Chloride Conductance Expressed by ΔF508 and Other Mutant CFTRs in *Xenopus* Oocytes

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The cystic fibrosis transmembrane conductance regulator (CFTR) is associated with expression of a chloride conductance that is defective in cystic fibrosis (CF). *Xenopus* oocytes injected with RNA coding for CFTR that contained mutations in the first nucleotide binding fold (NBF1) expressed chloride currents in response to raising adenosine 3′,5′-monophosphate (cAMP) with forskolin and 3-isobutyl-1-methylxanthine (IBMX). The mutant CFTRs were less sensitive than wild-type CFTR to this activating stimulus, and the reduction in sensitivity correlated with the severity of cystic fibrosis in patients carrying the corresponding mutations. This demonstration provides the basis for detailed analyses of NBF1 function and suggests potential pharmacologic treatments for cystic fibrosis.

CYSTIC FIBROSIS (CF), THE MOST common lethal, recessively inherited disease among Caucasians, affects nearly 1 in 2500 newborns (*1*). CF is caused by mutations in the gene encoding CFTR (*2–4*), a protein that has been associated with the expression of cAMP-stimulated Cl^- conductance in a variety of eukaryotic cell types (*5–8*).

Although a single mutation, deletion of the phenylalanine at position 508 (ΔF508), accounts for almost 70% of the CF alleles in the population (*4*), over 100 mutations have been identified in this gene (*9*). Patients homozygous for the ΔF508 mutation present a consistent clinical picture including elevated Cl^- concentrations in sweat, pulmonary disease, and pancreatic insufficiency, and are generally classified as severely affected (*10*). In contrast, the clinical profiles of patients with other mutations in CFTR vary in the severity of disease (*9–11*).

It is well established that Cl^- conductance is defective in cystic fibrosis, but it is not clear how disease-causing mutations in CFTR alter this conductance. We used *Xenopus* oocytes to compare the activation of cAMP-dependent Cl^- currents associated with the expression of wild-type CFTR and CFTRs with naturally occurring mutations in the first nucleotide binding fold (NBF1).

Membrane currents were recorded from oocytes injected with RNA from either wild-type or the ΔF508 mutant CFTR. Exposure of the oocytes injected with wild-type CFTR to a cocktail containing 10 μM forskolin (an activator of adenylate cyclase), 1 mM IBMX (a phosphodiesterase inhibitor), and 200 μM 8-chlorophenylthio cAMP (8-cpt cAMP) activated a large inward current (Fig. 1A). The same stimulus elicited no current in the uninjected oocyte or in oocytes injected with RNA from CFTR missing exon 9 (*12*). In the ΔF508-injected oocyte, stimulation induced an inward current, but the activation differed markedly from that seen in the oocyte injected with wild-type CFTR. The rate of increase of the current was slower and the final steady-state level significantly less for the mutant. The reduced current associated with the ΔF508 CFTR mutant was due, at least in part, to reduced sensitivity to cAMP. When the concentration of IBMX was raised above 1 mM, the current increased substantially in ΔF508-injected oocytes, but not in wild-type–injected oocytes (Fig. 1B). Maximal suppression of phosphodiesterase activity in *Xenopus* oocytes requires 5 mM IBMX (*13*), consistent with the interpretation that the increased activation of ΔF508 CFTR was a consequence of an elevation in cAMP concentrations (*14*).

We also examined three other, naturally occurring CFTR variants for which there is information pertaining to the severity of disease (Table 1). To facilitate comparisons between expression of Cl^- conductance and disease severity, we chose, where possible, mutations found in homozygotes so that only a single mutation contributes to the phenotype. If the stimulus was increased sufficiently (10 to 50 μM forskolin and 5 mM IBMX), even mutants associated with the most severe clinical symptoms exhibited a readily detectable Cl^- current (Table 2).

The sensitivity of the CFTR variants to forskolin and IBMX fell in the same rank order as the severity of disease in patients (Fig. 1B and Table 1). Oocytes expressing wild-type CFTR exhibited the greatest sensitivity, and the sensitivity of oocytes expressing F508C was only slightly reduced relative to wild type. F508C, a cysteine for phenylalanine substitution at position 508,

