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Downregulation of Epithelial Sodium Channel (ENaC) by CFTR Co-expressed in *Xenopus* Oocytes is Independent of Cl^- Conductance

H. Chabot, M.F. Vives, A. Dagenais, Cz. Grygorczyk, Y. Berthiaume, R. Grygorczyk
Centre de recherche, Centre hospitalier de l'Université de Montréal, Campus Hôtel-Dieu, 3850 Saint-Urbain, Montréal, Québec, Canada, H2W 1T8

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Abstract. Defective regulatory interactions between the cystic fibrosis conductance regulator (CFTR) and the epithelial sodium channel (ENaC) have been implicated in the elevated Na^+ transport rates across cystic fibrosis airway epithelium. It has recently been proposed that ENaC downregulation by CFTR depends on the ability of CFTR to conduct Cl^- into the cell and is negligible when Cl^- flows out of the cell. To study the mechanisms of this downregulation we have measured amiloride-inhibitable Na^+ current (I_{amil}) in oocytes co-expressing rat ENaC and human wild-type CFTR. In oocytes voltage-clamped to −60 mV, stimulating CFTR with 1 mM IBMX reduced I_{amil} by up to 80%, demonstrating that ENaC is inhibited when Cl^- is conducted out of the cell. Decreasing the level of CFTR stimulation in a single oocyte, decreased both the degree of I_{amil} downregulation and the CFTR-mediated plasma membrane Cl^- conductance, suggesting a direct correlation. However, I_{amil} downregulation was not affected when Cl^- flux across oocyte membrane was minimized by holding the oocyte membrane potential near the Cl^- reversal potential (67% ± 10% inhibition at −20 mV compared to 79% ± 4% at −60 mV) demonstrating that I_{amil} downregulation was independent of the amount of current flow through CFTR. Studies with the Ca^{2+}-sensitive photoprotein aequorin showed that Ca^{2+} is not involved in I_{amil} downregulation by CFTR, although Ca^{2+} injection into the cytoplasm did inhibit I_{amil}. These results demonstrate that downregulation of ENaC by CFTR depends on the degree of CFTR stimulation, but does not involve Ca^{2+} and is independent of the direction and magnitude of Cl^- transport across the plasma membrane.

Key words: Cystic fibrosis — CFTR — Epithelial Na^+ channel — Na^+ absorption — Amiloride — *Xenopus* oocytes

Introduction

In cystic fibrosis, mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR)[1] (Riordan et al., 1989) disrupt Cl^- transport across epithelia and lead to a variety of physiologic and pathologic abnormalities (Bye, Ewig & Quittell, 1994; Davis, Drumm & Konstan, 1996). It is difficult, however, to precisely relate all these abnormalities solely to the loss of CFTR Cl^- channel function. There is evidence that, in addition to serving as a cAMP-activated, ATP-dependent Cl^- channel, CFTR may have other functions including regulation of the epithelial sodium channel ENaC (Stutts et al., 1995), an outwardly rectifying Cl^- channel (Egan et al., 1992; Gabriel et al., 1993; Schwiebert et al., 1995) and membrane trafficking (Bradbury et al., 1992; Biversi, Emans & Verkman, 1996). Recent studies suggest that CFTR can also function as an efflux pathway for large cytoplasmic anions (Linsdell & Hanrahan, 1998).

Recent studies have established that ENaC (Canessa et al., 1994) is a major amiloride-sensitive Na^+-conducting pathway in tight epithelia and its biophysical properties resemble those of highly selective, low conductance Na^+ channel found previously e.g., in rat cortical collecting tubule (Palmer & Frindt, 1986; for recent review *see* Garty & Palmer, 1997; Barbry & Hofman, 1997). Na^+ absorption is increased by 2–3-fold in CF airway epithelia compared to epithelia from normal in-

Correspondence to: R. Grygorczyk

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na^+ channel; I_{amil}, amiloride-inhibitable Na^+ current

dividuals (Boucher et al., 1986) and this has been attributed to defective regulation by CFTR (Stutts et al., 1995; Stutts, Rossier & Boucher, 1997). When wild-type CFTR was co-expressed with rat ENaC (rENaC) in Madin Darby kidney (MDCK) cell monolayers, cAMP inhibited amiloride-sensitive short-circuit current (I_{sc}). In cells expressing ENaC in the absence of CFTR, cAMP-stimulation increased I_{sc}. This reversal of cAMP regulation was also observed in patch clamp studies on NIH 3T3 fibroblasts expressing ENaC; the open probability (P_o) of ENaC channels switched from a cAMP-stimulated increase, to a cAMP-stimulated decrease, when cells were cotransfected with wild-type CFTR (Stutts et al., 1997). cAMP inhibition of epithelial Na^+ conductance was also observed in native rat colonic crypt cells (Ecke, Bleich & Greger, 1996) and in the mouse M-1 kidney cell line expressing endogenous CFTR (Letz & Korbmacher, 1997). These results suggest that CFTR-induced Na^+ transport regulation by cAMP is physiologically relevant.

The mechanism of this regulation by CFTR has not yet been identified. It could potentially involve CFTR-dependent secretion of a regulatory factor that acts in an autocrine or paracrine fashion as proposed for ATP (Schwiebert et al., 1995; Al-Awqati, 1995). Alternatively, CFTR may regulate other channels through direct protein-protein interactions. Indeed, the activities of immunopurified bovine renal Na^+ and rat ENaC channels were both decreased by the presence of CFTR in planar bilayers (Ismailov et al., 1996). However in the bilayer studies, in the absence of CFTR, PKA-mediated phosphorylation increased activity of the bovine Na^+ channel whereas it had no effect on rat ENaC activity in bilayers or *Xenopus* oocytes (Ismailov et al., 1996). Regulation of ENaC by CFTR may also involve intermediate proteins, such as the ubiquitin-protein ligase Nedd 4 (Staub et al., 1996; Staub & Rotin, 1996) and NHE RF (Yun et al., 1997; Hall et al., 1998). CFTR may regulate ENaC by influencing vesicular insertion and retrieval at the plasma membrane and CFTR has been shown to regulate vesicular trafficking and endosomal fusion (Bradburry et al., 1992; Takahashi et al., 1996; Biversi et al., 1996). However, whether there is a link between these CFTR-dependent processes and ENaC recycling remains unknown.

It is well established that CFTR is a cAMP-activated Cl^- channel, therefore Cl^- flux through CFTR may be involved in ENaC downregulation. Indeed, it has recently been proposed that CFTR-mediated Cl^- transport is required for the effect (Briel, Greger & Kunzelmann, 1998). This hypothesis was based on the observation that the magnitude of I_{amil} downregulation was correlated with the ability of CFTR to conduct Cl^-, was reduced for CFTR mutants that had diminished membrane Cl^- permeability (G551D, ΔF508, R117H) and was abolished by inhibition of CFTR Cl^- conductance using diphenylamine-carboxylate (DPC; Mall et al., 1996; Briel et al., 1998). The degree of ENaC downregulation also depended on the direction of Cl^- flow and was more pronounced for Cl^- influx than efflux. These observations are compatible with the hypothesis that, raised intracellular Cl^- concentration is part of a feedback mechanism downregulating Na^+ channel activity (Dinudom, Young & Cook, 1993; Komwatana et al., 1998).

The present study examines the mechanisms responsible for CFTR-dependent reduction of amiloride-sensitive current (I_{amil}). In particular, we test the hypothesis that CFTR-mediated Cl^- conduction contributes to this effect when rat αβγ-ENaC and wild-type CFTR are co-expressed in *Xenopus* oocytes. Our data confirm that IBMX induces a CFTR-dependent reduction of I_{amil}. In contrast to previous reports, however, our data suggest that this regulation is independent of the magnitude and direction of Cl^- conduction across the plasma membrane. These results have been presented in preliminary form (Chabot et al., 1998).

Materials and Methods

Plasmid Construction

Two plasmids containing human CFTR cDNA were used in our experiments, pcDNAI-CFTR and pcDNAIII-CFTR. The pcDNAI-CFTR construct was obtained by excising a 6.2-kb *SmaI-EcoRV* fragment containing the coding sequence from pBQ6.2 (a gift from Drs. J.R. Riordan and X-B Chang, Mayo Clinic Scottsdale, Arizona), and subcloning it into the unique *EcoRV* site of expression vector pcDNAIamp (Invitrogen, San Diego, CA) in the correct orientation. For the pcDNAIII-CFTR construct, pBQ6.2 was cut with *Not I* and *Xho I* and inserted into the corresponding site in pcDNAIII. CFTR mCAP-RNA was generated using pcDNAI-CFTR and the STRATAGENE kit (La Jolla, CA).

