A Multifunctional Aqueous Channel Formed by CFTR

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The cystic fibrosis gene product (CFTR) is a complex protein that functions as an adenosine 3,5-monophosphate (cAMP)–stimulated ion channel and possibly as a regulator of intracellular processes. In order to determine whether the CFTR molecule contains a functional aqueous pathway, anion, water, and urea transport were measured in *Xenopus* oocytes expressing CFTR. Cyclic AMP agonists induced a Cl^- conductance of 94 microsiemens and an increase in water permeability of 4×10^{-4} centimeter per second that was inhibited by a Cl^- channel blocker and was dependent on anion composition. CFTR has a calculated single channel water conductance of 9×10^{-13} cubic centimeter per second, suggesting a pore-like aqueous pathway. Oocytes expressing CFTR also showed cAMP-stimulated transport of urea but not the larger solute sucrose. Thus CFTR contains a cAMP-stimulated aqueous pore that can transport anions, water, and small solutes. The results also provide functional evidence for water movement through an ion channel.

Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a 168-kD membrane-spanning glycoprotein containing regulatory and nucleotide binding domains (*1*). CFTR functions as a cAMP-stimulated channel for halides (*2*) and possibly for a wider variety of solutes including adenosine triphosphate. CFTR may also be an important intracellular regulator of trafficking and acidification in the endosomal and secretory pathways (*3*). It has been difficult to reconcile the pleiotropic cellular defects characteristic of cystic fibrosis with the unusual structure and function of CFTR (*4*). We examined whether CFTR contains a multifunctional aqueous pore that functions as a transporter not only of anions but also of water and of small solutes. The *Xenopus* oocyte was used as the expression system to examine the transporting properties of CFTR because (i) high levels of functional CFTR are expressed in oocytes injected with mRNA encoding CFTR (*5*); (ii) the oocyte has very low endogenous water and urea permeabilities (*6*); and (iii) water permeability can be measured accurately in oocytes by a real-time imaging method (*6*). This system has been used to characterize water channels from a variety of sources (kidney, reticulocyte, and toad bladder) (*7*) and the cloned erythrocyte and kidney proximal tubule water channels (*8*). *Xenopus* oocytes were injected with in vitro transcribed mRNA (~10 ng) encoding CFTR, human multidrug resistance protein (MDR1), or a mock transcription mixture (*9*) and then assayed for transport of Cl^-, water, or urea after 48 hours.

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The efficiency of CFTR expression was determined by voltage clamp (*10*). Addition of agonists that activate cAMP-dependent protein kinase caused a large increase in current in CFTR-expressing oocytes (Fig. 1A). This increase was slowly reversed by removal of the cAMP agonists and was more rapidly inhibited by 1 mM 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB) (*11*), but not inhibited by 0.1 mM NPPB, 1 mM diphenylamine-2-carboxylate, or 1 mM dinitrodisulfonic stilbene. Anion substitution confirmed that the current was a Cl^- current. Whole-oocyte conductances of 1 to 2 μS before and 94 ± 12 μS (SEM, $n = 5$) after addition of the cAMP agonists were measured (Fig. 1B). If CFTR has a unitary conductance of 8 pS and an open state probability of 0.5 (*2*), then these measurements indicate expression of $\sim 2 \times 10^7$ functional CFTR Cl^- channels in the oocyte plasma membrane.

Oocytes swell in response to the addition of hypotonic media (*12*). The rate of oocyte swelling in response to the osmotic gradient provides a quantitative measure of osmotic water permeability (P_f). Experiments were done at 10°C in order to minimize the endogenous lipid-mediated transport of water through the oocyte plasma membrane (*6*). Cyclic AMP agonists increased P_f in the CFTR-expressing oocytes but not in the mock-injected oocytes (Fig. 1C). Water permeability remained low in the CFTR-expressing oocytes in the absence of cAMP stimulation. In comparison,

