) were filled with solutions containing (in mM) 100 KCl, 1 MgCl2, 2 CaCl2, and 5 HEPES (pH 7.4 with KOH). Pipette tip resistance ranged from 3 to 5 MΩ. The Mg2+-free FVPP bath solution (in mM) 100 KCl, 10 EDTA, 2 fluoride, 1 vanadate, 1 pyrophosphate, and 10 HEPES, pH 7.4) has been described previously (14, 17, 18). Single-channel currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 1 kHz using an eight-pole Bessel filter, sampled every 0.1 ms (10 kHz) with Digidata-1200A interface, and stored directly onto a computer hard disk (120 GB) using pCLAMP9 software (Axon Instruments). Data were transferred to a CD for long-term storage. For analysis, event list files were generated and analyzed for open probability using pCLAMP9. Open probability was analyzed on segments of continuous recording (at least 2 min). For recording of the K+ channels in the CCD, patch-clamp recording was performed in split-open CCD from rats fed a normal-K+ diet as described (36).

Biochemical interactions of ROMK with syntaxin isoforms. Fusion proteins GST-S1A-ΔC, GST-Syn3-ΔC, and GST-Syn4 S1A-ΔC were incubated with purified H6-RKC and precipitated using glutathione Sepharose-4B beads (14, 18). The precipitates were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membranes, analyzed by immunoblot analysis using polyclonal antibodies against the COOH terminus of ROMK1 (1:1,000 dilution) (14) followed by horseradish peroxidase-coupled donkey anti-rabbit secondary antibody (1:5,000 dilution), and visualized using enhanced chemiluminescence (Amer sham Bioscience, Piscataway, NJ).

RESULTS

Inhibition of ROMK by syntaxin 1A and reversal of inhibition by synaptobrevin 2. The regulation of ROMK1 channels by syntaxin 1A was studied in X. laevis oocytes by two-electrode voltage-clamp recording. Coexpression of syntaxin 1A with ROMK1 in oocytes inhibited whole cell ROMK1 currents (Fig. 1A). In contrast, coexpression of neither syntaxin 3 nor syntaxin 4 inhibited ROMK1 currents. Figure 1B shows the characteristic weak inward rectification of the current-voltage relationship of ROMK currents and inhibition of the currents by extracellular barium. The inhibition of ROMK1...
currents by syntaxin 1A was dose dependent (Fig. 1C). The concentration of cRNA for syntaxin 1A required for inhibition of ROMK1 (5 and 20 ng for half-maximal and maximal inhibition, respectively) is similar to that for inhibition of voltage-gated Ca\(^{2+}\) channels (3, 37).

The v-SNARE synaptobrevin 2 forms a tight complex with syntaxin 1A during fusion of exocytotic vesicles with target membranes (31). We found that coexpression of synaptobrevin 2 prevented syntaxin 1A inhibition of ROMK1 (Fig. 2). By itself, synaptobrevin 2 did not inhibit ROMK1 currents. Synaptotagmin 2, another v-SNARE not involved in the formation of core complex with syntaxin 1A (31), did not affect syntaxin 1A-mediated inhibition of ROMK1 currents. Nor did synaptotagmin 2 by itself inhibit ROMK1 currents.

**Syntaxin 1A regulates ROMK1 in the plasma membrane.** The domain structure of syntaxin 1A can be separated into a NH\(_2\)-terminal cytoplasmic domain (amino acids 1–265) and COOH-terminal transmembrane domain (amino acids 266–288) (8). Syntaxin 1A regulates the voltage-dependent Ca\(^{2+}\) channels, CFTR, and ENaC via direct interactions with channel proteins (3, 16, 22–24, 29, 30, 33, 37). Inhibition of Ca\(^{2+}\) channels and CFTR by syntaxin 1A involves the NH\(_2\)-terminal cytoplasmic region. We made GST fusion proteins containing the cytoplasmic region of syntaxin 1A, 3, or 4 and examined direct interaction with ROMK1 using in vitro pull-down assays. GST fusion proteins were incubated with purified hexahistidine-tagged fusion proteins containing the COOH-terminal intracellular domain of ROMK1 (H\(_6\)-RKC) and precipitated by glutathione Sepharose-4B beads. As shown in Fig. 3, the cytoplasmic domain of syntaxin 1A, but not of syntaxin 3 or 4, interacted with the COOH-terminal intracellular domain of ROMK1.