The α-, β- and γ-rENaC cDNAs (gifts from Dr. B. Rossier, University of Lausanne, Switzerland) were also subcloned into pcDNAIII. The β- and γ-rENaC clones were digested with *Eco RI* and *Not I* and ligated to the vector in the correct orientation. For the α-rENaC clone the insert was generated by a *Eco I - Not I* digestion and ligation into the corresponding site of pcDNAIII. The missing 5′ end of the cDNA was restored by a *Kpn I* digestion of the original clone and ligation into the pcDNA III - partial clone of α-rENaC. Bacteria were grown overnight in Luria broth (100 μg/ml ampicilin) and the plasmids purified using a QIAGEN kit (Qiagen, Santa Clara, CA).

Oocyte Acquisition and Injection

Oocytes were taken from adult females of *Xenopus laevis* using standard surgical procedure (Colman, 1984). To remove follicle cells, oocytes were treated for 60–90 min with fresh collagenase solution (670 U/ml, type 2, Worthington Biochemical, Freehold, NJ) in Ca^{2+}- free Barth's solution (in mM: NaCl 90, KCl 3, $MgSO_4$ 0.82, Hepes 5, Na-pyruvate 2.5, Penicillin 80 U/ml, Streptomycin 80 μg/ml, ± 0.41 $CaCl_2$, ±$Ca(NO_3)_2$ 0.33, pH 7.6). The defolliculated, stage 5–6 oocytes were selected and maintained in Barth's solution. Oocyte nuclei were

injected by the blind method (Colman, 1984) with 0.5–5 ng of DNA in total volume of 14 nl/oocyte nucleus. In some experiments CFTR mCAP-RNA was injected into cytoplasm (10 ng/oocyte in 23 nl volume). Injections were performed using the motorized injector "Nanoject Variable" (Drummond Scientific, Broomall, PA). The injection pipettes (1 mm outer diameter, Drummond Scientific) were pulled in a single step (PP-97 puller, Sutton Instruments), mounted on a patch pipette microforge (MF-83 Narishige Scientific Instrument Laboratory, Tokyo, Japan), and the tips were broken off under microscope view to approximately 6–8 μm outer diameter. Injected oocytes prior to testing were incubated for 24 to 96 hr at 18°C (Ambio-Hi-Lo Incubator, Lab-Line Instruments, Melrose Park, IL). To prevent sodium loading, ENaC-expressing oocytes were maintained in low sodium (9.6 mM NaCl + 86 mM NMDG) solution or in Barth's containing 10 μM amiloride. Due to much faster expression of ENaC (12–24 hr) as compared to the expression of CFTR (2–4 days), oocytes were injected with CFTR mCAP-RNA on day 1 and reinjected with ENaC plasmids the night before the experiment (day 3 to 5) in order to achieve simultaneous high levels of expression.

Electrophysiology

For electrophysiological experiments, an oocyte was placed in a 0.5 ml perfusion chamber and voltage clamped using microelectrodes of 0.5–2.0 MΩ resistance filled with 3 M KCl and a GeneClamp-500 amplifier (Axon Instruments, Foster City, CA). All experiments were performed in ND96 solution (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, Hepes 5, Na-pyruvate 2.5, $CaCl_2$ 1.8, Penicillin 40 U/ml, Streptomycin 40 μg/ml, gentamycin 50 mg/liter, pH 7.6). For patch-clamp single-channel recording, oocytes were incubated for 10–15 min in hypertonic 600 mOsmol media (ND96 + sucrose) so that the vitelline layer could be removed using watch maker forceps. Single-channel recordings were performed in the cell-attached configuration using an Axopatch 1C amplifier (Axon Instruments).

Aequorin Luminescence

In aequorin luminescence experiments, recombinant aequorin (100 ng/oocyte; Molecular Probes, OR) was injected 1–3 hr before each experiment as described previously (Grygorczyk et al., 1996) and luminescence responses from individual oocytes were measured using a Bio-Orbit Luminometer (Fisher, Canada).

Data Analysis

The data shown in the bar graphs are means ± SEM. Statistical significance was evaluated using two tailed paired and unpaired Student's *t*-tests. A value of $P < 0.05$ was considered significant.

Results

Expression of Amiloride-Sensitive Na^+ Current in Oocytes Injected with (α,β,γ)-ENaC cDNA

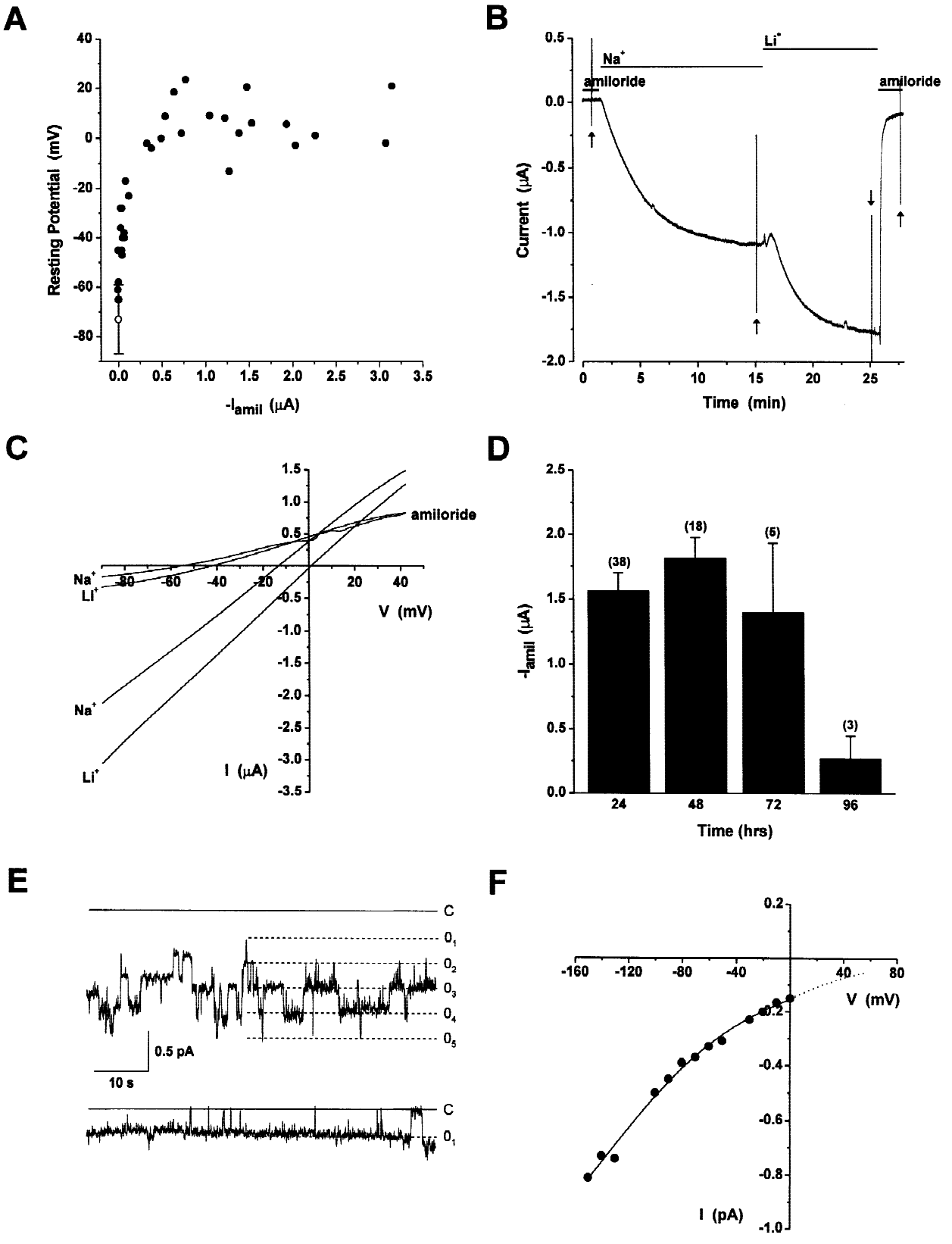
Oocytes injected with rat α,β,γ-ENaC cDNA following incubation for 16–24 hr had depolarized resting potentials (−15 to +15 mV), consistent with increased plasma membrane Na^+ permeability and intracellular Na^+ activity, Fig. 1*A*. Clamping oocytes at −60 mV, generated an inward, amiloride-inhibitable macroscopic current (I_{amil}, Fig. 1*B*). Replacement of Na^+ with Li^+ in the bath medium produced a larger inward current and shifted the current reversal potential to the right indicating higher permeability to Li^+ than Na^+ (Fig. 1*B, C*). Replacing Na^+ with K^+ abolished the inward current, as did the addition of benzamil (1 μM; *not shown*). These results demonstrate the expression of Na^+-selective, amiloride-sensitive conductance in oocytes injected with rat α,β,γ-ENaC cDNA, which was not observed in sham-injected control oocytes. I_{amil} persisted during the 48–72 hr period following cDNA injection, then oocytes gradually become degraded and I_{amil} declined, Fig. 1*D*. Na^+-selective channels with unitary conductance of 3 to 5 pS were observed in cell-attached patches on (α,β,γ)-ENaC cDNA-injected oocytes (Fig. 1*E, F*). The inward rectification and reversal potential of the single-channel current-voltage relationship are consistent with a Na^+ selectivity and ~10-fold lower Na^+ activity in the cytoplasm than in the bath. Single-channel current recordings also revealed slow gating, which is characteristic of the ENaC channel (Canessa et al., 1994).

