A functional CFTR-NBF1 is required for ROMK2-CFTR interaction

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Departments of 1Cellular and Molecular Physiology and 2Pediatrics, Yale University School of Medicine, New Haven, Connecticut 06520-8026; 3Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; 4Department of Physiology and Biophysics, University of Alabama Birmingham, Birmingham, Alabama 35294; and 5Division of Nephrology, Vanderbilt University Medical School, Nashville, Tennessee 37232-2372

McNicholas, Carmel M., Malcolm W. Nason, Jr., William B. Guggino, Erik M. Schwiebert, Steven C. Hebert, Gerhard Giebisch, and Marie E. Egan. A functional CFTR-NBF1 is required for ROMK2-CFTR interaction. Am. J. Physiol. 273 (Renal Physiol. 42):F843–F848, 1997.—In a previous study on inside-out patches of Xenopus oocytes, we demonstrated that the cystic fibrosis transmembrane conductance regulator (CFTR) enhances the glibenclamide sensitivity of a coexpressed inwardly rectifying K⁺ channel, ROMK2 (C. M. McNicholas, W. B. Guggino, E. M. Schwiebert, S. C. Hebert, G. Giebisch, and M. E. Egan. Proc. Natl. Acad. Sci. USA 93: 8083–8088, 1996). In the present study, we used the two-microelectrode voltage-clamp technique to measure whole cell K⁺ currents in Xenopus oocytes, and we further characterized the enhanced sensitivity of ROMK2 to glibenclamide by CFTR. Glibenclamide inhibited K⁺ currents by 56% in oocytes expressing both ROMK2 and CFTR but only 11% in oocytes expressing ROMK2 alone. To examine the role of the first nucleotide binding fold (NBF1) of CFTR in the ROMK2-CFTR interaction, we studied the glibenclamide sensitivity of ROMK2 when coexpressed with CFTR constructs containing mutations in or around the NBF1 domain. In oocytes coexpressed with ROMK2 and a truncated construct of CFTR with mutations in or around the NBF1 domain, we recently demonstrated that cystic fibrosis transmembrane conductance regulator (CFTR) enhances the glibenclamide sensitivity of ROMK2 to glibenclamide by CFTR. Glibenclamide inhibited K⁺ currents by 12% in oocytes expressing both ROMK2 and CFTR but only 11% in oocytes expressing ROMK2 alone. To examine the role of the first nucleotide binding fold (NBF1) of CFTR in the ROMK2-CFTR interaction, we studied the glibenclamide sensitivity of ROMK2 when coexpressed with CFTR constructs containing mutations in or around the NBF1 domain. In oocytes coexpressed with ROMK2 and a truncated construct of CFTR with mutations in or around the NBF1 domain, we recently demonstrated that cystic fibrosis transmembrane conductance regulator (CFTR) enhances the glibenclamide sensitivity of ROMK2 to glibenclamide by CFTR. Glibenclamide inhibited K⁺ currents by 12% in oocytes expressing both ROMK2 and CFTR but only 11% in oocytes expressing ROMK2 alone. To examine the role of the first nucleotide binding fold (NBF1) of CFTR in the ROMK2-CFTR interaction, we studied the glibenclamide sensitivity of ROMK2 when coexpressed with CFTR constructs containing mutations in or around the NBF1 domain. In oocytes coexpressed with ROMK2 and a truncated construct of CFTR with mutations in or around the NBF1 domain, we recently demonstrated that cystic fibrosis transmembrane conductance regulator (CFTR) enhances the glibenclamide sensitivity of ROMK2 to glibenclamide by CFTR. Glibenclamide inhibited K⁺ currents by 12% in oocytes expressing both ROMK2 and CFTR but only 11% in oocytes expressing ROMK2 alone. To examine the role of the first nucleotide binding fold (NBF1) of CFTR in the ROMK2-CFTR interaction, we studied the glibenclamide sensitivity of ROMK2 when coexpressed with CFTR constructs containing mutations in or around the NBF1 domain. In oocytes coexpressed with ROMK2 and a truncated construct of CFTR with mutations in or around the NBF1 domain, we recently demonstrated that cystic fibrosis transmembrane conductance regulator (CFTR) enhances the glibenclamide sensitivity of ROMK2 to glibenclamide by CFTR. Glibenclamide inhibited K⁺ currents by 12% in oocytes expressing both ROMK2 and CFTR but only 11% in oocytes expressing ROMK2 alone.

