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The CLIC1 Chloride Channel Is Regulated by the Cystic Fibrosis Transmembrane Conductance Regulator when Expressed in *Xenopus* Oocytes

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Abstract. CLIC proteins comprise a family of chloride channels whose physiological roles are uncertain. To gain further insight into possible means of CLIC1 channel activity regulation, this protein was expressed in *Xenopus* oocytes alone or in combination with the cystic fibrosis transmembrane conductance regulator (CFTR). Whole-cell currents were determined using two-electrode voltage-clamp methods. Expression of CLIC1 alone did not increase whole-cell conductance either at rest or in response to increased intracellular cyclic adenosine monophosphate (cAMP). However, expression of CLIC1 with CFTR led to increased cAMP-activated whole-cell currents compared to expression from the same amount of CFTR mRNA alone. IAA-94 is a drug known to inhibit CLIC family channels but not CFTR. In oocytes expressing both CLIC1 and CFTR, a fraction of the cAMP-activated whole-cell current was sensitive to IAA-94, whereas in oocytes expressing CFTR alone, the cAMP-stimulated current was resistant to the drug. Cell fractionation studies revealed that the presence of CFTR conferred cAMP-stimulated redistribution of a fraction of CLIC1 from a soluble to a membrane-associated form. We conclude that when expressed in *Xenopus* oocytes CFTR confers cAMP regulation to CLIC1 activity in the plasma membrane and that at least part of this regulation is due to recruitment of CLIC1 from the cytoplasm to the membrane.

Key words: Chloride channel — CLIC — Cystic fibrosis transmembrane conductance regulator — cAMP-activated chloride channel — Cystic fibrosis — NCC27 — CLIC1

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Introduction

CLIC1, also known as NCC27, is a member of the CLIC family of chloride channels (reviewed in Ashley 2003; Cromer et al., 2002). These proteins, which have significant structural homology with glutathione-*S*-transferase (Harrop et al., 2001), are atypical ion channels in that they exist in both a soluble and a membrane-inserted form *in vivo*. Essentially pure preparations of both recombinant CLIC1 and CLIC5A have been shown to function as anion-selective channels *in vitro*, and this activity can be inhibited by the drug indonyloxyacetic acid 94 (IAA-94) (Tulk et al., 2000; Berryman et al., 2004). Furthermore, CLIC1 prepared from bacteria in a soluble form in the absence of detergent is capable of direct insertion into preformed phospholipid membranes, where it functions as a channel (Tulk, Kapadia & Edwards, 2002; Warton et al., 2002).

Mechanisms that might regulate the channel activity or membrane insertion of the CLIC proteins *in vivo* or *in vitro* are poorly understood. Several parameters have been proposed to be relevant. First, insertional activity is clearly dependent on the lipid composition of target membranes, with an apparent requirement for negatively charged lipids (Tulk et al., 2002; Singh and Ashley, 2006). Second, pH has been reported to affect insertional activity, although different groups have reported different shapes of the pH-activity curve (Tulk et al., 2002; Warton et al., 2002). Third, the oxidation state of CLIC1 has been shown to affect activity, although, again, different groups have reported different patterns of dependence and it remains uncertain whether CLIC1 is oxidized *in vivo* (Singh and Ashley, 2006; Littler et al., 2004). Fourth, CLIC proteins can act as substrates of various kinases (Edwards, Tulk & Schlesinger, 1998), and, as has been shown for CLIC5B, tyrosine phosphorylation may effect activity (Edwards and

Kapadia, 2000; Edwards et al., 2006). Finally, CLIC proteins have been known to participate in a number of protein-protein interactions, and it has been proposed that these interactions, particularly with cytoskeletal components, might play an important role in regulation of CLIC activity *in vivo* (Berryman et al., 2004; Berryman and Goldenring, 2003; Shanks et al., 2002; Berryman and Bretscher, 2000; Qian et al., 1999).

Although CLIC proteins clearly can function as channels *in vitro*, their role in normal cell physiology remains elusive. Elucidation of mechanisms regulating CLIC activity and/or membrane insertion would help define the possible cellular roles of these proteins. Clearly, the regulation of partitioning between soluble and membrane-inserted forms of CLIC would be critical to regulation of channel activity.