Co-expression of CFTR with ENaC Has No Effect on I_{amil} in Unstimulated Oocytes

Previous co-expression studies suggested that CFTR can downregulate ENaC even in the absence of cAMP stimulation (Stutts et al., 1995; Ismailov et al., 1996), therefore we were interested if similar effects could be seen in *Xenopus* oocytes. Expression of CFTR alone had no effect on resting Cl^- current and membrane potential in the absence of cAMP stimulation (*not shown*). When both ENaC and CFTR were co-expressed, I_{amil} under basal conditions (i.e., in the absence of cAMP-stimulation) was similar to that of oocytes expressing ENaC alone, Fig. 2. Interestingly, CFTR-mediated Cl^- current during IBMX stimulation was significantly upregulated (~2.5-fold increase) in oocytes co-expressing ENaC when compared to oocytes expressing CFTR alone. Sham injection of water one day before the study did not cause such upregulation of CFTR; IBMX-stimulated CFTR-mediated Cl^- current was not different from that observed in oocytes that were not injected with water ($n = 5$, *not shown*).

Activating CFTR Reduces I_{amil} in Oocytes Co-expressing both Channels

In oocytes co-expressing ENaC and CFTR, 1 mM IBMX caused inhibition of I_{amil} and the degree of inhibition was proportional to the level of CFTR stimulation. This phenomena is illustrated on Fig. 3*A*; in the absence of IBMX, CFTR channels are inactive and most inward current at −60 mV is conducted by ENaC channels, as


A
Resting Potential (mV)
40
20
0
-20
-40
-60
-80
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
-Iamil (μA)
B
Na+
Li+
amiloride
amiloride
Current (μA)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0 5 10 15 20 25
Time (min)
C
amiloride
Na+
Li+
-80 -60 -40 -20 20 40
V (mV)
1.5
1.0
0.5
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Na+
Li+
I (μA)
D
-Iamil (μA)
2.5
2.0
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24 48 72 96
Time (hrs)
E
C
O1
O2
O3
O4
O5
0.5 pA
10 s
C
O1
F
0.2
-160 -120 -80 -40 40 80
V (mV)
-0.2
-0.4
-0.6
-0.8
-1.0
I (pA)

Fig. 1.

Fig. 1. Expression of ENaC in *Xenopus* oocytes. (*A*) Effect of rENaC expression on oocyte resting potential. The oocyte nucleus was injected 16 to 48 hr prior to the experiment with a mixture containing 0.5 ng of each plasmid subunit (α, β and γ in pcDNAIII). Oocytes were incubated during that time with 10 μM amiloride or in low Na^+ ND96 solution to minimize sodium loading (*see* Materials and Methods). Resting potential was determined by measuring the reversal potential of the ramp current-voltage relationship after washing off amiloride with ND96. The voltage was subsequently clamped at −60 mV and the amiloride sensitive current (i.e., current blocked by 10 μM amiloride; I_{amil}) was measured. Negative current corresponds to an inward flow of sodium ions with each point representing a pair of measurements (V_{rest} and I_{amil}) from an individual oocyte. For comparison, the mean (± SEM) resting potential of control noninjected oocytes ($n = 14$) is shown (○). (*B*) An example of the current trace recorded from an oocyte injected with α-, β- and γ-rENaC plasmids (0.5 ng each), 48 hr prior to the experiment. After placing an oocyte in the experimental chamber and clamping its membrane potential to −60 mV, amiloride was washed off, revealing the presence of approximately −1.1 μA of I_{amil} (*see* horizontal line "Na^+"). Complete replacement of Na^+ with Li^+ in the superfusate increased I_{amil} to approximately −1.7 μA, horizontal line "Li^+." The spikes on the trace (indicated by arrows) are due to the 1-sec voltage ramps used to determine current-voltage relationships (*see below*). Oocyte H980318003. (*C*) Current-voltage relationships (*I-V*) from the experiment shown in *B* obtained using a ramp voltage protocol. There were no significant changes in reversal potential of the *I-V* relationships during these experiments (20–30 min), indicating that under our experimental conditions Na^+ loading in the absence of amiloride was negligible. (*D*) Time course of amiloride-sensitive current expression in oocytes injected with α-, β- and γ-rENaC plasmids (0.5 ng each). The number of oocytes tested at each time point is shown above each bar. Significant deterioration of oocyte survival and I_{amil} was observed 48–72 hr post-injection. (*E*) Single channel ENaC currents recorded in the cell-attached configuration. Two current traces from a continuous 30-min recording that were taken ~11 min apart. The inward multichannel currents (O_1-O_5, shown as downward deflections) were recorded at +30 mV pipette potential; C - baseline current (all channels closed). Note the rundown of channel activity. Oocytes were injected with cDNAs for α-, β- and γ-rENaC (0.5 ng each) 48 hr prior to the experiment. The bath contained 150 K-gluconate and the pipette contained 150 Na-gluconate solution. Oocyte R7904000. (*F*) Single channel current-voltage (*I/V*) relationship from the experiment shown in *E*. The slope of the *I/V* relationship corresponds to a single channel conductance of 3–5 pS.

indicated by its sensitivity to 10 μM amiloride. In the example shown, this amiloride-inhibitable inward current I_{amil} was −2.2 μA, and is indicated by the horizontal line marked "1." After washing out amiloride, the oocyte was stimulated with 1 mM IBMX. This initially resulted in a small reduction of the inward current (by ~15%) followed by a large increase up to −3.2 μA due to activation of CFTR Cl^- channels. This current represents superimposed CFTR-mediated inward Cl^- current and ENaC-mediated inward Na^+ current. Subsequent application of amiloride in the continued presence of IBMX revealed that I_{amil} was only −0.7 μA under this condition, and was thus reduced by approximately 70% when compared to I_{amil} in the absence of IBMX, (compare response at horizontal line "2" to that at line "1"). Thus most of the remaining current (approximately −2.4 μA) could be attributed to CFTR-mediated inward Cl^- current. I_{amil} was not reduced due to rundown of ENaC channel activity, since amiloride responses gradually recovered after washing out IBMX. Several consecutive applications of amiloride during wash out demonstrated that I_{amil} gradually increased close to the level observed before IBMX application, (compare responses at horizontal lines 3, 4 and 5 in Fig. 3*A*). The inhibitory effect of CFTR activation was also seen when amiloride and IBMX were applied in reverse order, (horizontal line "5" in Fig. 3*A*). Here, with ENaC channels blocked by amiloride, application of 1 mM IBMX induced −2.0 μA of CFTR-mediated inward Cl^- current. Subsequent wash out of amiloride with IBMX still present increases the inward current by only −0.7 μA, confirming that I_{amil} is reduced by ~70% in the presence of 1 mM IBMX, similar to the reduction seen at "2." Thus IBMX inhibition is reversible and does not depend on the sequence of