To date, all of the channel regulator/drug binding subunits that have been identified are members of the ATP-binding cassette (ABC) transporter family and include SUR1, SUR2, and cystic fibrosis transmembrane conductance regulator (CFTR). SUR, first identified by Aguilar-Bryan and co-workers (1), is a regulator of insulin secretion, which couples with Kᵩ₁.₂ to form the Kᵩ channel of the pancreatic β-cell. Moreover, naturally occurring mutations of the SUR gene lead to familial hyperinsulinemic hypoglycemia of infancy (PHHI) (9, 30, 31).

ROMK2 (Kᵩ₁.₁b) is functionally similar (10, 15, 25, 35) to the small conductance ATP-sensitive K⁺ channel of the cortical collecting duct (11, 27, 33) and thick ascending limb of the loop of Henle (34) nephron segments. Using the Xenopus oocyte expression system, we recently demonstrated that ROMK2 is an ATP-sensitive K⁺ channel (25) that requires coinjection with CFTR for enhanced sulfonylurea sensitivity (23). CFTR not only enhances the sulfonylurea sensitivity of ROMK2 but also modulates the outwardly rectifying chloride channel in cultured airway cells (29) and inhibits the Na⁺ channel in epithelial cells (14). The purpose of the present study was to determine the region of CFTR that is important for the interaction between ROMK2 and CFTR that confers enhanced sulfonylurea sensitivity. We examined the role of the first nucleotide binding fold (NBF1) of CFTR by using...
two-microelectrode voltage-clamp techniques to measure glibenclamide-sensitive K⁺ currents in Xenopus oocytes coexpressing ROMK2 and CFTR with mutations in and around NBF1. We focused on NBF1 because naturally occurring mutations within the nucleotide binding folds of many ABC superfamily members are associated with pathophysiological states (2, 9, 13, 18, 30–32). Some of these data have been presented in abstract form (24).

METHODS

Preparation of oocytes for voltage-clamp experiments. Stages V-VI Xenopus laevis oocytes were isolated and injected (50 nl) as described previously (23). Oocytes were injected with 12.5 ng of ROMK2 cRNA, 50 ng of CFTR wild-type (CFTR-WT) cRNA, and/or 50 ng CFTR mutant cRNA. Experiments were performed on days 3–6 after injection.

Electrophysiological studies. We used the two-electrode voltage-clamp technique to measure whole cell currents from oocytes injected with either ROMK2 alone or CFTR (or CFTR mutants) alone, ROMK2 and CFTR (or CFTR mutants) coinjected, or H2O and uninjected controls. Recordings from either the Warner Oocyte Clamp (model OC-72513, Warner Instrument) or a GeneClamp 500 (Axon Instruments, Foster City, CA) were obtained from currents elicited by 20-ms test pulses from −100 to 40 mV in 20-mV increments (holding potential = −65 mV). Oocytes were bathed in a solution (pH 7.4) that contained (in mmol/l) 105 NaCl, 1 MgCl₂, 1 CaCl₂, and 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid with 2 BaCl₂ or 0.5 glibenclamide. Glibenclamide was diluted from a 100 mM stock solution dissolved in a 2:1 (vol/vol) ethanol-dimethyl sulfoxide (DMSO) mixture. In experiments where we tested the effect of glibenclamide on CFTR and mutant constructs expressed alone, we preincubated oocytes in a bath solution containing 100 µM forskolin (FSK) and 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical, St. Louis, MO). Stock solutions of FSK (20 mM) and IBMX (200 mM) were prepared with DMSO as the vehicle. Oocytes cojected with CFTR constructs and ROMK2 were not preincubated with FSK and IBMX. Microelectrode pipettes (Kimax-51, Kimble Products) typically had resistances of...
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0.5–2.0 MΩ when filled with 3 M KCl solution. Experiments were performed at 20–22°C.