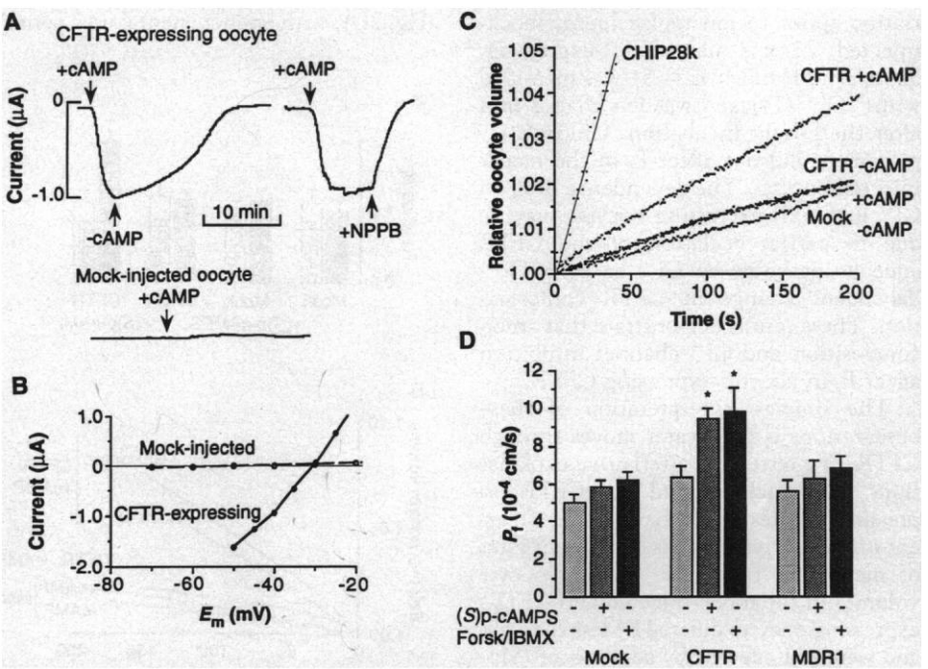

Fig. 1. Chloride conductance and water permeability in *Xenopus* oocytes expressing CFTR and MDR1. (**A**) Two-electrode voltage clamp measurements of mock-injected and CFTR-expressing *Xenopus* oocytes (*10*). Measurements were made at room temperature. Total oocyte current is shown at −50 mV (inside negative) clamp potential. Where indicated, the cAMP mixture (50 μM forskolin, 0.5 mM CPT-cAMP, and 0.5 mM IBMX) or NPPB (1 mM) was added. (**B**) Current-voltage curves for oocytes after cAMP stimulation. Data are average currents for five oocytes. (**C**) Time course of oocyte swelling in response to an osmotic gradient. Oocyte swelling was measured at 10°C. Where indicated, oocytes were incubated with 0.1 mM (*S*)p-cAMPS at 20°C for 5 min and then cooled to 10°C for 5 min before they were assayed for transport. CHIP28k corresponds to oocytes injected with mRNA (1 ng) encoding the proximal tubule water channel (*8*). (**D**) P_f [mean ± SEM (20 to 26 oocytes in each group)] measured in oocytes injected with mock transcription mixture or with mRNA (~10 ng) encoding CFTR or MDR1. *, $P < 0.01$ compared to the mock-injected control.

P_f was increased strongly by injection of mRNA (1 ng) encoding the kidney water channel CHIP28k (*8*). More than 10^9 CHIP28k water channels per oocyte were expressed as compared to $\sim 2 \times 10^7$ functional CFTR molecules estimated above.

The results of four sets of water transport measurements indicate that an increase in P_f occurred in CFTR-injected oocytes after addition of the cAMP agonist adenosine 3′,5′-monophosphorothioate [(*S*)p-cAMPS] or a mixture containing forskolin, CPT-cAMP, and IBMX (Fig. 1D). Oocytes expressing the structural homolog MDR1 showed no increase in P_f after cAMP addition, which is consistent with the result that the ion-transporting activity of MDR1 is volume-dependent rather than cAMP-dependent (*13*).

In order to determine whether water moves through the CFTR molecule, we measured oocyte P_f after modifying CFTR Cl^- channel function. The addition of 1 mM NPPB inhibited the cAMP-dependent increase in P_f in oocytes expressing CFTR (Fig. 2A). There was no effect of NPPB on P_f in oocytes injected with the mock transcription mixture. Depletion of oocyte intracellular Cl^- after a 6-hour incubation with Barth's buffer (gluconate or acetate replacing Cl^-) increased oocyte P_f (Fig. 2, B and C); oocyte Cl^- concentration (prior to ion replacement, mock-injected, 23 ± 3 mM; CFTR-expressing, 33 ± 3 mM; SEM, $n = 5$) was measured with $^{36}Cl^-$ (*14*) and was less than 2 mM after the 6-hour incubation. Chloride replacement did not affect P_f in the mock-injected oocytes. The dependence of P_f on Cl^- in CFTR-expressing oocytes may be due to partial occlusion of the CFTR aqueous pathway by Cl^- or to a Cl^--dependent change in CFTR conformation. These results demonstrate that anion composition and Cl^- channel inhibition affect P_f in oocytes expressing CFTR.