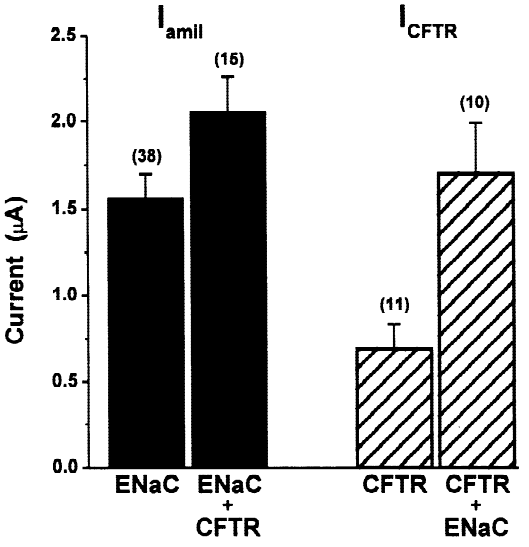

Fig. 2. I_{amil} is not affected by the presence of unstimulated CFTR in oocytes co-expressing ENaC and CFTR. Solid bars: comparison of I_{amil} measured at −60 mV in oocytes expressing ENaC alone, and in oocytes co-expressing ENaC and CFTR. Mean I_{amil} was not significantly different for the two groups of oocytes (two population *t*-test $P > 0.05$). Striped bars: comparison of IBMX-stimulated CFTR-mediated Cl^- current (I_{CFTR}) measured in oocytes expressing CFTR alone, and in oocytes expressing both ENaC and CFTR. Mean I_{CFTR} was significantly upregulated in oocytes co-expressing ENaC ($P = 0.004$). In oocytes co-expressing ENaC and CFTR, Cl^- current was determined as the IBMX-stimulated, amiloride insensitive current. CFTR mCAP-RNA was injected at 0 hr, and ENaC subunit plasmids were injected 48 hr later (*see* Materials and Methods). All electrophysiological experiments with oocytes co-expressing ENaC and CFTR that are summarized in this figure were performed at 72 hr, i.e., 24 hr after injecting α-, β- and γ-rENaC plasmids. Numbers above bars indicate number of oocytes tested for each condition.

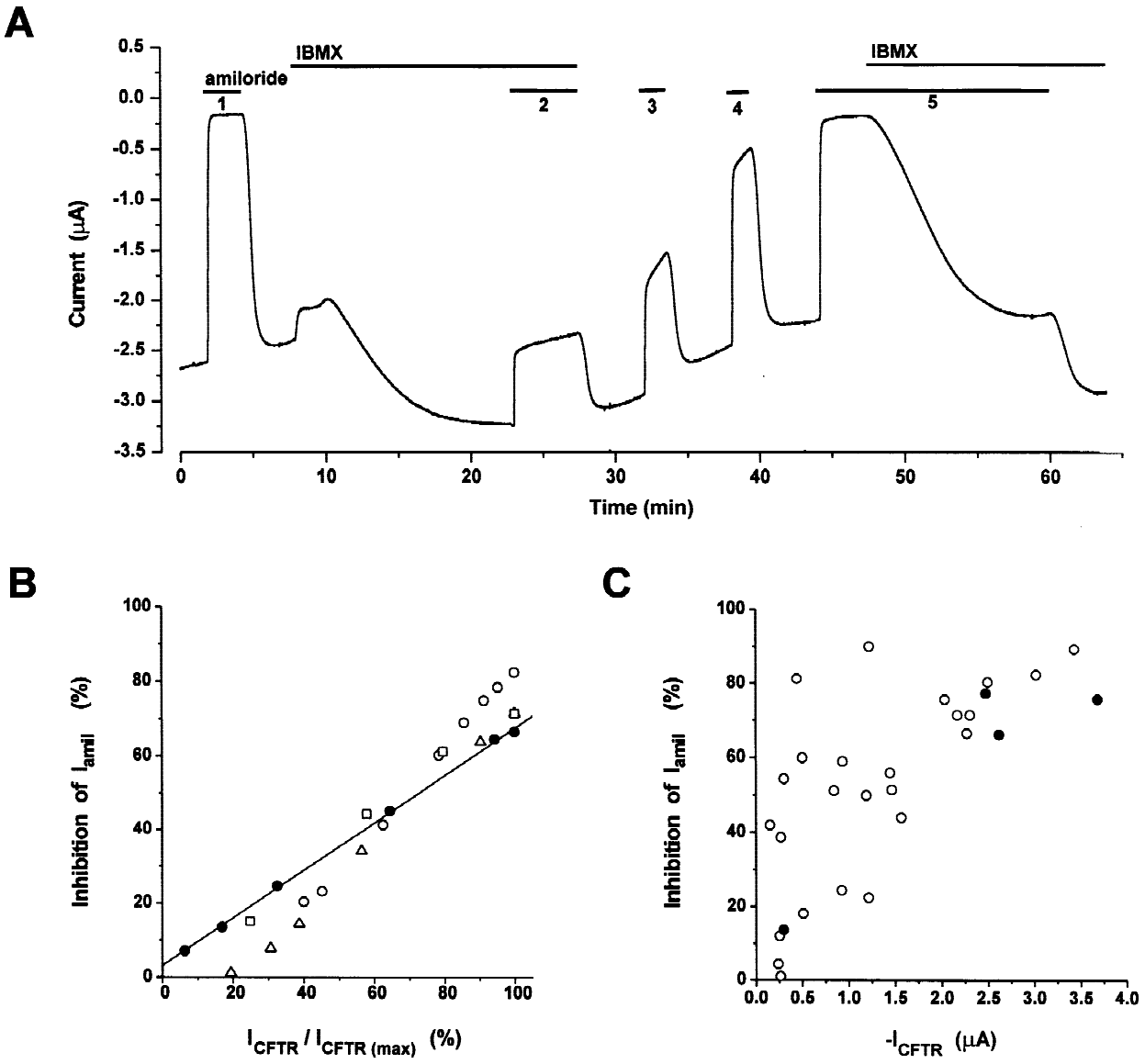

Fig. 3. IBMX stimulation reduces I_{amil} in oocytes co-expressing ENaC and CFTR. (*A*) An example of a continuous current trace recorded at −60 mV from an oocyte co-expressing rENaC and CFTR. Horizontal lines labeled 1–5 indicate sequential applications of 10 μM amiloride. Upper lines indicate exposure to 1 mM IBMX. Note the reduction in I_{amil} during exposure to 1 mM IBMX. This oocyte was injected with 10 ng of CFTR mCAP-RNA 4 days prior to the experiment, followed by nuclear injection of α-, β- and γ-rENaC plasmids 48 hr prior to the experiment. Oocyte H970617002. Representative of four experiments. (*B*) Relationship between inhibition of I_{amil} (in %) and stimulation of CFTR expressed as CFTR-mediated Cl^- current at −60 mV (I_{CFTR}) during wash out of IBMX, relative to the maximal current ($I_{CFTR(max)}$) observed in the presence of 1 mM IBMX. Data points from the experiment shown in part A are shown as (●) and are joined by a solid line; for comparison data points from three other experiments where similar protocol was used, are also shown (○, □, △). (*C*) Relationship between the reduction of I_{amil} (in %), and the maximum CFTR-mediated current during stimulation by 1 mM IBMX (○) or 5 mM theophylline (●), observed in different oocytes co-expressing ENaC and CFTR. Since a similar reduction of I_{amil} was observed with theophylline and IBMX, the data have been pooled. CFTR-mediated Cl^- currents were recorded at −60 mV in the presence of 10 μM amiloride to block Na^+ current.

IBMX/amiloride applications, although it does seem to be correlated with the level of CFTR stimulation.