To see whether inhibition of ROMK1 currents by syntaxin 1A occurs via direct interaction with channels in the plasma membrane, we examined the effects of syntaxins on ROMK1 in excised inside-out membranes. In inside-out membranes from oocytes expressing ROMK1, application of GST fusion protein of the cytoplasmic region of syntaxin 1A to the cytoplasmic face of the membrane decreased the activity of ROMK1 channel (Fig. 4A). Application of 0.3 and 1 \(\mu\)M syntaxin fusion protein inhibited ROMK1 activity by 51 and 80\%, respectively (Fig. 4B). As controls, GST alone (Fig. 4B) and fusion proteins of syntaxin 3 and 4 (not shown) had no effects. Overexpression of syntaxin 1A in oocytes may alter the stoichiometry of v-SNARE vs. t-SNARE proteins and thus decrease ROMK1 currents by preventing delivery of newly synthesized ROMK1 to plasma membranes. Our results, nevertheless, demonstrate that syntaxin 1A can directly regulate ROMK1 in the plasma membrane of oocytes.

**Effects of botulinum toxins on K\(^{+}\) channel in the CCD.** The physiological role of syntaxin 1A in the regulation of K\(^{+}\) channels in principal cells of rat CCD was examined using inside-out patch-clamp recording and application of botulinum toxin C1 to the cytoplasmic face of the membrane patches. Botulinum toxin C1 inactivates syntaxin 1A by cleaving at the junction between the cytoplasmic and transmembrane domains (21, 31, 32). The direct inhibition of CFTR by syntaxin 1A is reversed by botulinum toxin C1 (23).

After formation of a GΩ seal and initial recording in the cell-attached configuration, inside-out membrane was excised. Inside-out recording is necessary because the entry of toxins across the plasma membrane of nonneuronal cells is limited. As reported by others (26), we found that the incidence of K\(^{+}\) channels per patch from animals on a normal-K\(^{+}\) diet is \(\sim 28\%\) [16 of 58 patches contain active channels; average number of channels times open probability (\(NP_o\)) 0.35 \(\pm 0.05, n = 58\)]. In the representative experiment shown in Fig. 5A, channel activity was not detected during recording in the cell-attached configuration (not shown) or after excision of inside-out membranes (Fig. 5A). However, the activity of channels was revealed by application of botulinum toxin C1 (1 U/ml) to the cytoplasmic face of the inside-out membrane. In multiple experiments, average activity of the K\(^{+}\) channels (measured as...
NP₀ was increased about fourfold by toxin C1 over 4 min (Fig. 5B). Botulinum toxin B inactivates synaptobrevin but not syntaxin (31). In separate experiments, application of botulinum toxin B to the cytoplasmic face of inside-out membranes did not cause an increase in the activity of K⁺/H⁺ exchangers in the CCD (Fig. 6), demonstrating the specificity of toxin C1. Furthermore, these results agree with the finding that synaptobrevin 2 has no direct inhibitory effect on ROMK. Insertion of channels is unlikely in excised inside-out membranes. These results thus suggest that a pool of K⁺/H⁺ exchangers in the apical membrane of CCD is tonically inhibited by membrane-anchored syntaxin 1A. Botulinum toxin C1 cleaves syntaxin 1A at the junction between the

**Fig. 4.** Effects of S1A on ROMK1 channels in the plasma membrane of oocytes. Single-channel activity was recorded from *X. laevis* oocytes expressing ROMK1. GST fusion protein of the cytoplasmic domain of syntaxin 1A (S1A-C) was applied to the cytoplasmic face of the excised inside-out patches. **A**: representative recording shown in compressed (20 s/bar) and expanded (200 ms/bar) time course. Addition of S1A-C is indicated by arrow. Current bar is 4 pA. Dotted line indicates current level with 0 channel opening. **B**: dose-dependent inhibition by S1A-C. Activity is shown as number of channels times open probability (NP₀); *n = 8. *P < 0.05 after vs. before application of toxin by paired t-test.

**Fig. 5.** Effect of botulinum toxin C1 on inside-out membranes of rat CCD. **A**: representative recording shown in compressed (20 s/bar) and expanded (300 ms/bar) time course. Addition of toxin C1 is indicated by arrow. A time gap of 150 s is shown. C, 1, 2, 3, and 4: current level with 0 (closed), 1, 2, 3, or 4 channels opening, respectively. Current bar is 4 pA. **B**: results of 9 experiments. NP₀ before and after toxin from the same experiment are connected by solid lines. *P < 0.05 by paired t-test.

**Fig. 6.** Effect of botulinum toxin B on inside-out membranes of rat CCD. **A**: representative recording shown in compressed (2 min/bar) time course. Addition of toxin B is indicated by arrow. C and O indicate closed and open state, respectively. **B**: results of 6 experiments. NP₀ before and after toxin from the same experiment are connected by solid lines. NS, not significant by paired t-test.
cytoplasmic and transmembrane domains (21, 31, 32). The cytoplasmic domain of syntaxin 1A causes inhibition of ROMK1 (see Fig. 4 above). However, the concentration of the cytoplasmic domain of syntaxin 1A released by toxin into the cytoplasmic solution is likely much lower than the level sufficient to cause inhibition of the channel. Inhibition of CFTR involves the cytoplasmic region of syntaxin 1A (23, 24). Similarly, injection of toxin C1 into oocytes releases the inhibition of CFTR by syntaxin 1A (23).