The cystic fibrosis transmembrane conductance regulator (CFTR) is the protein which is defective in cystic fibrosis (reviewed in Jentsch, Maritzen & Zbedik, 2005; Nilius and Droogman, 2003). One of the physiological defects in cystic fibrosis is a failure of cyclic adenosine monophosphate (cAMP)-regulated chloride conductance in a variety of epithelia. CFTR is known to function as a cAMP-regulated chloride channel itself, and clearly at least some of the defect in cystic fibrosis is due to loss of CFTR channel activity. Unlike CLIC channels, the CFTR channel is not significantly inhibited by IAA-94 (Walsh and Wang, 1996; Husted et al., 1995). In addition to functioning as a channel itself, CFTR is a regulator of other channels. CFTR regulates the activity of the epithelial sodium channel, the ROMK2 potassium channel, the sodium-proton exchanger, aquaporins and others (reviewed in Nilius and Droogman, 2003; Kunzelman and Schrieber, 1999), including at least one other chloride channel with properties distinct from the CFTR channel (Ogura et al., 2002; Schwiebert et al., 1995).

To investigate whether CFTR might play a role in the regulation of CLIC proteins, we used a *Xenopus* oocyte system to express CLIC1 and CFTR, both singly and together, and to assess for whole-oocyte conductance at baseline and in response to increased intracellular cAMP. We found that expression of CLIC1 alone had no effect on basal or cAMP-activated conductance. As expected, expression of CFTR alone resulted in robust cAMP-activated conductance which was not inhibited by IAA-94. Expression of CLIC1 with CFTR enhanced the cAMP-activated conductance compared to CFTR alone, and this conductance was partially and reversibly inhibited by IAA-94. Finally, fractionation of oocytes revealed that the fraction of CLIC1 that is membrane-associated is enhanced by raising intracellular cAMP in oocytes that express both CFTR and CLIC1 but not in oocytes that express CLIC1 alone. We conclude that CFTR confers cAMP regulation to CLIC1, resulting in increased plasma membrane chloride conductance and increased membrane association.

Materials and Methods

Plasmids and Drugs

pGEMHECFTR, encoding full-length human CFTR downstream of the T7 promoter and flanked by *Xenopus* β-globin 5′ and 3′ untranslated regions, was provided by Dr. Colin Nichols at Washington University (St. Louis, MO). TFpG11 was generated by insertion of the human CLIC1 coding region from cDNA clone PG11 (Tulk et al., 2000) into the *Xenopus* expression vector SP64T (Krieg and Melton 1984). Cesium chloride gradients were used to prepare plasmid DNA for transcription.

Isobutylmethylxanthine (IBMX), forskolin and 8-Br-cAMP were purchased from Sigma (St. Louis, MO). IAA-94 was from Biomol (Plymouth Meeting, PA).

Preparation of RNA and Oocyte Injection

pGEMHECFTR was linearized with restriction endonuclease *Nhe*I; TFpG11 was linearized with *Bam*HI. Template DNAs were transcribed using the mMessage mMachine kit (Ambion, Austin TX), and the RNA yield was determined by incorporation of α-^{32}P uridine triphosphate (UTP). Products were dissolved in nuclease-free water at 0.5 μg/μl.

Stage IV-V oocytes were isolated from female *Xenopus laevis* frogs, digested with collagenase and then stored at 15°C in ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES, pH 7.2]) plus 1.8 mM $CaCl_2$ using standard protocols (Goldin, 1992). Healthy-appearing oocytes were selected for injection, and oocytes were injected and handled using published methods (Soreq and Seidman, 1992). Injection pipettes were mounted on a microprocessor-controlled positive displacement syringe (Nanoliter 2000; World Precision Instruments, Sarasota, FL). Fifty nanoliters of appropriate RNA solution or water were injected into each oocyte. Injected oocytes were incubated in ND96 with 1.8 mM Ca at 15°C for 36–60 h prior to study.

Oocyte Recordings

Recordings were performed with an OC725c voltage clamp (Warner Instruments, Hamden, CT) interfaced through an Axon Digidata 1200 analog-digital converter to a personal computer running Clampex7 (Axon Instruments, Foster City, CA) following published protocols (Stuhmer, 1992, 1998). The head stage was connected to the oocyte recording chamber through agarose salt bridges in 3 M KCl. The chamber was continuously perfused using a gravity-driven system with manual solution changer and flow rate controller. Individual oocytes were selected, mounted in the oocyte chamber and continuously perfused with ND96 (no calcium). Microelectrode pipettes with resistances of 5–10 megaohms were prepared with a P-30 vertical pipette puller (Sutter Instruments, Novato, CA), filled with 3 M KCl and mounted on the voltage and current intracellular electrodes. The pipettes were inserted into the oocyte, and initial resting potential was determined. Oocytes with resting potentials less negative than -10 mV were discarded.