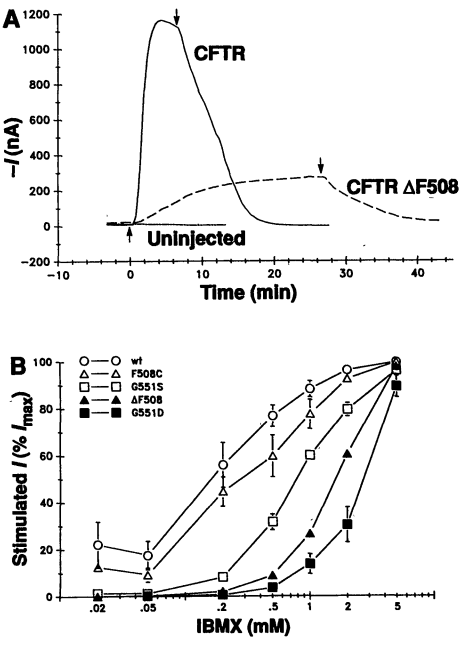

Fig. 1. (**A**) Membrane currents recorded from *Xenopus* oocytes at a holding potential of −60 mV. Records are from oocytes injected 3 days previously with 15 ng of RNA transcribed from wild-type or ΔF508 CFTR and, for comparison, an uninjected oocyte. A stimulation cocktail of 10 μM forskolin, 1 mM IBMX, and 200 μM 8-cpt cAMP was added at the upward arrow and washed out at the downward arrows. These conditions were used for initial screening of CFTR mutants because they elicited high-level activation of wild-type CFTR injected oocytes (*24*). (**B**) Steady-state activation of inward current as a function of IBMX concentration for the oocytes described in Table 2. Each oocyte was exposed to a series of IBMX concentrations in the continuous presence of 10 μM forskolin. The 8-cpt cAMP was omitted in these experiments to allow graded increases in cytosolic cAMP concentration. Results are expressed as percent of maximum activation for each oocyte, and values represent mean ± SEM for three to six oocytes. At 0.5 and 1 mM IBMX, the values for all mutants except F508C differ significantly from wild type, and G551S is different from all others (ANOVA; $P < 0.01$). These results were obtained with three batches of oocytes removed from the same frog at 2-week intervals. All of the CFTR variants were assayed in each batch. Follicular cells were removed from *Xenopus* oocytes obtained as described (*24*), and oocytes were injected with 10 to 15 ng of RNA. Three to 12 days after injection, oocytes were assayed for activatable membrane currents with a two-electrode voltage clamp. Outward membrane current was defined as positive. During experiments, oocytes were continuously perfused with amphibian Ringer containing 100 mM NaCl, 2 mM KCl, 5 mM Hepes, 1.8 mM $CaCl_2$, and 1 mM $MgCl_2$ at pH 7.4.

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Table 1. Comparison of CF patient genotype with clinical phenotype. Patient genotypes are listed in order of disease severity and are designated by both alleles. The ΔF508/F508C compound heterozygotes are clinically unaffected; G551S homozygotes are mildly affected, and the G551D and ΔF508 homozygotes are severely affected.

CF patient genotype (reference)	Sweat Cl^-	Pulmonary disease	Pancreatic status
ΔF508/F508C (*15*)	Normal	None	Sufficient
G551S/G551S (*16*)	Normal	Mild	Sufficient
ΔF508/ΔF508 (*10*)	High	Severe	Insufficient
G551D/G551D (*16, 17*)	High	Severe	Insufficient

has been found in combination with ΔF508 in an unaffected individual and is considered to be phenotypically neutral (*15*). Next was G551S, a serine substituted for glycine at position 551, which is associated with mild disease (*16*). Accordingly, the expression of Cl^- conductance, although less than that seen with wild-type CFTR, was substantial at 1 mM IBMX. The greatest reduction in sensitivity was seen with ΔF508 and G551D (*16, 17*) (an aspartate substituted for glycine), both of which are associated with severe disease (*10, 16*).

The Cl^- selectivity of the oocyte membranes was examined by comparing current-voltage (*I-V*) relations in the presence of normal extracellular Cl^- (106 mM) to those obtained after reducing external Cl^- concentration to 4 mM (Fig. 2). With both wild-type CFTR and ΔF508 CFTR, reducing the external Cl^- shifted the reversal potential in the positive direction by about 50 mV and reduced the conductance at the reversal potential by about 50%. Similar results were obtained with the other mutants, indicating that all of these NBF1 mutants were associated with the expression of a Cl^- conductance.

These data indicate that four naturally occurring mutations in NBF1 of CFTR do not abolish the function of the protein in *Xenopus* oocytes but rather result in reduced sensitivity to cAMP. The similarity in the Cl^- conductance properties associated with expression of the five forms of CFTR suggests that, if CFTR functions as a channel (*5, 7, 18*), then the conduction pathway per se is not compromised by these mutations.

Although the normal sequence of events linking elevation of cytosolic cAMP to channel activation is not completely clear, some elements of the process have been identified. Phosphorylation of at least one serine residue in the putative regulatory (R) domain (*3*) appears to be necessary for activation of the Cl^- conductance in intact cells transfected with wild-type CFTR constructs (*19*). In excised membrane patches from such cells, presence of the catalytic subunit of protein kinase A (PKA) and adenosine triphosphate (ATP) is sufficient for channel activation (*20, 21*), and activation involves at least two discrete steps (*21*): phosphorylation by PKA, and ATP binding and hydrolysis, presumably involving the nucleotide binding folds.