For water-injected or uninjected controls, we did not observe significant inward/outward currents. For oocytes injected with ROMK2 cRNA, we selected only those cells expressing >2 µA of whole cell current for a minimum of 5 min at the beginning of each experiment. Experiments were discarded if a stable baseline was not obtained.

Data were compared using a paired Student’s t-test within a single experiment or with ROMK2-injected oocytes using one-way analysis of variance. P < 0.05 were considered significant.

Method of mutagenesis. Site-directed mutagenesis was performed as described by Kunkel et al. (21). Mutations were created in the CFTR clone pBQ4.7 by standard oligonucleotide-directed mutagenesis of single-stranded DNA using the MutaGene Phagemid In Vitro Mutagenesis Kit (Bio-Rad, Richmond, CA) as described previously (12, 26). The oligonucleotides used for mutagenesis were CFTR-G551D:5’ GAGTGGAGATCAACGAG 3’, CFTR-A455E:5’ GTGTGGAGGGTTGGTGG 3’, CFTR-K370X:5’ GCAATAACTAAATACAGGATATCTTAC 3’, and CFTR-K593X:5’ CTGTAACTGATGCTAGCAAATAG 3’. The mutations were confirmed by DNA sequencing. To prepare cRNA, plasmids were linearized with appropriate restriction enzymes and transcribed in vitro using T7 RNA polymerase in the presence of capped GTP and nucleotide mixtures as described previously (15, 26).

RESULTS

Coexpression of CFTR-WT and ROMK2. The two-microelectrode voltage-clamp technique was used to examine the currents elicited by injecting Xenopus oocytes with either ROMK2 alone or ROMK2 and CFTR. To measure ROMK2 K⁺ currents, we executed an experimental protocol in which oocytes were exposed to BaCl₂ for 2–3 min to inhibit ROMK2, then exposed to glibenclamide for 15 min, and finally exposed again to BaCl₂ for 2–3 min. Figure 1A depicts a representative experiment in which a Xenopus oocyte expressed ROMK2 alone. In these experiments (n = 7), the BaCl₂-sensitive outward currents only decreased by 11.4 ± 2.5% (P = 0.13) after oocytes were exposed to glibenclamide (Fig. 1A, A, C, and D). In contrast, for oocytes coexpressed with ROMK2:CFTR-WT (n = 12), the BaCl₂-sensitive outward currents were reduced by 56.0 ± 10.0% (P = 0.001) after a similar exposure to glibenclamide (Fig. 1B, A and C). This percentage of decrease in whole cell K⁺ current is significantly different from the attenuated currents (~11%) observed in oocytes expressing ROMK2 alone (P = 0.003) (Fig. 1D). The effect of glibenclamide on the BaCl₂-sensitive currents was not reversible, a result which is similar to previous findings (3, 17). Thus these data complement our previous results in which we demonstrated that CFTR enhances the sensitivity of ROMK2 to glibenclamide (23).

Coexpression of mutant CFTR and ROMK2. The highest density of cystic fibrosis-causing mutations occur within NBF1 of CFTR (32). Given the importance of NBF1 for the normal function of CFTR, we hypothesized that this domain of CFTR is also necessary for the ROMK2:CFTR interaction, which results in the glibenclamide sensitivity of K⁺ channel currents. To test our hypothesis, we measured the glibenclamide sensitivity of the K⁺ currents (using the experimental protocol described above) when ROMK2 was coexpressed with two engineered CFTR-mutant constructs, CFTR-K593X or CFTR-K370X, or two naturally occurring CFTR-mutant constructs, CFTR-G551D or CFTR-A455E (see Fig. 2). Oocytes injected with these mutant constructs (in contrast to uninjected cells) display a FSK: IBMX-stimulated chloride conductance, evidence that the constructs are functional proteins at the oocyte plasma membrane (Table 1). These findings are similar to the previously reported values of chloride currents generated by CFTR-mutants (8, 12, 26, 28, 29).