Current-voltage relationships were determined using a voltage step protocol in which the membrane was held for 0.5 s at potentials between −100 and + 100 mV in 10-mV increments and the resultant current recorded. The average current and voltage were determined for the latter 400 ms of each sweep (after capacitance

transients had dissipated). Data are reported as mean ± standard error of the mean.

Oocytes were stimulated by perfusing the chamber with ND96 (no calcium) supplemented with 100 μM IBMX, 10 μM forskolin and 250 μM 8-Br-cAMP. Inhibition solution was identical with the addition of 250 μM IAA-94. Stock solutions of drugs were made in dimethyl sulfoxide (DMSO) as follows: IBMX 100 mM, forskolin 10 mM, 8-Br-cAMP 500 mM, IAA-94 500 mM. Each perfusion solution was adjusted to contain identical amounts of DMSO throughout the experiment.

Western Blotting

To test expression, individual oocytes were placed in 100 μl of loading buffer (50 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 20 mM dithiothreitol, 10% glycerol, 0.002% bromophenol blue), vigorously vortexed and then heated to 95°C for 5 min. Five microliters were loaded onto a 10% SDS-polyacrylamide gel. For fractionation experiments, groups of five oocytes were suspended in 1 ml of homogenization buffer (150 mM NaCl, 20 mM Tris [pH 8.0], 1 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM phenylmethyl sulfonyl fluoride [PMSF]) and homogenized with a Teflon® Dounce homogenizer on ice. The solution was centrifuged at 40,000 rpm (100,000 x *g*) at 4°C for 1 h in a Ti70 Beckman (Fullerton, CA) rotor. The supernatant was taken as the soluble fraction. The pellet was washed by resuspension in 1 ml of homogenization buffer, followed by centrifugation as before. The pellet was then dissolved in 0.2 ml of 150 mM NaCl, 20 mM Tris (pH 8.0), 1% nonidet P40 and 0.1 mM PMSF and centrifuged at 14,000 rpm at 4°C for 30 min in an Eppendorf microcentrifuge. The supernatant was taken as the membrane fraction. Protein concentration was determined with the bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotting to nitrocellulose sheets was performed using standard methods. Western blots were developed using the AP823 antibody specific for CLIC1 (Tulk and Edwards, 1998) with Supersignal chemiluminescent reagents (Pierce Biochemicals) and detected by autoradiography.

Results

Expression of CLIC1 in Oocytes

Fifty nanoliters of nuclease-free water or mRNA encoding human CLIC1 (0.5 μg/μl) were injected into oocytes and the oocytes incubated for 48 h at 15°C. Individual oocytes were solubilized in loading buffer, and equal fractions were separated on 12% SDS-PAGE gels, blotted to nitrocellulose and probed with affinity-purified antibody raised against CLIC1. The results are shown in Figure 1. Lanes 1–6 are from six individual oocytes injected with CLIC1 mRNA. Lane 7 is a pooled sample from five water-injected oocytes. No CLIC antigen is detected in the water-injected oocytes, while each of the six CLIC1 mRNA-injected oocytes expressed the expected CLIC protein with an apparent molecular weight of 34 kDa by SDS-PAGE. There appears to be some variability in the level of expression among the individual oocytes, although these samples were not normalized for total protein or oocyte size.

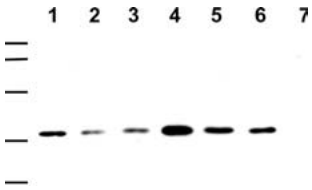

Fig. 1. Expression of CLIC1 in oocytes. Total protein from six individual oocytes injected with CLIC1 mRNA (*lanes 1–6*) or from the same fraction of total protein pooled from five oocytes injected with water (*lane 7*) were separated by SDS-PAGE, blotted to nitrocellulose and probed for CLIC1. *Bars to the left* indicate migration position of molecular size standards of 96, 66, 45, 31 and 21 kDa (top to bottom).

