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WNK1 and WNK4 modulate CFTR activity

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP-gated chloride channel. WNK kinases are widely expressed modulators of ion transport. WNK1 and WNK4, two WNK kinases that are mutated in familial hyperkalemic hypertension (FHHt), are co-expressed with CFTR in several organs, raising the possibility that WNK kinases might alter CFTR activity *in vivo* or that CFTR could be involved in the pathogenesis of FHHt. Here, we report that WNK1 co-localizes with CFTR protein in pulmonary epithelial cells. Co-expression of WNK1 or WNK4 with CFTR in *Xenopus laevis* oocytes suppresses chloride channel activity. The effect of WNK4 is dose dependent and occurs, at least in part, by reducing CFTR protein abundance at the plasma membrane. This effect is independent of WNK4 kinase activity. In contrast, the effect of WNK1 on CFTR activity requires intact WNK1 kinase activity. Moreover WNK1 and WNK4 exhibit additive CFTR inhibition. Previous reports suggest that patients with FHHt exhibit mild changes in nasal potential difference that resemble the more severe changes that occur in cystic fibrosis. We report that the FHHt-causing mutant WNK4 Q562E is a more potent inhibitor of CFTR activity than is the wild-type WNK4. Taken together, these results suggest that WNK1 and WNK4 may modulate CFTR activity; they further suggest that WNK kinases may be potential therapeutic targets for cystic fibrosis.

Keywords: Cystic fibrosis; Chloride channels; WNK kinases; Na channels; Pseudohypoaldosteronism type II; Familial hyperkalemic hypertension; Lung mutation

The WNK (for With No Lysine (K)) kinases are unique serine/threonine kinases with the catalytic lysine in subdomain I rather than subdomain II [1]. Mutations in two WNK kinases, WNK1 and WNK4, cause familial hyperkalemic hypertension, FHHt (also known as pseudohypoaldosteronism type II or Gordon's syndrome), a kidney-related disease of hypertension with hyperkalemia [2]. The mechanisms by which WNK mutations cause disease are not fully understood, but WNK kinases have been implicated in the regulation of ion transport by divergent mechanisms. Although FHHt has a renal phenotype, WNK kinases are widely expressed essential proteins. Notably, the predominant sites of WNK expression are epithelia that transport chloride [3]. The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-gated chloride channel produced by the gene that is mutated in cystic fibrosis (CF). WNK kinases are co-expressed with the CFTR in a variety of tissues [4], raising the possibility that either WNK1 or WNK4 may affect the CFTR [4]. Accordingly, the current experiments were designed to test the hypothesis that WNK kinases affect CFTR activity.

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Materials and methods

Antibody preparation. Constructs encoding amino acid residues 2–167 of mouse WNK4 and residues 2–126 of rat WNK1 were generated by polymerase chain reaction and subcloned into pGEX6-P-1 (Amersham Pharmacia Biotech). GST-WNK4 2–167 and GST-WNK1 2–126 fusion proteins were purified from *Escherichia coli* by standard methods. The proteins were used to immunize rabbits. The resulting serum was purified using GST and fusion protein affinity columns, dialyzed in phosphate-buffered saline, and concentrated.

DNA constructs. Mouse WNK4, rat WNK1 and human CFTR were generated as described [5–7]. The full-length human WNK3 cDNA clone was purchased from OriGene Technologies, Inc. (Rockville, MD). WNK3 was subcloned into the mammalian/*Xenopus* oocyte expression vector PMO by PCR amplification. The DNA was sequenced confirming the absence of PCR errors.

In vitro transcription. The CFTR cRNAs for *Xenopus* oocyte injection were synthesized using the *in vitro* transcription kit, mMessage Machine (Ambion, Inc.), as described [5].

Electrophysiological recordings. Oocyte preparation was as described [5]. Oocytes were co-injected with ~0.15 ng of CFTR cRNA and 5 ng of WNK4, WNK1, WNK1 D368A or WNK4 Q562E, unless otherwise indicated. Three days following injection, CFTR-mediated chloride current was measured using two-electrode voltage-clamp (TEVC) as described [8]. CFTR channels were activated using a cocktail containing 1 mM isobutylmethyl xanthine (IBMX, Sigma, St. Louis, MO), a phosphodiesterase inhibitor, and 10 μM isoproterenol (ISOP, Sigma, St. Louis, MO), an agonist for β-adrenergic receptors or forskolin, a cAMP agonist. The majority of the experiments were done using isoproterenol and IBMX (Isop + IBMX) where oocytes were also co-injected with the cRNA of β-adrenergic receptor.

Data were acquired as described [9]. Briefly, oocytes were continuously perfused with frog Ringer's solution (in mM: 98 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 5 Hepes-SemiNa). The volume of the perfusion chamber was ~100 μl and the flow rate to the chamber was 4 ml/min at 24 °C. The two-electrode voltage-clamp system and the pClamp data acquisition program (Axon Instruments, Inc.) were used. Oocytes were normally kept under open circuit conditions in experimental chambers. At the time of interest, the membrane potential was ramped from −120 to +60 mV in a period of 1.8 s to construct the whole-cell *I–V* plots.

