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

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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, glibenclamide inhibited K+ currents by 46%. However, in oocytes coexpressed with ROMK2 and a CFTR mutant truncated immediately before NBF1 (CFTR-K370X), glibenclamide inhibited K+ currents by 12%. Also, oocytes expressing both ROMK2 and CFTR mutants with naturally occurring NBF1 point mutations, CFTR-K551D or CFTR-A455E, display glibenclamide-inhibitable K+ currents of only 14 and 25%, respectively. Because CFTR mutations that alter the NBF1 domain reduce the glibenclamide sensitivity of the coexpressed ROMK2 channel, we conclude that the NBF1 motif is necessary for the CFTR-ROMK2 interaction that confers sulfonylurea sensitivity.

Cystic fibrosis transmembrane conductance regulator (CFTR); inwardly rectifying potassium channel (IRK); potassium channel, ROMK2 (Kir1.1b); sulfonylurea; cystic fibrosis transmembrane conductance regulator; cystic fibrosis transmembrane conductance regulator (CFTR); first nucleotide binding fold (NBF1) of CFTR; inwardly rectifying potassium channel, ROMK2 (Kir1.1b); nucleotide binding fold.
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) co-injected, 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 CFTR 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 co-injected 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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Fig. 1. Effect of glibenclamide on whole cell $Ba^{2+}$-sensitive currents for ROMK2- and ROMK2:CFTR-WT-injected oocytes. Time courses showing whole cell $K^+$ currents at a holding potential ($V_{hold}$) = –60 mV for Xenopus oocytes expressing ROMK2 (A) or ROMK2 and CFTR-WT (B) obtained using 2-microelectrode voltage-clamp techniques. Shown are outward currents from $V_{hold}$ = –60 mV plotted against time. After an initial equilibration period (5 min), oocyte was perfused with a solution containing 2 mM BaCl2; thereafter, BaCl2 was removed, and oocyte was exposed to a solution containing 0.5 mM glibenclamide (Glib.). Note: in coexpressing oocytes, glibenclamide inhibition of $K^+$ currents was irreversible for at least 25 min after the removal of glibenclamide from the bath solution. Second application of 2 mM BaCl2 determined residual $K^+$ currents at the end of experiment. For time course display, we plot $Ba^{2+}$-sensitive currents elicited at $V_{hold}$ = –60 mV against time. For summary data, we graph %$Ba^{2+}$-sensitive currents inhibited by 0.5 mM glibenclamide for an average of 2 current measurements at $V_{hold}$ = –60 mV immediately prior to the second BaCl2 application. Note all voltage-clamp experiments where ROMK2, which is constitutively active, is expressed alone or when coexpressed with the CFTR constructs were performed in the absence of forskolin and 3-isobutyl-1-methylxanthine (IBMX). This is done to ensure that there is no activation of CFTR-dependent chloride currents in oocytes that coexpress ROMK2 and CFTR. C: representative family of whole cell currents from oocytes injected with either ROMK2 or ROMK2:CFTR-WT. Oocytes were held at –60 mV and thereafter pulsed for 20 ms from –100 to 40 mV in 20-mV increments, and currents elicited are shown for control, during application of BaCl2 and after glibenclamide (immediately prior to the second application of BaCl2). There was no significant difference between initial the mean BaCl2-sensitive currents ($V_{hold}$ = –60 mV) in ROMK2 (11.35 ± 3.3 µA, n = 7) vs. ROMK2:CFTR-WT (8.29 ± 0.9 µA, n = 12) injected oocytes. D: mean data showing effect of glibenclamide in several similar experiments expressed as %$Ba^{2+}$-sensitive current (no. of experiments in parentheses).
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 Kunzel et al. (21). Mutations were created in the CFTR gene using 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’-GAGTGGGAGTCAACGAG 3’, CFTR-A455E:5’-GGTTGGAGGTTGCTGG 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 Ba2+ for 2–3 min to inhibit ROMK2, then exposed to glibenclamide for 15 min, and finally exposed again to Ba2+ for 2–3 min. Figure 1A depicts a representative experiment in which a Xenopus oocyte expressed ROMK2 alone. In these experiments (n = 7), the Ba2+-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 co-injected with ROMK2:CFTR-WT (n = 12), the Ba2+-sensitive outward currents were reduced by 56.0 ± 10.0% (P = 0.001) after a similar exposure to glibenclamide (Fig. 1B, B 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 Ba2+-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 Ba2+-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