To analyze this correlation in more detail, we took advantage of the observed slow wash out of IBMX and corresponding slow decline in CFTR activity as shown in Fig. 3*A* (and also Fig. 5 below). This time course allowed several brief applications of amiloride, so that changes in I_{amil} could be followed during the decline of CFTR. Figure 3*B* demonstrates the strong linear relationship between degree of I_{amil} inhibition and level of CFTR stimulation. We examined whether a similar relationship exists when the stimulus remains constant and

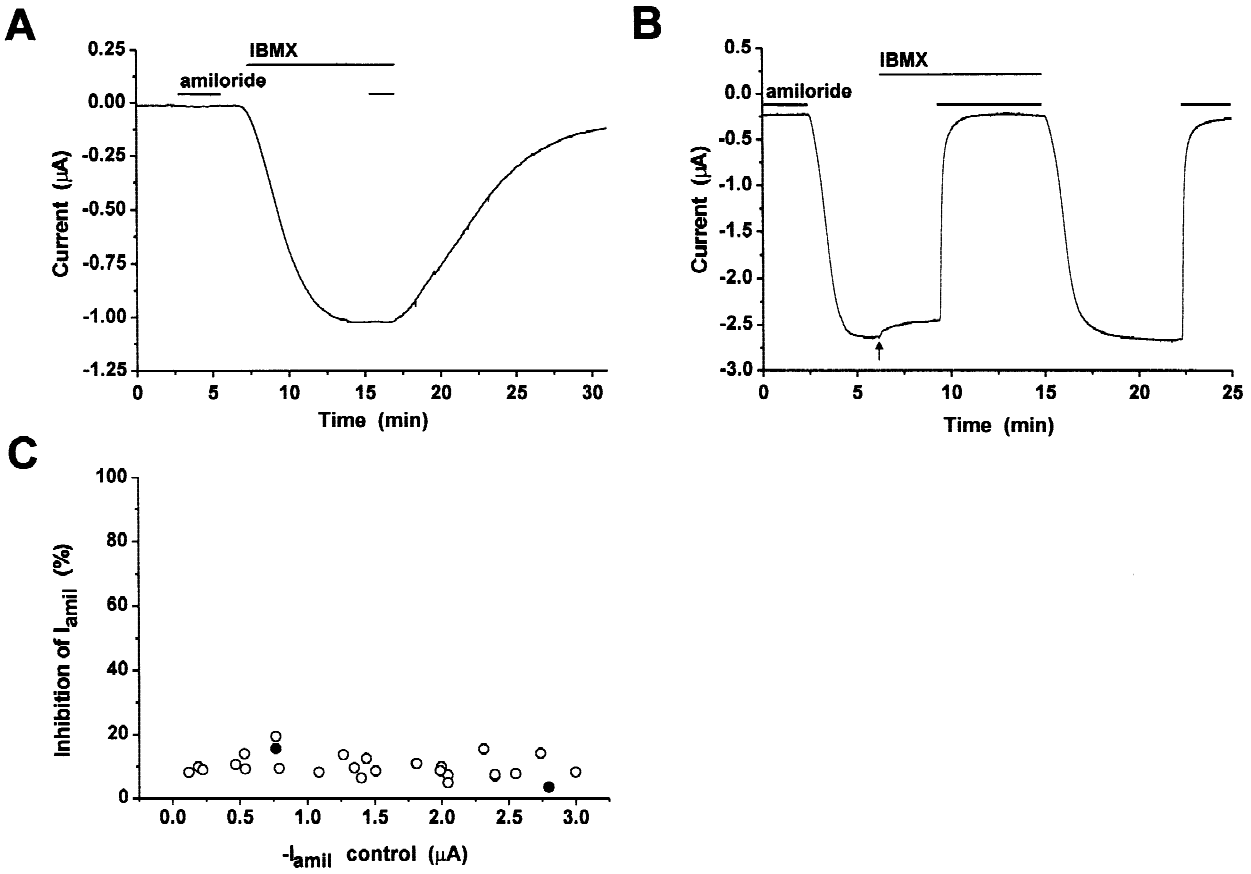

Fig. 4. Amiloride does not affect CFTR current; IBMX induces a small inhibition of ENaC current. (*A*) In oocytes expressing only CFTR and voltage clamped to −60 mV, 10 μM amiloride had no detectable effect on basal oocyte current or IBMX-stimulated (CFTR-mediated) Cl^- current. In the example shown, the oocyte was injected with 2.5 ng of pcDNAIII-CFTR 3 days prior to the experiment. Oocyte H970422012. (*B*) Example of the effect of 1 mM IBMX on I_{amil} in an oocyte expressing ENaC alone. Note the slight inhibition of I_{amil} by IBMX (arrow). Oocyte H970502003. (*C*) Relationship between the inhibition of I_{amil} (in %) caused by 1 mM IBMX (○) or 5 mM theophylline (●) and the level of I_{amil} expression in individual oocytes tested.

the level of CFTR expression is varied. Inhibition of I_{amil} was compared between different oocytes that happened to express different amounts of CFTR Cl^- current when maximally stimulated with 1 mM IBMX or other xanthine, theophylline (5 mM). These data are summarized in Fig. 3*C*. Despite large variability due to the use of oocytes from different frogs and during different seasons, there is a clear trend towards greater inhibition of I_{amil} with increasing expression of CFTR Cl^- current.

In control experiments we tested the effect of 10 μM amiloride on oocytes expressing CFTR alone, and 1 mM IBMX on oocytes expressing ENaC alone. Figure 4*A* shows that amiloride had no effect on basal current in CFTR-expressing oocytes either in the absence or presence of IBMX stimulation. However, as indicated by ↑ in Fig. 4*B*, IBMX did cause a small but reproducible reduction of I_{amil} in oocytes expressing ENaC alone, which averaged 10 ± 5% (n = 27 preparations). This effect did not depend on the level of ENaC expression and was similar when another xanthine, theophylline, was used. These xanthine effects on ENaC are summarized in Fig. 4*C*. The relatively small inhibitory effect of IBMX observed in the absence of CFTR clearly would not explain the large (up to 80%) reduction of I_{amil} seen in oocytes co-expressing CFTR and ENaC. Thus CFTR must play some role in mediating the inhibition of ENaC during IBMX stimulation.

Role of CFTR-Mediated Cl^- Conductance in Downregulation of I_{amil}

The above results indicate that I_{amil} downregulation is directly proportional to the level of CFTR channel stimulation and suggest a possible dependence on CFTR-mediated Cl^- transport and/or local Cl^- concentration changes that could arise during activation of CFTR. To test this, experiments were performed on oocytes voltage-clamped near the presumed Cl^- reversal potential (−20 mV, Dascal, 1987) to minimize net Cl^- fluxes and