In our initial experiments with the mutant CFTR constructs, we coexpressed ROMK2 with either CFTR truncated after NBF1 (CFTR-K593X, Fig. 2) or CFTR truncated before NBF1 (CFTR-K370X, Fig. 2). Similar to the effect observed with the coexpression of wild-type CFTR and ROMK2, coexpressing ROMK2:CFTR-K593X elicited BaCl₂-sensitive currents that were decreased by 45.8 ± 8.1% (n = 8) after the oocytes were exposed to glibenclamide (Figs. 3A and 4). This inhibition of K⁺ current is significantly different from that observed in oocytes expressing ROMK2 alone (P = 0.0008). Moreover, these glibenclamide-sensitive K⁺ currents were similar (P = 0.52) to those in oocytes injected with ROMK2 and CFTR-WT (Fig. 4). Therefore, the mutant CFTR-K593X is similar to CFTR-WT in conferring glibenclamide sensitivity on ROMK2. Because mutant CFTR-K593X is a truncated version of CFTR-WT that lacks the latter half of the protein [including the regulatory (R) and NBF2 domains, as well as transmembrane regions 7–12 (see Fig. 2)], this portion of the
Table 1. Sensitivity of CFTR Cl− currents to glibenclamide

<table>
<thead>
<tr>
<th>Construct</th>
<th>Whole Cell Current, nA</th>
<th>% Inhibition By Glibenclamide</th>
<th>n</th>
<th>P</th>
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<tr>
<td>CFTR-WT</td>
<td>560 ± 150</td>
<td>51.9</td>
<td>9</td>
<td></td>
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<tr>
<td>CFTR-K593X</td>
<td>190 ± 31</td>
<td>50.1</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>CFTR-K370X</td>
<td>183 ± 85</td>
<td>44.1</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>CFTR-G551D</td>
<td>334 ± 80</td>
<td>49.6</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>CFTR-A455E</td>
<td>299 ± 27</td>
<td>63.2</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Uninjected</td>
<td>26 ± 10</td>
<td>0</td>
<td>5</td>
<td>0.02</td>
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Values are means ± SE; n is no. of experiments. Average whole cell currents (V_m = +40 mV) observed in oocytes injected with designated cystic fibrosis transmembrane conductance regulator (CFTR) construct after maximal stimulation with 100 µM forskolin and 1 mM 3-isobutyl-1-methylxanthine. Note: stimulated currents were not significantly different from those observed in uninjected cells (P = 0.002). Basal currents in unstimulated oocytes injected with CFTR were not significantly different from those observed in uninjected cells. Percent inhibition (%) obtained using 2-microelectrode voltage-clamp techniques. Summary of data obtained for ROMK2, ROMK2:CFTR-WT, and ROMK2:mutant CFTR channels as indicated on x-axis. No. of experiments are shown in parentheses. *Significant difference from ROMK2 control (P < 0.05). Average Ba2+-sensitive whole cell currents generated by each CFTR construct that are inhibited from glibenclamide with the % of wild-type CFTR (CFTR-WT) whole cell currents inhibited by Ba2+. Observed currents for each CFTR construct were significantly different from those observed in uninjected cells (P = 0.013, Fig. 1) or with CFTR-K593X (P = 0.013, Fig. 3A) but similar to that observed when ROMK2 was expressed alone (P = 0.73, Fig. 4). Therefore, the alteration of NBF1 at amino acid 551 abolishes the CFTR-ROMK2 interaction that leads to increased sulfonylurea sensitivity.
A455E (Fig. 2). Coexpressing ROMK2 with CFTR-A455E resulted in Ba\(^{2+}\)-sensitive outward currents that were not significantly inhibited by glibenclamide (n = 10) (Fig. 4). The observed 25.2 ± 5.6% reduction in the outward current is similar to the attenuated currents (~11%) observed in oocytes expressing ROMK2 alone (P = 0.07) (Fig. 1D). The data from oocytes coexpressed with ROMK2 and either CFTR-G551D or CFTR-A455E demonstrate that at least two amino acids in NBF1 are necessary for the ROMK2-CFTR interaction. In summary, these observations strongly suggest that the interaction between these two distinct proteins involves, at least, the NBF1 of CFTR.