Whole-Cell Currents in Oocytes Expressing CLIC-1, CFTR or Both

To avoid excessive CFTR-dependent current, each preparation of CFTR mRNA was titrated to find a concentration which produced small but easily detectable cAMP-dependent activation of whole-cell currents on the order of approximately 10 μA at 100 m*V*. This was typically with CFTR mRNA at approximately 0.005 μg/ml. Using this concentration of CFTR RNA, oocytes were injected with 50 nl of either water, 1:1 mixture of CFTR mRNA and water, CLIC mRNA (0.5 μg/ml) and water or CFTR mRNA and CLIC mRNA. With this protocol, the mass of each specific RNA should be identical in each of the groups of oocytes that received that RNA. The oocytes were incubated at 15°C for 24–48 h. The oocytes were mounted in the perfusion chamber and impaled with electrodes, and resting potential was determined while the chamber was continuously perfused with ND96. Oocytes with acceptable resting potential were subjected to an *I-V* step protocol to determine resting whole-cell conductance. The chamber was then perfused with ND96 with 10 μM forskolin, 100 μM IBMX and 250 μM 8-Br-cAMP. After 10 min, another *I-V* step protocol was performed. Results are shown in Figure 2. Water-injected oocytes ($n = 3$) had low resting plasma membrane conductance and no significant response to stimulation (Fig. 2A, triangles). Oocytes injected with CLIC1 mRNA alone ($n = 11$) also had low resting conductance with no significant response to stimulation (Fig. 2A, squares). Thus, expression of CLIC1 in *Xenopus* oocytes does not lead to increased plasma membrane ionic permeability either at baseline or in response to activation of the cAMP pathway.

Oocytes injected with CFTR mRNA alone ($n = 21$) showed low basal conductance that was not different from that of water-injected oocytes. As expected, stimulation with cAMP led to increased whole-cell conductance due to activation of the