CFTR surface protein biotinylation. Human CFTR cDNA was subcloned into pgh19. Five nanogram cRNA of CFTR was injected with and without 10 ng cRNA of WNK4 or WNK1 per oocyte. Surface proteins were biotinylated, as described [5], and CFTR was immunoprecipitated using a monoclonal anti-CFTR antibody (R&D Systems, Minneapolis, MN).

Immunocytochemistry. Immunocytochemistry was performed on formaldehyde-fixed lung tissue of young female Sprague–Dawley rats. During surgery, lung collapse was prevented by applying positive airway pressure via nasal ventilation with a polyethylene tube. After opening the left atrium, the lungs were flushed for 20 s with a cacodylate-buffered sucrose solution (0.1 M sodium cacodylate, 300 mOsm, pH 7.4) and subsequently perfused with a fixative containing 3% paraformaldehyde in cacodylate-buffered sucrose solution. After fixation lung tissue was removed and processed for paraffin embedding. For immunofluorescence 3-μm paraffin sections were immersed in xylol to remove the paraffin and then rehydrated in decreasing concentrations of ethanol. Sections were then boiled for 6 min in an antigen retrieval solution containing 16.4 mM sodium citrate and 3.6 mM citric acid (pH 6). To block unspecific protein-binding sites sections were incubated for 1 h with 5% skimmed milk in PBS (pH 7.4). Primary antibodies were applied in 5% skimmed milk in PBS (CFTR 1:200, WNK1 1:100). Sections were incubated for 2 h at room temperature and then overnight at 4 °C. Bound antibody was detected using the respective secondary Cy2- or Cy3-labeled secondary antibodies. Double labeling was achieved by repeating the aforementioned procedures on the single labeled sections. Tissue distribution of the antigens was evaluated using a Leica DMRB microscope equipped with a fluorescence module.

Data analysis. The TEVC data were analyzed using an analysis program developed in the Dawson laboratory [10] and are presented as *I–V* plots. The conductance reported here was calculated from the slope of the *I–V* plot at the reversal potential ($V_m = E_{rev}$) using a voltage range from $V_m = E_{rev} = 10$ mV to $V_m = E_{rev} + 10$ mV. Data are reported as means ± SEM.

Results

WNK1 and WNK4 are expressed in lung and kidney

Antibodies directed against WNK1 and WNK4 recognized these proteins specifically, when the proteins were expressed in *Xenopus* oocytes (Fig. 1A). Both WNK1 and WNK4 are detected in kidney (Fig. 1B) and in calu-3 cells, a human lung carcinoma cell line that expresses CFTR [11]. Fig. 1C shows that pulmonary ciliary cells express both WNK1 protein, within the cytoplasm, and CFTR protein, at the apical membrane.

WNK1 inhibits CFTR activity by a kinase-dependent mechanism

Channels in oocytes expressing CFTR along with a WNK kinase were activated by isoproterenol or forskolin and IBMX (Isop + IBMX) as described [8]. Fig. 2A shows that co-expression with WNK1 reduced CFTR conductance by more than 40%, compared with oocytes expressing CFTR alone ($P < 0.01$). This effect of WNK1 on CFTR activity was dependent, at least in part, on intact kinase activity, because the effects of a kinase-dead WNK1 (WNK1 (D368A)) were significantly less than those of the wild-type WNK1 (Fig. 2A). To establish the specificity of this effect, CFTR was co-expressed with an unrelated membrane protein, the thiazide-sensitive Na–Cl co-transporter, NCC. Fig. 2A shows that co-expression with NCC had no effect on CFTR activity. WNK1 itself did not enhance chloride conductance, indicating that it does not function as a chloride channel (Fig. 2A). In view of the inhibiting effect of WNK1, a serine/threonine kinase, on CFTR activity, we tested whether an unrelated serine/threonine kinase would also affect CFTR activity. Co-expression with sgk1 stimulated CFTR activity significantly in the current experiments. Stimulation of CFTR activity by sgk1 has been previously reported [12] (see Fig. 2A). Finally, we determined whether the effects of WNK1 were a non-specific effect of all WNK kinases by co-expressing CFTR with WNK3. Fig. 2B shows that WNK3 had no effect on CFTR conductance. Together, these data indicate that the ability of WNK1 to suppress CFTR activity is not the result of translational interference (cf. NCC experiments), is not a non-specific serine/threonine kinase effect (cf. sgk and WNK3 experiments), and is not a generic effect of WNK kinases (cf. WNK3 experiments).

Fig. 2C shows representative *I–V* plots for oocytes expressing CFTR alone and with WNK1. WNK1 did not affect the shape of the *I–V* curve. It should be noted that