Glibenclamide sensitivity of CFTR mutants. To eliminate the possibility that the observed differences of glibenclamide sensitivity in the ROMK2-CFTR coexpression experiments are secondary to alterations in the sulfonylurea sensitivity of the CFTR mutants per se, we assessed the glibenclamide sensitivity of each CFTR mutant construct. Because the extent of CFTR-ROMK2 interaction in our experimental assay relies on measuring a change in the glibenclamide sensitivity of potassium currents, it is essential that the mutant CFTR constructs and CFTR-WT are equally sensitive to glibenclamide. Indeed, there was no significant difference in glibenclamide sensitivity of any of the CFTR constructs compared with wild-type currents (Table 1). Thus the changes in glibenclamide sensitivity in the coexpression experiments were due to differences in protein-protein interactions between ROMK2 and CFTR and not to a reduced sensitivity of the individual CFTR constructs to glibenclamide.

**DISCUSSION**

\(K_{ATP}\) channels result from a complex of at least two subunits: a \(K^+\) channel subunit and a sulfonylurea receptor. We have demonstrated that ROMK2 (Kir1.1b) can interact with CFTR to form a sulfonylurea-sensitive channel (23). Similarly, other \(K_{ATP}\) channels interact with subunits (e.g., SUR1 and SUR2) that bind sulfonylureas (3, 16, 17, 19). The hypothesis that CFTR is the sulfonylurea receptor of the distal nephron is supported by the observation that CFTR localizes to the apical membrane of the distal nephron (7). Furthermore, pseudo-Bartter’s syndrome, a disease characterized by metabolic alkalosis and hypokalemia, is observed in some patients with cystic fibrosis (20). Although the resultant hypokalemia is likely to be caused by excessive sweat losses combined with insufficient salt replacement, there may be subtle renal abnormalities that contribute to these findings, such as excessive potassium secretion in the distal nephron.

Although CFTR is a good candidate for the sulfonylurea receptor of the distal nephron, it may not be the only secondary protein that can functionally link with ROMK and its isoforms (5). In particular, an isoform of the sulfonylurea receptor, SUR2B, has been identified in the kidney (6, 19) but an interaction with ROMK has yet to be demonstrated. The mechanism by which sulfonylureas, such as glibenclamide, inhibit ROMK2 and other \(K_{ATP}\) channels is not presently known. It is unlikely that these compounds act as channel pore blockers, given their effects on cloned \(K_{ATP}\) channels are only observed when the pore-forming subunit is coexpressed with ABC proteins such as SUR1, SUR2, or CFTR (3, 16, 17, 23). In addition, the inhibition of ROMK2-CFTR whole cell currents by glibenclamide occurs slowly (i.e., over a period of minutes) compared with the rapid effect (i.e., within seconds) of the pore blocker barium (Fig. 1B). Taken together, these data indicate a complex mechanism by which sulfonylureas inhibit potassium channels.

To elucidate the regions necessary for CFTR to function as a channel regulator, we have evaluated the glibenclamide sensitivity of ROMK2 when coexpressed with CFTR constructs containing a truncated or mutated NBF1 domain. We focused on this domain because the interactions of CFTR and SUR with ion channels can be modulated by naturally occurring mutations of the NBF domains (9, 14, 22, 29–31). Also, mutations within the NBF domains of other members of the ABC family lead to pathophysiological states. For example, loss-of-function mutations in either NBF1 or NBF2 of the SUR gene have been linked to PHH1 (9, 30, 31) and non-insulin-dependent diabetes mellitus (18). Mutations in similar regions of another ABC transporter, ABCR, a photoreceptor cell-specific ATP-binding transporter, is associated with Stargardt’s disease (macular dystrophy) (2). Furthermore, mutations within NBF1 and NBF2 of CFTR are associated with cystic fibrosis (32).

In summary, we confirm our previous finding that coexpression of ROMK2 with CFTR significantly enhances the sensitivity of the \(K^+\) channel to glibenclamide. In addition, we demonstrate that the interaction between the two proteins requires an intact nucleotide binding fold (NBF1) of the CFTR protein. Last, the implication of NBF1 in this interaction may suggest the underlying mechanism involves an ATP-dependent process such as phosphorylation.

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**REFERENCES**