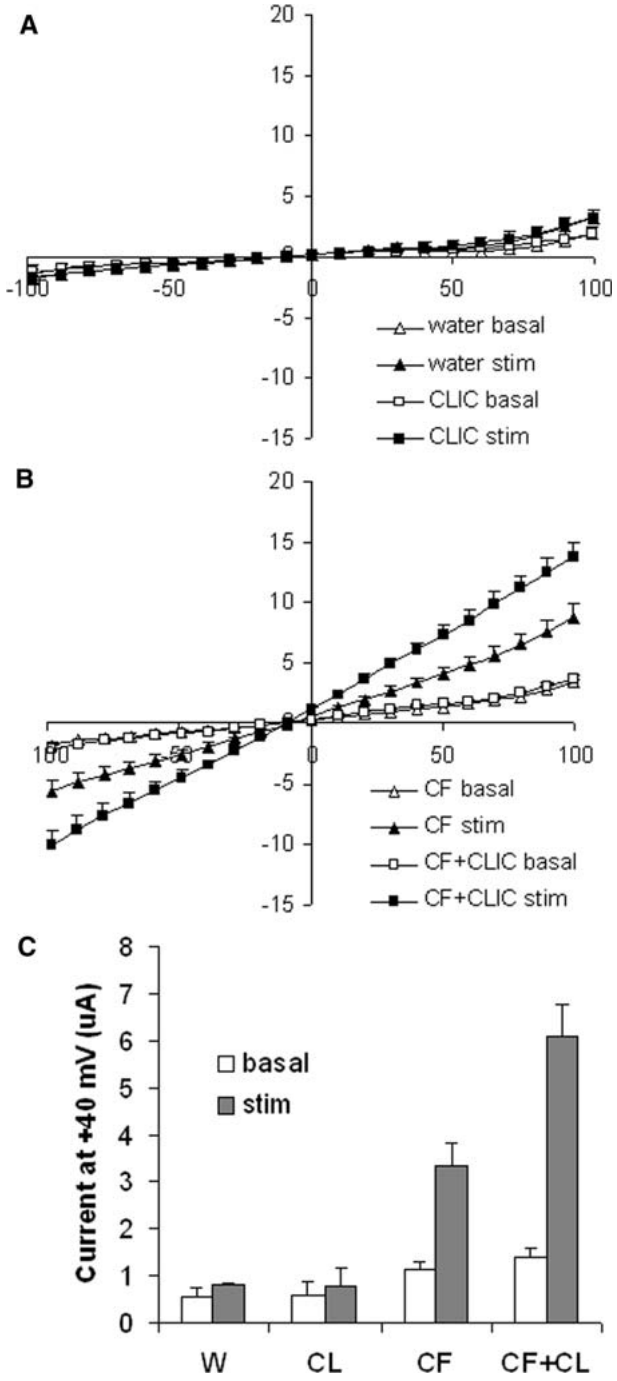

Fig. 2. Current-voltage relationships of oocytes expressing CLIC1 and CFTR alone and in combination. Current-voltage relationships were determined for oocytes injected with water, CLIC1, CFTR or CLIC1 + CFTR before (*basal*) and after (*stim*) exposure to 8-Br-cAMP, forskolin and IBMX. Plots represent the average of each group of oocytes. Error bars represent standard error of the mean for each data point; error values smaller than the symbols are not visible. (*A*) Water-injected oocytes (*triangles*, $n = 3$) and CLIC1-injected oocytes (*squares*, $n = 11$) under basal conditions (*open symbols*) or following stimulation (*closed symbols*). (*B*) CFTR- injected oocytes (*triangles*, $n = 21$) or CFTR + CLIC1-injected oocytes (*squares*, $n = 21$) under basal condition (*open symbols*) or following stimulation (*closed symbols*). (*C*) Average current at + 40 m*V* holding potential for each group of oocytes labeled as follows: *W*, water-injected ($n = 3$); *CL*, CLIC1-injected ($n = 11$), *CF*, CFTR-injected ($n = 21$) and *CF + CL*, CFTR + CLIC1-injected ($n = 21$). *Open bars* represent current under basal condition; *closed bars* represent current following stimulation. Error bars represent standard error of the mean. Values of means and standard errors are presented in the text.

CFTR chloride channel (Fig. 2B, triangles). Oocytes injected with an identical amount of CFTR mRNA as the previous group plus CLIC1 RNA ($n = 21$) also showed low basal current and large cAMP-stimulated current (Fig. 2B, squares). The cAMP-stimulated current was markedly greater than that supported by CFTR mRNA alone.

Average whole-cell currents for each group of ooctyes at 40 m*V* are shown in Figure 2C. Water-injected oocytes showed basal current of 0.55 ± 0.21 μA and cAMP-stimulated current of 0.82 ± 0.03 μA ($n = 3$). Oocytes injected with CLIC1 mRNA alone showed basal current of 0.59 ± 0.31 μA and cAMP-stimulated current of 0.79 ± 0.41 μA ($n = 11$). Oocytes injected with CFTR mRNA alone showed basal current of 1.16 ± 0.15 μA and cAMP-stimulated current of 3.34 ± 0.50 μA ($n = 21$). Oocytes injected with both CFTR and CLIC1 mRNA showed basal currents of 1.42 ± 0.17 μA and stimulated currents of 6.11 ± 0.65 μA ($n = 21$). The difference between cAMP-stimulated currents of CFTR only and CFTR plus CLIC oocytes was highly significant ($P < 0.001$). The difference in basal currents among the groups did not reach significance with any pairwise comparison (significance determined using analysis of variance).

IAA Inhibition of cAMP-Activated Currents

The above results indicate that expression of CLIC1 alone does not lead to increased basal or cAMP-induced cell membrane conductance but that expression of CLIC1 does enhance the CFTR-dependent, cAMP-induced current. One potential explanation for this observation would be that CFTR confers cAMP regulation to CLIC1. If a portion of the cAMP-activated current in the oocytes expressing both proteins is actually carried through a CLIC1 channel, that portion of the current should be inhibitable with IAA-94. Alternatively, if the enhanced current in the presence of CLIC1 is due to increased chloride transit through CFTR itself, the current should not be inhibited by the drug.

Oocytes expressing CFTR alone or CFTR plus CLIC1 were studied as above. Since we wanted to have roughly equal total stimulated current in the two groups of oocytes, we used approximately 1.5-fold more CFTR RNA in the oocytes expressing