The simplest interpretation of these observations is that water moves through CFTR. We tested the alternative explanations that mock-injected and CFTR-expressing oocytes might have either different osmotic driving forces or different rates of membrane turnover. Average oocyte volumes in the mock-injected and CFTR-expressing oocytes differed by less than 5% and were not affected by addition of (*S*)p-cAMPS, indicating similar osmotic driving forces. Average oocyte Cl^- concentrations, determined by $^{36}Cl^-$ incorporation, were changed by less than 5% by addition of (*S*)p-cAMPS (*14*). The surface area of oocyte plasma membrane, and thus the rate of membrane turnover, was not affected by (*S*)p-cAMPS addition as determined by a microfluorimetry assay (*15*). These results indicate that differences in osmotic driving forces or membrane turnover cannot account for the cAMP-dependent increase in P_f observed in oocytes expressing CFTR.

If water moves through a CFTR pore, then the single channel water permeability (p_f)—estimated from the cAMP-dependent increment in oocyte P_f (4×10^{-4} cm/s), the oocyte surface area (0.045 cm^2), and the number of functional CFTR molecules in the oocyte plasma membrane (2×10^7)—is 9×10^{-13} cm^3/s. This p_f is greater than the value of 6.8×10^{-14} cm^3/s for the CHIP28k water channel (or 3×10^{-13} cm^3/s if the functional water transporting unit of CHIP28k is a tetramer), which does not transport ions or small solutes such as urea (*8*, *16*), and also is greater than the values of 1 to 10×10^{-14} cm^3/s for the gramicidin A, nystatin, and amphotericin B channels, all of which do conduct ions (*17*). The high single channel water permeability associated with CFTR suggests a pore-like conduit that may be large enough to pass small solutes. In order to test this possibility, we measured transport of [^{14}C]-urea, [^{3}H]*O*-methylglucose, and [^{14}C]sucrose in mock-injected and CFTR-expressing oocytes (*18*). Addition of (*S*)p-cAMPS did not affect the uptake of methylglucose or sucrose, whereas it did increase the uptake of a small hydrophilic solute, urea (Fig. 2D), with an incremental urea permeability (P_{urea}) of 3.2×10^{-7} cm/s. The relative increase in the ratio of P_f to P_{urea} was ~1000 for CFTR, much greater than the ratio of ~40 for the amphotericin B pore (*17*), but less than that for the biological water channel CHIP28k which does not transport urea. The CFTR pore is thus wide enough at its most narrow point to pass some urea; however, the aqueous pathway associated with CFTR cannot be a simple right cylindrical pore like amphotericin B or a narrow channel like CHIP28k.

These studies suggest that CFTR contains a cAMP-regulated pore that can transport Cl^-, water, and small hydrophilic solutes. The water permeability of CFTR provides direct support for the unproven assumption that a continuous aqueous pathway exists in ion channels when they are open (*19*). The presence of an aqueous pathway in a membrane protein might allow a single protein to carry out complex transport functions. The aqueous pore characteristic of CFTR is relevant to proposed models of CFTR topology and might provide an explanation for the multiple functions ascribed to CFTR. The water-transporting function of CFTR might function in solute-solvent coupling to facilitate hydration of epithelial cell secretions.