**DISCUSSION**

Besides its role in vesicle docking and fusion, syntaxin 1A has been shown to directly regulate ion channels, including voltage-gated Ca\(^{2+}\) channels, CFTR Cl channels, and ENaC in plasma membranes (3, 22–24, 29, 30, 33, 37). In the present study, we report that syntaxin 1A regulates ROMK in *X. laevis* oocytes and in the CCD. Initially thought to be exclusively in neuronal tissues, syntaxin 1A was later shown to colocalize with CFTR in colonic and airway epithelial cells (22–24). Probably due to low abundance, localization of syntaxin 1A to apical membranes of renal tubules has not been demonstrated. Nevertheless, syntaxin 1A regulates ENaC in oocytes and in the A6 epithelial cell line (29) and regulates H\(^{+}\)-ATPase in cultured and native inner medullary collecting duct cells (1). Together, these results suggest that syntaxin 1A has a general role in direct regulation of ion channels in many tissues, including airway and renal epithelia.

The direct regulation of voltage-dependent Ca\(^{2+}\) channels by SNARE proteins is important for control of neurotransmitter release in the neuronal synapses (3, 30, 33, 37). In the resting state, Ca\(^{2+}\) channels are inhibited by binding to syntaxin 1A. During fusion of vesicles to the target membrane, syntaxin, SNAP-25, and synaptobrevin interact to form a very tight complex, called the core complex (4, 31, 32). The core complex forms the receptor for binding by α-SNAP (for soluble NSF attachment protein; different from SNAP-25), which in turn recruits the ATPase NEM-sensitive fusion protein (NSF). ATP hydrolysis by NSF dissociates the SNAREs, permitting subsequent fusion to occur. Formation of the core complex from docking of vesicles to the presynaptic surface membrane activates Ca\(^{2+}\) channels and raises local Ca\(^{2+}\) concentration to facilitate fusion of vesicles.

What is the potential physiological role of the direct regulation of ROMK by syntaxin 1A? AVP increases K\(^{+}\) secretion in the CCD (5, 9). The increase in K\(^{+}\) secretion by AVP is also associated with an increase in the number of active K\(^{+}\) channels, as measured in cell-attached patch-clamp recording (5). The mechanism for the increase in active channels is not known. Botulinum toxin C1 inactivates syntaxin 1A specifically (31). We find that botulinum toxin C1 increases activity (measured as *NP*\(_{\text{p}}\)) of ROMK by about fourfold in inside-out membranes where insertion of channels is unlikely. Single-channel open probability of ROMK is ~0.9 at intracellular pH 7.4 (6, 17), suggesting that the increase in *NP*\(_{\text{p}}\) by toxin C1 is due to an increase in the number of active channels. Thus some of the K\(^{+}\) channels in the apical membrane of principal cells are inactive (i.e., silent channels) because of binding to syntaxin 1A. AVP increases water permeability in the kidney collecting duct by promoting plasma membrane insertion of the water channel aquaporin 2 (34). The increase in water permeability by AVP parallels with the increase in K\(^{+}\) channel density. Synaptobrevin 2 is present in aquaporin 2-containing vesicles (25). We find that synaptobrevin 2 releases syntaxin 1A-induced inhibition of the channels (Fig. 2). Thus reversal of syntaxin 1A-induced inhibition of K\(^{+}\) channels by synaptobrevin 2 may account for the increase in the density of active channels by AVP.

Syntxin 3 and 4 are expressed in renal collecting ducts (19, 20). Syntaxin 4 is localized to the apical membrane of collecting ducts and proposed to be involved in fusion of aquaporin 2-containing vesicles to the apical membrane (19). Interestingly, we find that neither syntxin 3 nor syntxin 4 regulates ROMK. We have no obvious explanation for this apparent difference at the moment.

ROMK channels are critical for K\(^{+}\) secretion in the CCD and Na\(^{+}\) reabsorption through the Na-K-2Cl cotransporter in the thick ascending limb of Henle’s loop. Multiple factors regulate ROMK channels (9, 11, 13). Some of these factors (besides AVP-induced vesicle fusion) may also modulate syntaxin 1A inhibition of ROMK. For example, phosphorylation of ROMK by protein kinases may alter syntaxin 1A inhibition of ROMK. Future studies will investigate this possibility. Finally, AVP also stimulates Na\(^{+}\) reabsorption in the CCD (10). It would be interesting to investigate whether synaptobrevin modulates inhibition of ENaC by syntaxin 1A and, if so, whether it underlies the increase in Na\(^{+}\) transport by AVP.

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