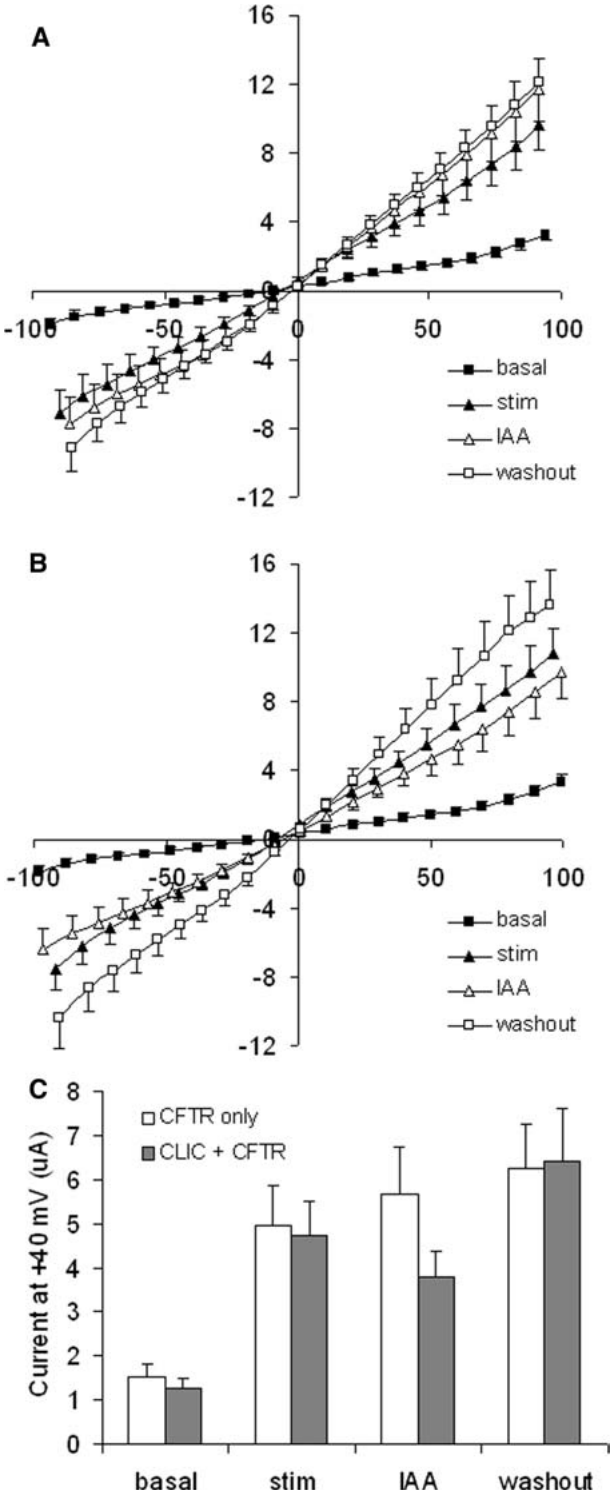

Fig. 3. Effect of IAA-94 on stimulated currents of oocytes expressing CFTR alone or CFTR plus CLIC1. *I-V* plots derived from averages of each group of oocytes following treatment as described in text. For both *A* and *B*, symbols are as follows: *closed squares*, basal state prior to stimulation; *closed triangles*, following 10-min stimulation with 8-Br-cAMP, forskolin and IBMX; *open triangles*, following 10-min exposure to 250 μM IAA-94 in the continued presence of stimulatory cocktail; *open squares*, following 10-min washout of IAA-94 in the continued presence of stimulatory cocktail. Plots represent the averages from each group of oocytes. Error bars represent the standard error of the mean for each data point; error values smaller than the symbols are not visible. (*A*) Oocytes expressing CFTR alone. (*B*) Oocytes expressing CFTR and CLIC1. (*C*) Average total current at + 40 m*V* holding potential for each group of oocytes after each treatment period, labeled as follows: *basal*, basal state prior to stimulation; *stim*, after 10-min exposure to stimulatory cocktail; *IAA*, after 10-min exposure to 250 μM IAA-94 in continued presence of cocktail; *washout*, following 10-min washout of IAA-94 in continued presence of cocktail. Error bars represent standard errors of the mean. See text for values of means, standard errors and significance differences between individual pairs of means.