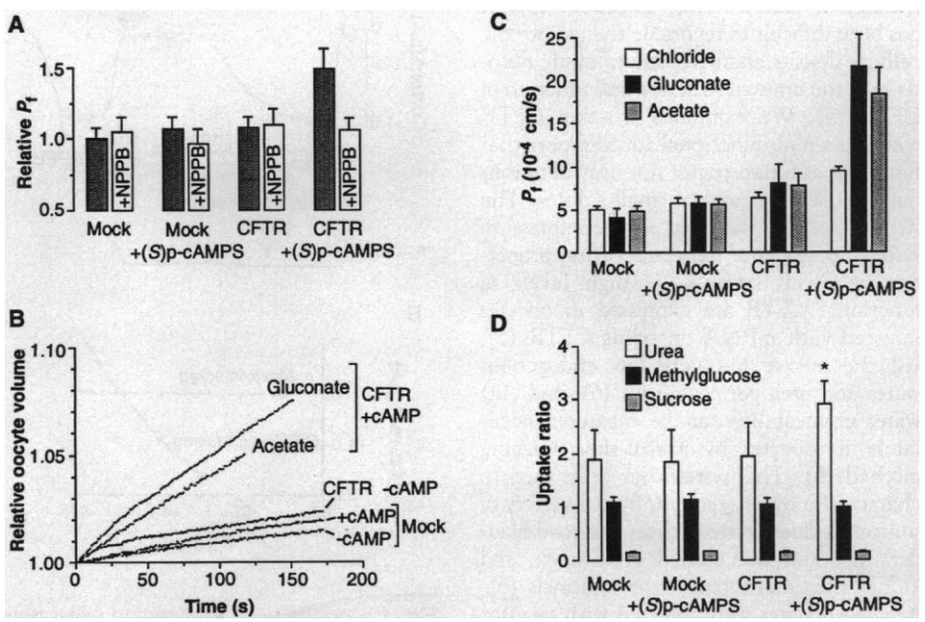

Fig. 2. Water and urea permeability properties of oocytes expressing CFTR. (**A**) Effect of NPPB on oocyte water permeability. NPPB was present for 3 min before and throughout the assay. Data expressed as mean ± SEM of measured P_f for 12 to 18 oocytes in each group. (**B**) Effect of anion substitution on oocyte P_f. Chloride was replaced by incubating oocytes for 6 hours in modified Barth's buffer in which Cl^- was replaced by gluconate or acetate. Water permeability was measured in the same Cl^--free solutions diluted with two volumes of distilled water. Representative data are shown for cAMP-stimulated CFTR-injected oocytes in which Cl^- was replaced by gluconate or acetate, and for mock-injected and CFTR-injected (not cAMP-stimulated) oocytes in which Cl^- was replaced by gluconate. (**C**) P_f (mean ± SEM) for 15 to 20 oocytes in each group. (**D**) Uptake ratios (mean ± SEM, 10 to 15 oocytes) for 10-min influx of [^{14}C]urea, [^{3}H]*O*-methylglucose, and [^{14}C]sucrose at 10°C (*18*). *, $P < 0.02$ compared to mock.

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9. Full-length CFTR cDNA (derived from plasmid pBQ4.7) was cloned into plasmid SP64T to generate pSP CFTR. To maximize expression efficiency, the CFTR 5′ untranslated region was removed by digestion of pSP CFTR with Ava I and Nhe I. Into this vector was ligated an Ava I–Nhe I–digested PCR fragment prepared with oligonucleotides (sense: AGGATCTGGCTAGCGATGACC; antisense: TGTCCTCGGGGATCTGCCAAAGTTGAGCGTTTATT) that converted the Bgl II site of the *Xenopus* globin 5′ untranslated region to an Ava I site. The resulting plasmid, pSP CFTR^{HP}, contained the *Xenopus* globin 5′ untranslated region located 10 bp upstream of the CFTR ATG initiation codon, which enhanced CFTR translation efficiency. MDR1 cDNA (plasmid 2000XS) was digested with Bst UI and Eco RI. A 1200-bp fragment was ligated into SP64T following sequential digestion with Bgl II, Klenow, and Eco RI to generate plasmid MDRA2. A 3060-bp Eco RI fragment from plasmid 2000XS was then ligated into MDRA2 to reconstruct the full-length coding region of MDR1. The mRNA encoding CFTR or MDR1 was generated with 4 μg of linearized plasmid per 10 μl of buffer containing 5 mM magnesium acetate, 0.5 mM diguanosine triphosphate, 10 mM dithiothreitol, calf liver tRNA (0.2 mg/ml), ribonuclease inhibitor (0.8 U/μl), SP6 polymerase (0.4 U/μl), and 40 mM tris (pH 7.5). After transcription for 1 hour at 40°C, the mixture was stored at −70°C. Mock-transcription mixture was prepared in the same manner but lacked plasmid DNA.
10. Electrophysiological measurements were made with a Dagen Instruments TEV-200 two-electrode voltage clamp amplifier. De-folliculated oocytes were prepared as for water permeability measurements and impaled with glass microelectrodes filled with 3 M KCl and having resistances of 0.5 to 5 megaohms. Oocytes were superfused continuously with Barth's buffer (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, and penicillin (10 μg/ml) and streptomycin) at room temperature. The cAMP-activating mixture contained 50 μM forskolin, 0.5 mM 8-chlorophenylthio-cAMP (CPT-cAMP), and 0.5 mM isobutylmethylxanthine (IBMX). In anion substitution experiments in which solution Cl^- was decreased to 5 mM (replaced by gluconate), the reversal potential moved in the positive direction by 40 to 60 mV.
11. NPPB was synthesized by a modification of the published procedure [Wangemann *et al.*, *Pfluegers Arch.* **407**, 128 (1986)]. 2-Chloro-5-nitrobenzoic acid was reacted with excess 3-phenylpropyl-1-amine at 100°C for 2 hours, and the product was purified.
12. Stage V and VI oocytes were microinjected with 50-nl mock transcription mixture or in vitro transcribed mRNA encoding CFTR or MDR1 (~0.2 μg/μl). Oocytes were incubated at 18°C for 48 hours in Barth's buffer with a buffer change at 24 hours. Oocytes were then de-folliculated with collagenase (2 mg/ml, 2 hours, 20°C) and incubated for 3 to 6 hours before measurements were made. P_f was measured from the time course of oocyte swelling in response to a threefold dilution of Barth's buffer with distilled water. Oocyte volume was estimated from cross-sectional area measured by transmission light microscopy and image analysis. P_f was calculated from oocyte surface to volume ratio (S/V_o, 50 cm^{-1}), the initial rate of oocyte swelling [$d(V/V_o)/dt$], and the osmotic gradient according to the equation, $P_f = [d(V/V_o)/dt]/[S/V_o)V_w\Delta Osm]$, where V_w = 18 cm^3/mol (*7*).
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14. Oocyte Cl^- activity was estimated by incubation of de-folliculated oocytes for 24 hours in Barth's buffer containing $Na^{36}Cl$ (1 μCi/ml) at 18°C. Oocytes were washed in ice-cold buffer and oocyte-associated radioactivity was determined; Cl^- activity was calculated from the specific activity of extracellular Cl^- and oocyte aqueous volume.
15. Oocyte plasma membrane–associated fluorescence of trimethylammonium-diphenylhexatriene (TMA-DPH) was used as a measure of plasma membrane surface area [D. Illinger *et al.*, *Cell Biophys.* **14**, 17 (1989)]. De-folliculated oocytes (mock-injected or CFTR-expressing) were incubated for 5 min with (*S*)p-cAMPS (0 or 0.1 mM) in the presence of TMA-DPH (1 μM). Membrane-associated fluorescence was quantified with a Leitz epifluorescence microscope with ultraviolet filter set and Photometrics cooled CCD camera detector.
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18. We measured uptake of [^{14}C]urea, [^{3}H]*O*-methylglucose, and [^{14}C]sucrose by incubating oocytes with each isotope (2 μCi/ml) in Barth's buffer for 10 min at 10°C. Influx was terminated by washing oocytes three times with ice-cold Barth's buffer; individual oocytes were dissolved in 1 ml of 2% SDS, and total uptake was measured by scintillation spectrometry. Uptake was expressed as the oocyte radioactivity divided by the radioactivity contained in the oocyte aqueous volume (~450 nl) at equilibrium.
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20. We thank J. Biwersi for synthesis of NPPB, L.-C. Tsui for plasmid pBQ4.7, M. Gottesman and I. Pastan for plasmid 2000XS, and A. Finkelstein for helpful suggestions. Supported by the National Cystic Fibrosis Foundation and NIH grants HL42368, DK35124, DK43840, DK16095, and DK39354. H.H. is a fellow of the National Kidney Foundation, W.S. the recipient of Physician-Scientist award CA01614, and A.S.V. an established investigator of the American Heart Association.