The scheme in Fig. 3 incorporates previous results and our data into a kinetic model for the role of CFTR in the activation of Cl^- conductance. Phosphorylation, and ATP binding and hydrolysis, are presumed to precede channel activation. Mutations in NBF1 could alter the rate of ATP binding or hydrolysis, thereby reducing channel activation. Elevated cytosolic cAMP (and hence catalytic subunit of PKA) might partially overcome this defect by increasing the abundance of the phosphorylated form of CFTR.

In COS cells some mutant forms of CFTR (including ΔF508 but not G551D) are trapped in the endoplasmic reticulum (*22*), and introduction of the ΔF508 construct into mammalian cells is not associated with the expression of an activated conductance (*6–8, 22*), although a small response could not be excluded (*6, 7*). These discrepancies with our results may reflect fundamental differences between the expression systems, but could also result from inadequate elevation of cAMP in the previous studies. Although the maximal currents elicited by forskolin and IBMX in oocytes (Table 2) suggest quantitative differences in protein expression among the CFTR variants, the shapes of the IBMX dose-response curves suggest that even at the highest dose of IBMX the ΔF508 and G551D mutants were not fully activated (Fig. 1B). Thus, the differences in maximal currents observed among the CFTR variants did not reflect alterations in delivery of mature CFTR to the cell membrane. In fact, normal amounts of CFTR protein may be present in the plasma membrane of respiratory epithelial cells from individuals homozygous for ΔF508 (*23*).

Table 2. Maximal currents activated by forskolin and IBMX. Currents (mean ± SEM) were activated in oocytes injected with RNA (*26*) encoding the five forms of CFTR (*27*) described in the text. Maximal currents were defined as those elicited by 5 mM IBMX and 50 μM forskolin.

Genotype	$-I$ (nA)	n
Wild type	595 ± 54	6
F508C	860 ± 34	6
G551S	470 ± 60	5
ΔF508	370 ± 43	6
G551D	205 ± 51	5
Uninjected	11 ± 3	7

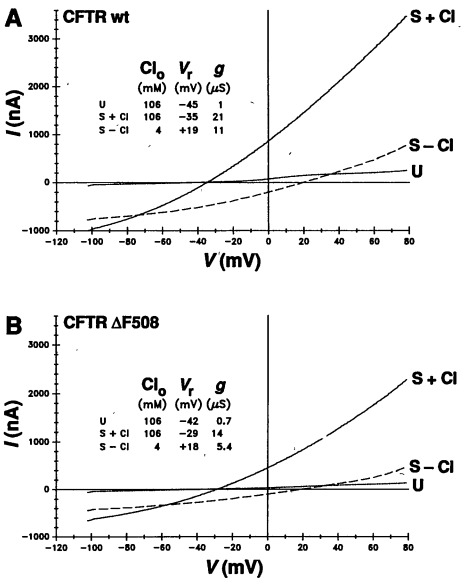

Fig. 2. Representative *I-V* relation for oocytes injected with either (**A**) wild-type or (**B**) ΔF508 RNA. The command potential was changed from −100 mV to +80 mV at 100 mV per second. Records were corrected for the small capacitative component of the current. Records were obtained prior to activation by forskolin and IBMX (U) and during maximal activation before (*S* + Cl) and after (*S* − Cl) reducing external Cl^- from 106 to 4 mM (102 mM aspartate). The small outward current in (A) at depolarizing potentials prior to activation was also in uninjected oocytes and appears to represent an endogenous Cl^- conductance activated as the result of voltage-dependent Ca^{2+} entry (*25*). This current was eliminated by perfusing the oocyte with a nominally Ca^{2+}-free Ringer solution. Because this change had no effect on the activation of the Cl^- conductance in injected oocytes, all *I-V* relations [except the unstimulated condition in (A)] were obtained in the Ca^{2+}-free condition. Cl_o, extracellular Cl^- concentration; V_r, reversal potential; and g, conductance.

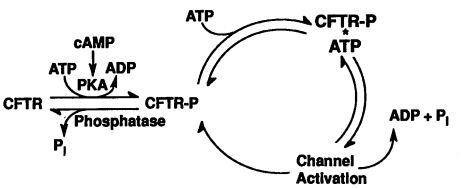

Fig. 3. Model for the role of CFTR channel activation. cAMP-activated Cl^- channel function requires phosphorylation of CFTR by PKA (*3, 18, 21*) to give CFTR-P. Subsequent ATP binding and hydrolysis (*21*) are coupled to channel activation, although the intermediates have not been defined. The second step is depicted as a reaction cycle to account for the reversible effects of ATP after PKA-mediated phosphorylation (*21*).