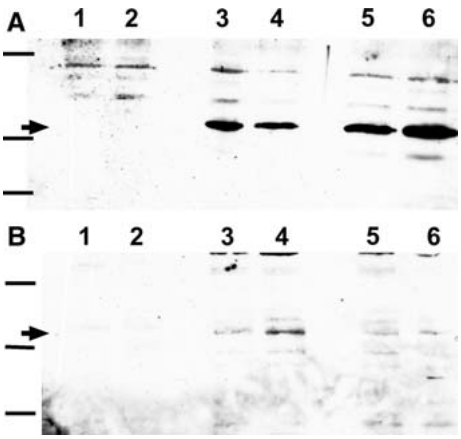

Fig. 4. CFTR-dependent, cAMP-stimulated redistribution of CLIC1 in oocytes. Groups of oocytes expressing CLIC1 alone or CFTR plus CLIC1 were exposed to control medium or stimulated with 8-Br-cAMP, forskolin and IBMX and then separated into soluble and membrane fractions. Four micrograms of soluble protein (*A*) or 20 μg of membrane protein (*B*) were separated by SDS-PAGE, blotted to nitrocellulose and probed for CLIC1. In each panel, lanes are as follows: *1*, water-injected, unstimulated; *2*, water-injected, stimulated; *3*, CFTR + CLIC1-injected, unstimulated; *4*, CFTR + CLIC1-injected, stimulated; *5*, CLIC1-injected, unstimulated; *6*, CLIC1-injected, stimulated. *Bars to the left* in each panel indicate migration positions of molecular weight markers of 45, 31 and 21 kDa. *Arrowheads* indicate migration position of CLIC1.

CFTR alone than in the oocytes expressing both CFTR and CLIC1. After determining resting potential, a baseline *I-V* relationship was determined. Oocytes were exposed to the cAMP cocktail for 10 min, and a second *I-V* curve was collected. The oocyte chamber was then perfused for 10 min with ND96 with stimulatory cocktail plus 250 μM IAA-94, and another *I-V* curve was recorded. Finally, IAA-94 was washed out with the continued presence of stimulatory cocktail for 10 min, and a final *I-V* relationship was obtained. Average *I-V* relationships for oocytes expressing CFTR alone or CFTR plus CLIC1 are shown in Figure 3A and 3B, respectively. In both sets of oocytes, cAMP stimulatory cocktail

led to increased whole-cell conductance. In cells expressing CFTR alone (Fig. 3A), the whole-cell conductance continued to increase slightly after 10-min exposure to IAA in the continued presence of cocktail. Conductance did not change significantly with subsequent washout of the IAA during continued cAMP stimulation. In contrast, cells expressing both CLIC1 and CFTR (Fig. 3B) showed a decrease in whole-cell conductance following exposure to IAA-94 in the continued presence of stimulatory cocktail. Furthermore, conductance returned to levels above the initial stimulated level following washout of the drug.

Average whole-cell currents at 40 mV for each group of oocytes are shown in Figure 3C. The CFTR-only oocytes ($n = 21$) had an average basal current of 1.54 ± 0.28 μA, a stimulated current of 4.96 ± 0.91 μA, an IAA-inhibited current of 5.70 ± 1.03 μA and a postwashout current of 6.25 ± 0.99 μA. In contrast, the CLIC1 plus CFTR oocytes ($n = 18$) exhibited an average basal current of 1.26 ± 0.23 μA, a stimulated current of 4.73 ± 0.79 μA, an IAA-inhibited current of 3.80 ± 0.58 μA and a postwashout current of 6.43 ± 1.20 μA. Using a paired t-test, the IAA-inhibited current was significantly different from both the stimulated and postwashout currents in oocytes expressing both CLIC1 and CFTR ($P < 0.05$).

CFTR-Dependent, cAMP-Driven Redistribution of CLIC1 in Oocytes

Groups of five oocytes injected with water, CLIC1 mRNA alone or CLIC1 plus CFTR mRNA were incubated in ND96 or ND96 with stimulatory cocktail for 10 min at room temperature. Each group was then separated into soluble and membrane fractions. Five micrograms of each soluble sample and 20 μg of each membrane fraction were separated by SDS-PAGE, blotted to nitrocellulose and probed with affinity-purified antibodies to CLIC1. Results are shown in Figure 4. No CLIC1 was detected in either fraction of resting or stimulated cells which had been injected with water (Fig. 4A and B, lanes 1 and 2). In cells expressing CLIC1 alone, the great majority of the protein was in the soluble fraction (Fig. 4A, lanes 5 and 6). However, a faint band was detectable in the membrane fraction in unstimulated cells (Fig. 4B, lane 5), and the intensity of this band did not change with cAMP stimulation (Fig. 4B, lane 6). In cells expressing CFTR plus CLIC1, the distribution in resting cells was similar, with the vast majority of the protein in the soluble fraction (Fig. 4A, lane 3) and a faint signal in the insoluble fraction (Fig. 4B, lane 3). However, in response to cAMP stimulation, the fraction of CLIC in the insoluble fraction increased (Fig. 4B, lane 4).