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Porins in the Cell Wall of Mycobacteria

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The cell wall of mycobacteria is an efficient permeability barrier that makes mycobacteria naturally resistant to most antibiotics. Liposome swelling assays and planar bilayer experiments were used to investigate the diffusion process of hydrophilic molecules through the cell wall of *Mycobacterium chelonae* and identify the main hydrophilic pathway. A 59-kilodalton cell wall protein formed a water-filled channel with a diameter of 2.2 nanometers and an average single-channel conductance equal to 2.7 nanosiemens in 1 M potassium chloride. These results suggest that porins can be found in the cell wall of a Gram-positive bacterium. A better knowledge of the hydrophilic pathways should help in the design of more effective antimycobacterial agents.

The outer membrane of Gram-negative bacteria plays an important role in the intrinsic resistance of these organisms to antibiotics by decreasing the flow of antimicrobial agents into the cell. In contrast, Gram-positive bacteria do not contain an outer membrane in their cell wall, and consequently such organisms are, in general, more sensitive to antibiotics. Mycobacteria are Gram-positive bacteria that are highly resistant to a wide range of antibiotics and are major human pathogens. Tuberculosis, caused by *Mycobacterium tuberculosis*, kills 3 million people yearly worldwide (*1*). Leprosy, caused by *M. leprae*, is a major health problem in developing countries (*2*). Mycobacterial infections caused by *M. avium-intracellulare* complex and *M. tuberculosis* are among the most frequent opportunistic infections in patients with acquired immunodeficiency syndrome (AIDS) (*3, 4*). The mycobacteria have a distinct cell wall that contains a thick conventional peptidoglycan covalently linked to arabinogalactan polysaccharides that are esterified to mycolic acids (branched, long-chain fatty acids). It also includes complex lipids, lipopolysaccharides anchored in the cell wall, and proteins.

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