Fig. 2. Schematic representation of CFTR protein showing mutations studied here. A: two naturally occurring first nucleotide binding folds (NBF1) mutants, CFTR-G551D and CFTR-A455E. B: CFTR-K593X, a mutant truncated at residue 593, has an intact NBF1. C: CFTR-K370X is truncated at residue 370 prior to NBF1. Each of these mutants were coexpressed with wild-type ROMK2, and functional interaction was assayed as glibenclamide sensitivity of Ba2+-sensitive K+ currents.
GLIBENCLAMIDE SENSITIVITY OF ROMK2 REQUIRES CFTR-NBF1

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 inhibited by Ba²⁻. Observed currents for each CFTR construct were significantly different from those observed in uninjected cells (P = 0.002). Basal currents in unstimulated oocytes injected with CFTR were not significantly different from uninjected cells. Percent inhibition is whole cell current inhibited by application of 0.5 mM glibenclamide after a 10-min incubation. P values compare % of whole cell currents generated by each CFTR construct that are inhibited by glibenclamide with the % of wild-type CFTR (CFTR-WT) whole cell currents that are glibenclamide sensitive. NS, not significantly different from CFTR-WT. NS, not significant.

Fig. 3. Effect of glibenclamide on Ba²⁻-sensitive currents. A: Time course showing whole cell currents at V_m = −60 mV for Xenopus oocytes expressing ROMK2:CFTR-K593X (A) and ROMK2:CFTR-G551D (B) obtained using 2-microelectrode voltage-clamp techniques. Experiments were performed as described in Fig. 1.

Fig. 4. Effect of glibenclamide on Ba²⁻-sensitive currents obtained from Xenopus oocytes 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 for each condition are as follows: ROMK2 alone = 11.35 ± 3.3 µA, ROMK2:CFTR-WT = 8.29 ± 0.9 µA, ROMK2:CFTR-G551D = 5.57 ± 0.66 µA, ROMK2:CFTR-K593X = 2.37 ± 0.7 µA, ROMK2:A455E = 6.26 ± 1.39 µA, and ROMK2:K370X = 5.57 ± 0.66 µA.

CFTR does not appear necessary for the CFTR-ROMK2 interaction that leads to glibenclamide sensitivity.

To examine whether CFTR-NBF1 is the important region for this interaction and not the transmembrane domains, we coexpressed ROMK2 with CFTR-K370X (Fig. 2). In this CFTR construct, a premature termination codon at amino acid residue 370 results in a polypeptide that contains the first six transmembrane helices but does not contain NBF1. When ROMK2 and CFTR-K370X were coexpressed, the observed Ba²⁻-sensitive K⁺ currents decreased by only 12.3 ± 3.3% (n = 12) after oocytes were exposed to glibenclamide. This decrease is similar (P = 0.8) to that observed in oocytes expressing ROMK2 alone (Fig. 4) and provides further support that an intact NBF1 is essential for the enhanced glibenclamide response.

Next, we coexpressed ROMK2 with naturally occurring CFTR mutations within NBF1 (CFTR-G551D or CFTR-A455E). As shown in Fig. 3B, coexpressing ROMK2 with CFTR-G551D resulted in Ba²⁻-sensitive outward currents both before and after the oocyte was exposed to 0.5 mM glibenclamide for 15 min. The Ba²⁻-sensitive outward currents decreased by only 13.8 ± 6.7% after glibenclamide exposure (n = 6). This minimal reduction in the Ba²⁻-sensitive current following glibenclamide treatment was significantly less than that observed when ROMK2 was coexpressed with CFTR-WT (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.

To determine whether the change in CFTR-ROMK2 interaction was specific to the CFTR-G551D mutation or whether other CFTR-NBF1 mutations would produce a similar response, we examined the effect of coexpressing ROMK2 with another naturally occurring disease-causing NBF1-CFTR mutant construct, CFTR-
A455E (Fig. 2). Coexpressing ROMK2 with CFTR-A455E resulted in BaCl2-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<sub>ATP</sub> channels result from a complex of at least two subunits: a K<sup>+</sup> 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<sub>ATP</sub> 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<sub>ATP</sub> channels is not presently known. It is unlikely that these compounds act as channel pore blockers, given their effects on cloned K<sub>ATP</sub> 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 PHHI (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<sup>+</sup> 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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Glibenclamide Sensitivity of ROMK2 Requires CFTR-NBF1