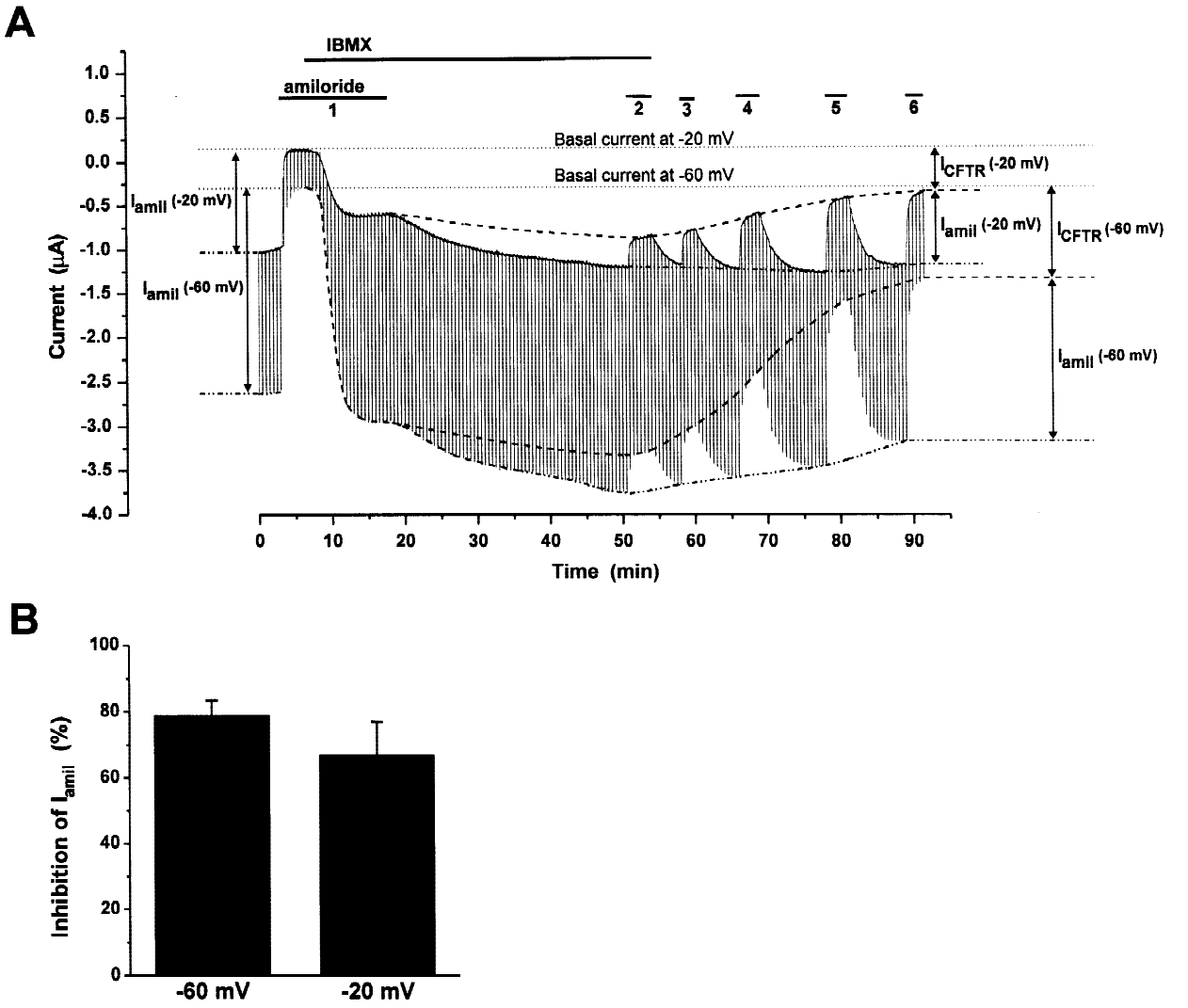

Fig. 5. Chloride flux through activated CFTR channels does not affect I_{amil}. (*A*) To minimize Cl^- flux through activated CFTR channels, oocytes co-expressing ENaC and CFTR were voltage clamped near the presumed Cl^- reversal potential of −20 mV and current was recorded continuously, solid line. To compare oocyte currents with those in previous experiments, such as the one shown in Fig. 3, hyperpolarizing voltage steps of 1-sec duration were applied each minute to measure the inward current at −60 mV, (indicated by tops of the spikes). For clarity, broken lines were drawn to outline the amiloride-inhibitable current at −60 mV, I_{amil}(−60 mV); amiloride-inhibitable current at −20 mV, I_{amil}(−20 mV); CFTR-mediated current at −60 mV, I_{CFTR}(−60 mV); and CFTR-mediated current at −20 mV, I_{CFTR}(−20 mV). Horizontal lines ("1" through "6") above the trace indicate sequential applications of 10 μM amiloride. Note the reversible reduction of I_{amil} at both voltages (−20 and −60 mV) induced by IBMX stimulation of CFTR, similar to that shown in Fig. 3. Oocyte H980203003. Similar results were obtained with four oocytes from two different batches where this protocol was used. (*B*) Comparison of I_{amil} inhibition by IBMX-stimulated CFTR in the same oocytes at −60 and −20 mV. The data are mean ± SEM from four experiments like the one shown in *A*. The values of I_{amil} inhibition at −60 and −20 mV are not significantly different (two populations unpaired *t*-test, $P = 0.33$).

thus minimize any local changes in Cl^- concentration. Short (1 sec) hyperpolarizing voltage steps to −60 mV were applied at one-minute intervals to measure the current at the same membrane potential as during previous experiments (e.g., Fig. 3). In the example illustrated in Fig. 5, holding oocytes near the Cl^- reversal potential did not prevent the inhibition of I_{amil} following IBMX stimulation. In fact, amiloride-inhibitable current recorded during brief voltage pulses to −60 mV, labeled "I_{amil}(−60 mV)" in Fig. 5*A*, had similar kinetics and a similar degree of downregulation (~78%) as in the experiment shown in Fig. 3, when the membrane potential was continuously held at −60 mV. Thus, despite a significant reduction in net Cl^- flux through CFTR, downregulation of I_{amil} was unchanged. The data in Fig. 5*A* allow us also to compare downregulation at −60 and −20 mV (labeled as I_{amil}(−60 mV) and I_{amil}(−20 mV), respectively). The results from four preparations are summarized in Fig. 5*B*.

CFTR induced inhibition of I_{amil} is not different at −20 and at −60 mV ($P = 0.3$), demonstrating that it is not related to Cl^- flux across the oocyte plasma membrane *per se* or local Cl^- concentration changes.

Cytoplasmic Injections of Ca^{2+} Inhibits I_{amil} by a CFTR-Independent Mechanism

Figure 6*A* shows that injecting Ca^{2+} into the cytoplasm (<100 μm underneath the plasma membrane) of oocytes expressing ENaC alone (300–1,000 pmol) induced a transient inward Cl^- current, I_{Cl}(Ca), mediated by oocyte's native Ca^{2+}-activated Cl^- channels. The slow decline of the current allowed the application of amiloride to test for ENaC-mediated I_{amil} in the presence of Cl^- flux through Ca^{2+}-activated Cl^- channels. Figure 6*A* shows that cytoplasmic Ca^{2+} injection inhibited I_{amil} by ~40% (compare response at horizontal line "2" with the recovery from amiloride after line "1"). Similar inhibition was observed when the membrane voltage was clamped near Cl^- reversal potential to minimize Cl^- flux through the channels (*data not shown*). Interestingly, I_{amil} remained inhibited long after I_{Cl}(Ca) had fully declined (horizontal line "3"). Thus, I_{amil} can be inhibited by elevating cytoplasmic Ca^{2+} in the absence of CFTR and does not require Cl^- flux through I_{Cl}(Ca). Spatial distribution of intracellular Ca^{2+} can also play a role in this effect as indicated by the experiments such as that shown on Fig. 6*B*. Injection of Ca^{2+} deep into oocyte cytoplasm i.e., approximately 200–300 μm away from the plasma membrane, did not stimulate I_{Cl}(Ca). Nevertheless, it induced significant run-down of I_{amil}, supporting the view that Ca^{2+}-dependent inhibition of I_{amil} is independent of Cl^- flux across the plasma membrane. On average, under our experimental conditions, Ca^{2+} injection increased rate of I_{amil} rundown more than 2-fold when compared to control oocytes not injected with Ca^{2+}, Fig. 6*C*.

Is Ca^{2+} Involved in CFTR-Dependent I_{amil} Downregulation?

In view of the Ca^{2+} effects described above, we considered the possibility that downregulation of ENaC during stimulation of CFTR by IBMX might be CFTR-dependent, but still result from an IBMX-induced elevation of intracellular Ca^{2+}, for example via nonspecific interactions with adenosine receptors. However, this was not the case, since Ca^{2+}-activated Cl^- channels were not activated by IBMX in control, uninjected oocytes or in oocytes expressing ENaC alone (*data not shown*). Moreover, Ca^{2+} transients were not detectable in aequorin-loaded oocytes exposed to IBMX, although subsequent exposure to 5% bovine serum produced a robust luminescence response through activation of endogenous lysophosphatydic acid receptors, which are coupled to the IP_3-Ca^{2+} signaling pathway (Fig. 6*D*; Tigyi & Miledi, 1992). Taken together, these data indicate that CFTR-independent inhibition of I_{amil} by Ca^{2+} and CFTR-dependent inhibition by IBMX occur through different mechanisms.

Discussion

Upregulation of Na^+ absorption in CF airways has been attributed to defective regulatory interactions between ENaC and mutant CFTR. The recent observations by Briel et al. (1998) that downregulation of I_{amil} was correlated with the ability of CFTR to conduct Cl^- into the cell lead them to conclude that CFTR-mediated Cl^- transport is required for this effect. Consistent with this hypothesis, our Fig. 3*B* indicates that when CFTR stimulation is varied in a single oocyte, it causes proportional changes in Cl^- current and inhibition of I_{amil}. We found, however, that Cl^- current and I_{amil} inhibition are not causally related; the degree of I_{amil} inhibition did not depend on the magnitude of the current. Experiments like the one shown on Fig. 5 revealed that I_{amil} downregulation is not changed when CFTR-mediated Cl^- current is reduced at a constant level of IBMX stimulation by clamping the oocyte membrane close to the presumed reversal potential for Cl^-. From the relation shown on Fig. 3*B*, one might expect that a 5-fold reduction of inward Cl^- current would result in a similar reduction of I_{amil} inhibition, i.e., approximately from 80% to 15%, but this was not observed. Instead as Fig. 5*B* shows, it was not significantly different at −20 mV (67 ± 10%) and at −60 mV (79 ± 4%). This demonstrates that neither Cl^- transport nor the voltage across oocyte membrane are involved in regulating ENaC. Furthermore, the data indicate that stimulation of CFTR has independent effects on oocyte plasma membrane Cl^- conductance and I_{amil} inhibition.