Our results raise the possibility that the activity of mutant CFTRs in epithelial cells might, by appropriate pharmacological intervention, be increased sufficiently to ameliorate disease symptoms that appear to be largely related to insufficient Cl^- secretion. It is estimated that 92% of CF patients have at least one ΔF508 allele (*10*); therefore pharmacologic activation of this mutant protein would be of benefit in the management of CF. The demonstration of Cl^- conductance in association with the defective CFTR variants also sets the stage for detailed structure-function analysis of the mutant proteins.

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26. To synthesize RNA encoding CFTR, 1 μg of linearized plasmid was incubated with 50 U T7 RNA polymerase (BRL), 40 U RNAsin (Promega), and 2 mM each ATP, UTP, CTP, and GTP in buffer supplied by the enzyme manufacturer at 37°C for 60 to 90 min. RNA was phenol-extracted, ethanol-precipitated, and then resuspended in water.
27. Mutation constructs were generated by oligonucleotide-mediated, site-specific mutagenesis of a 1.7-kb cDNA fragment containing the first third of the CFTR coding sequence. This fragment was cloned into the pSelect vector (Promega), and oligonucleotides corresponding to the desired mutation were synthesized and used for second strand priming as described by the supplier. After sequencing to verify the presence of the mutation, the fragment was transferred into a full-length cDNA construct in the vector pBluescript (Stratagene).

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Inhibition of HIV Replication in Acute and Chronic Infections in Vitro by a Tat Antagonist

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The human immunodeficiency virus-1 (HIV-1) trans-activator Tat is an attractive target for the development of antiviral drugs because inhibition of Tat would arrest the virus at an early stage. The drug Ro 5-3335 [7-chloro-5-(2-pyrryl)-3*H*-1,4-benzodiazepin-2(*H*)-one], inhibited gene expression by HIV-1 at the level of transcriptional trans-activation by Tat. The compound did not inhibit the basal activity of the promoter. Both Tat and its target sequence TAR were required for the observed inhibitory activity. Ro 5-3335 reduced the amount of cell-associated viral RNA and antigen in acutely, as well as in chronically infected cells in vitro (median inhibition concentration 0.1 to 1 micromolar). Effective inhibition of viral replication was also observed 24 hours after cells were transfected with infectious recombinant HIV-1 DNA. The compound was active against both HIV-1 and HIV-2 and against 3′-azido-3′-deoxythymidine (AZT)–resistant clinical isolates.

AMONG THE REGULATORY GENE products encoded by HIV-1 is a strong positive regulator, Tat, that is required for HIV-1 replication (*1*) and has no apparent cellular homologs. Deleting the gene or disabling its initiation codon renders a provirus nonreplicative (*2*). Tat increases gene expression directed by the HIV-1 long terminal repeat (LTR) promoter. Stimulation of gene expression is due to an increase in steady-state mRNA and also possibly to an increase in the efficiency of mRNA use (*3*). Tat functions through the cis-acting Tat-responsive sequence (TAR) immediately downstream of the transcription start site (*4, 5*). The protein stabilizes elongation of transcription and may also increase transcription initiation (*6*).

To screen for Tat inhibitors, we put an indicator gene encoding secreted alkaline phosphatase (SeAP) under the control of the HIV-1 LTR promoter in the plasmid construct pBC12/HIV/SeAP (7). The *tat* gene was similarly controlled in a second plasmid pBC12/HIV/Tat. Cotransfection of COS cells with the two plasmids led to production of alkaline phosphatase, which was secreted into the culture medium. Alkaline phosphatase was quantitated by a colorimetric assay (7) that is amenable to automation and has a high throughput. Quantities of the two plasmids used in the transfection assay were adjusted so that SeAP production was linear in response to input pBC12/HIV/Tat (*8*). To minimize the inherent variability of transfecting cells in monolayers, we transfected COS cells in bulk suspension before plating them on culture dishes for drug testing.

One of the compounds in the repository of Hoffmann–La Roche, Ro 5-3335, reduced the alkaline phosphatase activity in the culture media of COS cells transfected with the plasmids pBC12/HIV/SeAP and pBC12/HIV/Tat (Fig. 1A). Reduction of enzyme activity was due to inhibition of SeAP expression from the HIV-1 LTR promoter because the compound did not inhibit the expression of SeAP driven by the Tat-insensitive LTR promoter of Rous sarcoma virus (RSV). Inhibition of SeAP expression was not 100% in these assays because the compound was added 24 hours after transfection; at this point, SeAP mRNA and enzyme activity could already be detected, and the enzyme was stable up to

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