Discussion

In this report, we demonstrate that coexpression of CLIC1 with CFTR in *Xenopus* oocytes leads to increased CFTR-dependent, cAMP-activated plasma membrane conductance compared to expression of CFTR alone. Furthermore, expression of CLIC1 confers sensitivity to IAA-94, a drug which is known to inhibit CLIC channels but not the CFTR channel, to a fraction of the CFTR-dependent, cAMP-activated current. Finally, CFTR confers cAMP-stimulated redistribution of CLIC1 to the membrane fraction. These results support the hypothesis that CFTR regulates CLIC1 in a cAMP-dependent manner, leading to membrane insertion and plasma membrane channel activity of this protein.

We found increased cAMP-stimulated conductance in oocytes expressing CLIC1 plus CFTR compared to oocytes expressing CFTR alone, and this difference reached a high level of statistical significance. However, cAMP-stimulated conductance of oocytes injected with CFTR can be variable. Several aspects of our experimental protocol contribute to our confidence that this difference is indeed real. First, these results come from recordings made over multiple days, using oocytes from different frogs and different, independently synthesized mRNA preps. Second, oocytes from each experimental group were studied each day and were alternated to avoid any unintended systematic differences in levels of expression, changes in solution temperature or flow rates, etc., during the course of the day. Third, the strategy of generating 1:1 mixtures of RNA stocks with each other or with water prior to injection and the use of a microprocessor-controlled microinjector ensured that the same amount of RNA was injected into each oocyte. Fourth, to maximize reproducibility, we intentionally used relatively low levels of CFTR mRNA, keeping the cAMP-stimulated current in a range that did not result in enormous conductances which could have deleterious effects on the oocytes during prolonged recording. To complement the low overall level of CFTR expression, we used a potent cocktail of forskolin, 8-Br-cAMP and IBMX to maximize the activation of the CFTR that was present. With these considerations and in light of the high level of significance using standard statistical analysis, we are confident that this difference is meaningful.

Having found that CLIC potentiates the CFTR-dependent, cAMP-stimulated conductance, we then used a pharmacological tool to characterize the nature of this effect. One key question is whether the cAMP-stimulated conductance is entirely due to CFTR or whether some of the current could be carried by CLIC itself. To distinguish these two possibilities, we stimulated oocytes with cAMP cocktail and then challenged the oocytes with exposure to

IAA-94 followed by washout of the drug. We found a clear difference in oocyte response, with CFTR-only oocytes continuing to increase plasma membrane conductance with the continued presence of stimulatory cocktail despite the addition of IAA-94. Moreover, the washout of IAA-94 had little effect other than continued activation. This pattern is consistent with the entire cAMP-activated current being carried by CFTR itself. In contrast, oocytes expressing CFTR and CLIC1 showed decreased whole-cell conductance in the presence of IAA-94 despite continued presence of cocktail and a dramatic increase of conductance following IAA washout. The simplest explanation for these observations is that some of the current in the coexpressing oocytes is carried by the CLIC1 protein and that CFTR confers cAMP regulation to CLIC1 channels. IAA-94 inhibits the fraction of the current which is carried by CLIC, leaving the CFTR current intact, while washout of the drug unmasks the continued cAMP activation occurring in the continued presence of cocktail, resulting in a higher level of current after the washout than before.

Although we believe this interpretation of the data is the simplest, other explanations for the observations are compatible with the data. For instance, through unknown mechanisms, CLIC1 could enhance cAMP activation of CFTR and IAA could block this effect rather than directly blocking channel channel activity. Alternatively, CLIC could enhance expression or plasma membrane targeting of CFTR. However, the rapid effect of the inhibitor seems incompatible with effects on synthesis of the protein.

If CFTR confers cAMP regulation to CLIC1 channel activity, it could do so in at least two distinct ways: it could activate CLIC1 already residing in the membrane, or it could cause soluble CLIC1 to be translocated from the cytoplasm to the membrane, where it could function as a channel. The cell fractionation studies reported here indicate that in the presence, but not the absence, of exogenous CFTR, the fraction of CLIC1 which is membrane-associated is enhanced by cAMP. Thus, CFTR may increase CLIC1 channel activity at least partly by causing membrane insertion and hence increasing the amount of CLIC1 protein in the plasma membrane.

The regulation of CLIC1 by CFTR suggests that some aspects of the cystic fibrosis disease process may be due to failure to activate CLIC channel activity. However, until the roles of CLIC1 in normal physiology are known, there is little basis for speculation as to what part of the cystic fibrosis phenotype may be attributable to dysregulation of CLIC1. Whether CFTR regulates other members of the CLIC family remains to be seen.

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