Our finding that significant inhibition of I_{amil} occurs at −60 mV, i.e., when Cl^- is conducted out of the cell, differs from those of Briel et al. (1998), who reported that inhibition by CFTR was more pronounced at positive membrane potentials (between +10 and +40 mV; Cl^- influx) and negligible at negative potentials (between −20 and −90 mV; Cl^- efflux). Although, it is not entirely clear why I_{amil} downregulation would be reduced at negative voltages, our Ca^{2+}-injection experiments suggest possible explanation for enhanced inhibition of I_{amil} at positive potentials. Repetitive strong depolarizations (up to +40 mV) used in their experimental protocol may have produced significant Ca^{2+} influx via oocyte native voltage-gated Ca^{2+} channels, which might lead to inhibition of I_{amil} by Ca^{2+}.

In the present study we found that cytoplasmic Ca^{2+} injection stimulates Ca^{2+}-activated Cl^- current, I_{Cl}(Ca),

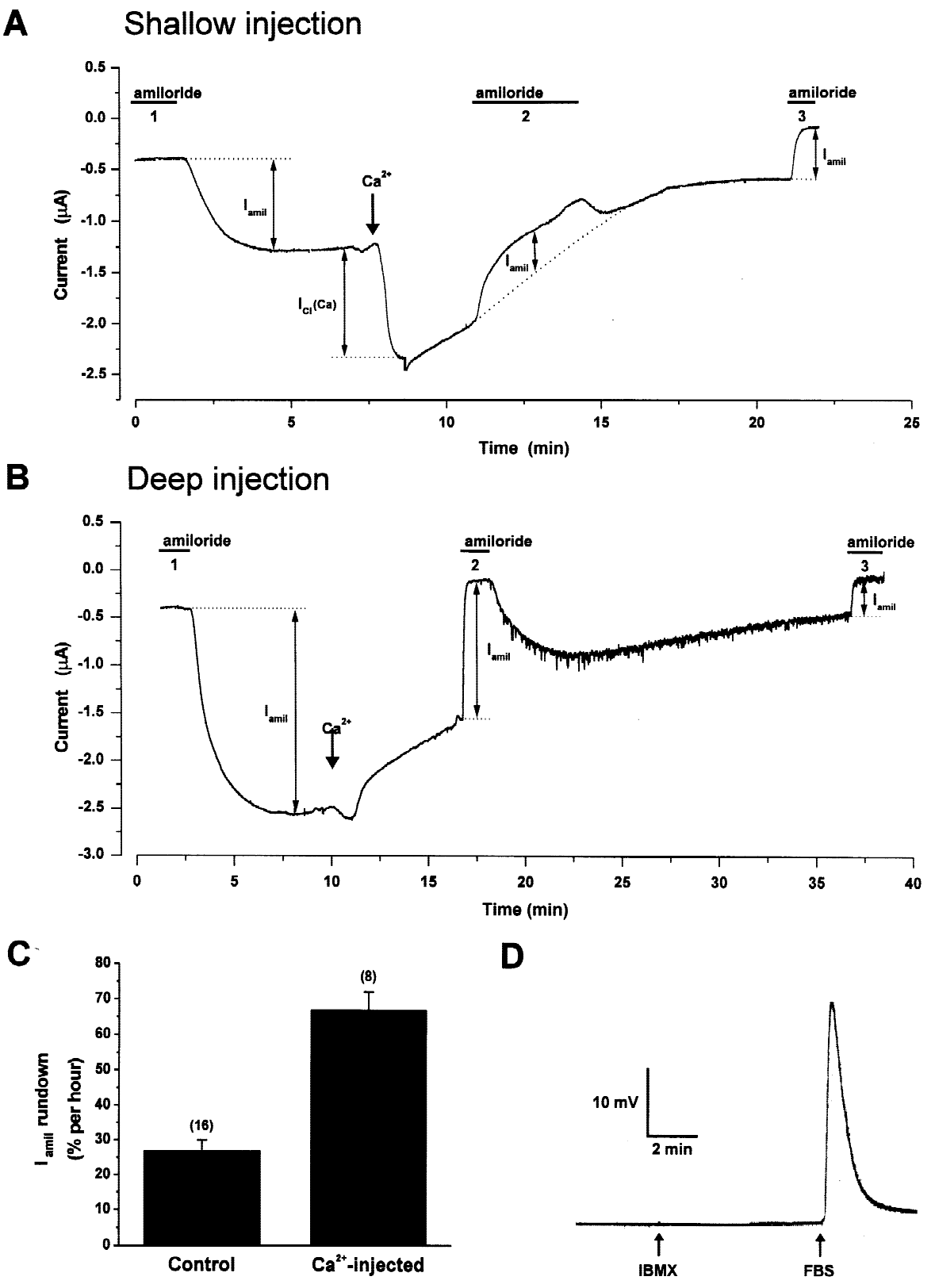
A
Shallow injection
amiloride 1
amiloride 2
amiloride 3
I_{amil}
Ca^{2+}
$I_{Cl}(Ca)$
Current (μA)
Time (min)
B
Deep injection
amiloride 1
amiloride 2
amiloride 3
I_{amil}
Ca^{2+}
Current (μA)
Time (min)
C
I_{amil} rundown (% per hour)
(16)
(8)
Control
Ca^{2+}-injected
D
10 mV
2 min
IBMX
FBS

Fig. 6.

Fig. 6. Effects of cytoplasmic Ca^{2+} on I_{amil}. (*A*) Effect of shallow (less than 100 μm underneath the plasma membrane) Ca^{2+} injection on I_{amil}. Continuous current recording from an oocyte expressing ENaC alone, voltage-clamped at −60 mV. Cytoplasmic injection of $Ca(NO_3)_2$ (~1 nmol) activated inward Cl^- current, I_{Cl}(Ca), which slowly declined during the next 10 min. Application of 10 μM amiloride during that time period revealed a significant reduction in I_{amil} (horizontal line "2") when compared to that before Ca^{2+} injection (horizontal line "1"). Oocyte R97O01001. Representative of three experiments. (*B*) Effect of deep (approximately 200–300 μm underneath the plasma membrane) Ca^{2+} injection on I_{amil}. All other conditions the same as in *A*. Oocyte H981007006. (*C*) Rate of I_{amil} rundown induced by deep Ca^{2+} injection compared to control oocytes not injected with Ca^{2+}. The data represent mean ± SEM from $n = 16$ and $n = 8$ for control and Ca^{2+} injected oocytes respectively. (*D*) Aequorin luminescence experiment showing that IBMX (1 mM) has no effect on intracellular Ca^{2+} in CFTR-expressing oocyte. As a positive control for the assay, subsequent addition of 5% fetal bovine serum (FBS) induced large luminescence response, demonstrating functionality of the injected aequorin. Oocytes used in these experiments were injected with CFTR mCAP-RNA three days before experiments. To ensure that the oocytes were expressing CFTR, they were allowed to recover for 1 hr in ND96 solution after luminometry experiments and then tested for CFTR expression by stimulating with 1 mM IBMX under voltage-clamp. The data are representative of eight preparations. The output signal of the luminometer is in mV and represents relative units of luminescence intensity.

in oocytes expressing ENaC alone and inhibits I_{amil}. Several repetitive injections of Ca^{2+} (100–400 pmol each dose) were used in other studies without adverse effects on oocytes (Boton, Singer & Dascal, 1990; Dascal & Boton, 1990; Grygorczyk et al., 1996), but resulted in gradual potentiation of I_{Cl}(Ca) response due to loading of intracellular Ca^{2+} stores. In this study we used a single comparable dose of Ca^{2+} (300–1,000 pmol) to elicit a prolonged I_{Cl}(Ca) response. The resulting I_{amil} inhibition is similar to that observed during IBMX-stimulation of oocytes co-expressing CFTR and is not influenced by membrane potential or Cl^- flux. Therefore cytoplasmic Ca^{2+} directly downregulates I_{amil} independently of CFTR or Ca^{2+}-dependent Cl^- channels. This conclusion is consistent with observation by Ishikawa, Marunaka & Rotin (1998), who also found that cytoplasmic Ca^{2+} (1–10 μM), inhibits the activity of ENaC channels expressed in MDCK cells. Conversely, we found no evidence that downregulation of I_{amil} by CFTR involves Ca^{2+} elevation, supporting the view that I_{amil} downregulation by Ca^{2+} and by CFTR involve different mechanisms. These findings differ from those reported by Briel et al. (1998). They did not observe I_{amil} inhibition after stimulating Ca^{2+}-activated Cl^- channels using Ca^{2+}-ionophore ionomycin, and concluded that ENaC downregulation is specific to CFTR-mediated Cl^- conductance. The apparent discrepancy may result, in part, from the procedures used to elevate cytoplasmic Ca^{2+}. Physiological responses in oocyte may depend on the source of Ca^{2+} and its spatial distribution within the cytoplasm. For example, Ca^{2+}-activated Cl^- channels inactivate rapidly when oocytes are stimulated with Ca^{2+}-ionophore, but not when Ca^{2+} is injected or photoreleased using caged compounds (Boton et al., 1990; Parker & Yao, 1994). Furthermore, as discussed above, Ca^{2+} influx through endogenous voltage-gated Ca^{2+} channels may have inhibited I_{amil} and diminished the effect of subsequent exposure to ionomycin.

Although several reports show that cAMP stimulation of CFTR downregulates ENaC (Ecke et al., 1996; Mall et al., 1996; Letz & Korbmacher, 1997; Briel et al., 1998), the effects of basal (unstimulated) CFTR activity on ENaC seems to vary with the expression system used. We found that co-expression of ENaC and CFTR in *Xenopus* oocytes had no effect on ENaC-mediated I_{amil} under basal conditions; i.e., in the absence of cAMP-stimulation. This agrees with the finding of Briel et al. (1998), who also used the same expression system, but contrasts with those of Stutts et al. (1995), who found that expression of wild-type CFTR in MDCK cells reduced sodium current in the absence of cAMP stimulation. In addition, CFTR reversed the direction of ENaC regulation from a cAMP-dependent increase to a cAMP-dependent decrease, although the latter effect was much weaker (<10%) than the inhibition observed in oocytes (both our present study and Briel et al., 1998). These variable results may reflect differences between the two expression systems and also the experimental protocols used. We measured macroscopic currents under two-electrode voltage clamp in oocytes co-expressing CFTR and ENaC. In the study of Stutts et al., vectorial transport of Na^+ and Cl^- was measured using transfected MDCK cells in Ussing chambers. Furthermore, MDCK cells were treated for 24 hr with dexamethasone and butyrate to induce ENaC expression, which may increase expression of other proteins. A reduction in basal Na^+ transport in cells overexpressing both ENaC and CFTR could result from competition for common factors during transcription, translation, post-translational processing or delivery to the plasma membrane. In our experiments this could have been minimized by injecting CFTR plasmid 48 hr before the ENaC plasmid. A reduction of CFTR expression was observed previously in oocytes when it was co-expressed simultaneously with human PGE_2 receptor (Grygorczyk et al., 1995). In the present study we found that ENaC co-expression actually boosted the level of CFTR current (Fig. 2). Thus the effects of co-expression vary depending on the type of protein co-expressed or protocol used.

The present results restrict the range of possible models for regulatory interactions between ENaC and CFTR to those which are independent of plasma membrane Cl^- conduction. CFTR-dependent release of cellular ATP has been postulated as the autocrine mecha-

nism by which CFTR regulates other channels. Secreted extracellular ATP is proposed to act on other channels through interactions with a purinergic receptors (Schwiebert et al., 1995). However, these data remain controversial. Initial reports that CFTR is an ATP-conducting channel or transporter (Reisin et al., 1994; Pasyk & Foskett, 1997) have not been confirmed by other groups (Grygorczyk, Tabcharani & Hanrahan, 1996; Reddy et al., 1996; Li, Ramjeesingh & Bear, 1996; Grygorczyk & Hanrahan, 1997; Watt, Lazarowski & Boucher, 1998). There are data supporting a revised hypothesis, in which CFTR gates an associated ATP permeable channel (Sugita, Yue & Foskett, 1998; Jiang et al., 1998). It also has been recently suggested that distinct domains of CFTR are responsible for Cl^- transport and for facilitating the release of ATP (Schwiebert et al., 1998).

Alternative mechanisms include direct or indirect protein-protein interactions in the plasma membrane. Yeast two hybrid analysis of CFTR and rat ENaC identified a cytosolic domain of wild-type CFTR (first nucleotide binding domain and regulatory domain, amino acids 351–830) and the C-terminus of α-rENaC as possible interacting domains (Kunzelmann et al., 1997). The interaction was not observed if the CFTR peptide fragment contained the CF mutation G551D, and co-expression of ENaC with each CFTR peptide fragment in *Xenopus* oocytes suggested that ENaC was downregulated by wild type but not G551D peptide. These findings appear to conflict with more recent report by the same group, which indicate that downregulation requires functional CFTR channels capable of conducting Cl^- (Briel et al., 1998). Several proteins that interact with CFTR or ENaC have been recently identified. WW domains of the ubiquitin-protein ligase Nedd4 have been shown to interact with proline-rich PY motifs in the C terminals of β and γ subunits of the ENaC and to regulate the rate of proteolysis and stability of ENaC channels in the plasma membrane (Staub et al., 1996; Staub & Rotin, 1996). Furthermore, it was recently proposed that Nedd4-mediated feedback control of ENaC is modulated by elevation of intracellular Na^+ activity (Dinudom et al., 1998; Komwatana et al., 1998; Kellenberger et al., 1998). Using random peptide display technique it was demonstrated that PDZ1 domain of the Na^+/H^+ exchanger regulatory factor (NHE-RF; Yun et al., 1997; Hall et al., 1998) is capable of binding to the CFTR C-terminus, suggesting a potential regulatory role of this protein in CFTR function (Wang et al., 1998). An interplay between the membrane-anchored syntaxin 1A and syntaxin-binding protein of the Munc 18 protein family has recently been suggested to regulate CFTR (Naren et al., 1997). Whether these or similar regulatory proteins are involved in CFTR-ENaC interactions remains to be established.

Another plausible mechanism for ENaC downregulation by CFTR involves interactions at a common point along the secretory pathway of the two proteins. Cell surface expression of ENaC is a dynamic process that involves continuous recycling of the protein. ENaC normally has a short half-life time at the cell surface in vivo (~1 hr; Staub et al., 1997) and must be replaced by membrane insertion. Moreover, there is mounting evidence that CFTR influences membrane recycling and endosomal fusion in CFTR-expressing epithelial cells and in *Xenopus* oocytes (Bradbury et al., 1992; Biversi et al., 1996). It has been suggested that these processes determine plasma membrane expression and activity of other ion channels, perhaps due to cohabitation of multiple channel types in trafficking vesicles (Takahashi et al., 1996). Both CFTR and ENaC channels have been identified in endocytic clathrin-coated vesicles (Bradbury et al., 1994; Shimkets, Lifton & Canessa, 1997). Our observation that downregulation of I_{amil} does not correlate with CFTR-mediated Cl^- conductance at the plasma membrane, is compatible with the hypothesis that CFTR-dependent membrane recycling is involved. Furthermore, our observation that co-expression of ENaC increases CFTR Cl^- conductance by more than twofold in oocytes (Fig. 2) suggests that ENaC-CFTR interactions may be reciprocal (i.e., CFTR → ENaC and ENaC → CFTR), and emphasizes the need for further studies of protein-protein interactions and ENaC recycling in the regulation by CFTR.

In summary this study demonstrates that ENaC downregulation is linked to the presence of CFTR and to the degree of CFTR stimulation, but does not depend on CFTR Cl^- channel function at the plasma membrane. Furthermore, we show that the inhibitory effects of CFTR and cytoplasmic Ca^{2+} on ENaC are mediated by independent mechanisms. Further studies are needed to establish the alternative mechanisms for ENaC downregulation.

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