Expression of Cystic Fibrosis Transmembrane Regulator Cl⁻ Channels in Heart

Paul C. Levesque, Pádraig J. Hart, Joseph R. Hume, James L. Kenyon, and Burton Horowitz

Cyclic AMP (cAMP)-dependent chloride channels modulate changes in resting membrane potential and action potential duration in response to autonomic stimulation in heart. A growing body of evidence suggests that there are marked similarities in the properties of the cAMP-dependent chloride channels in heart and cystic fibrosis transmembrane regulator (CFTR) chloride channels found in airway epithelia or in cells expressing the CFTR gene product. We isolated poly A⁺ mRNA from rabbit ventricle and converted it to cDNA for amplification using the polymerase chain reaction (PCR). A fragment corresponding to the nucleotide-binding domain 1 (NBD1) of the CFTR transcript was cloned. Comparison of the amino acid sequence of NBD1 of human CFTR with the deduced sequence of the rabbit heart PCR product indicated 98% identity. Northern blot analysis, using the heart amplification product as a cDNA probe, demonstrated expression of homologous transcripts in human atrium, guinea pig and rabbit ventricle, and dog pancreas. Xenopus oocytes injected with poly A⁺ mRNA extracted from rabbit and guinea pig ventricle or dog pancreas expressed robust time-independent chloride currents in response to an elevation of cAMP. We conclude that CFTR chloride channels are expressed in heart and are responsible for the observed cAMP-dependent chloride conductance. (Circulation Research 1992;71:1002–1007)

Key Words • cardiac muscle • chloride channels • cyclic AMP • cystic fibrosis transmembrane regulator

The existence of cyclic AMP (cAMP)-dependent chloride channels in heart was first reported in the guinea pig in 1989,1,2 and subsequently studies3–7 have verified their presence in other species and have provided insight into the possible physiological role of these autonomically regulated anion channels. Activation of these channels can modulate resting membrane potential and shorten the cardiac action potential plateau and duration. Thus, cAMP-dependent Cl⁻ channels may represent a new potential target site for the development of antiarrhythmic agents.

Although there appears to be diversity in the properties of Cl⁻ channels in many different preparations, the cAMP-activated Cl⁻ conductance in cardiac muscle8 has many properties in common with the Cl⁻ conductance observed in epithelial cells9,10 or Xenopus oocytes11,12 expressing cystic fibrosis transmembrane regulator (CFTR). Both the CFTR-mediated9,11 and cardiac Cl⁻ currents9 are time independent and exhibit similar rectification properties. The cAMP-activated Cl⁻ channels from heart13 have the same anion selectivity (bromide > chloride > iodide > fluoride) as the CFTR-generated channel,10,14 and both channels are regulated through the cAMP-dependent protein kinase A pathway.1,2,10 A novel dependence on nucleoside triphosphates has recently been demonstrated for chloride channel activation in both epithelial cells13 and heart.16 Finally, cardiac and CFTR chloride channels exhibit a similar sensitivity to chloride channel blockers1,2,5,17,18 and have similar single channel conductances (8–13 pS).10,11,16,19 These similarities raise the important question of whether CFTR chloride channels and cardiac cAMP-dependent chloride channels are structurally equivalent proteins.

Our results provide the first molecular evidence that CFTR is expressed in human, guinea pig, and rabbit heart. Furthermore, the expression of an exogenous cAMP-dependent chloride conductance in Xenopus oocytes after injection of mRNA from heart strongly suggests that CFTR is responsible for the cAMP-dependent chloride conductance observed in native cardiac cells.

Materials and Methods

mRNA Preparation and cDNA Synthesis

Poly A⁺ mRNA was prepared from freshly dissected rabbit and guinea pig ventricle and dog pancreas using the FAST TRACK kit (Invitrogen, San Diego, Calif.) according to the manufacturer’s instructions. First-strand cDNA synthesis was carried out on approximately 0.5 μg poly A⁺ mRNA. The following reagents were combined: 200 units of Moloney murine leukemia viral reverse transcriptase, 1 unit of RNase inhibitor, 1 mM deoxynucleotidetriphosphates, and 100 pmol oligo(dT) primers in a final volume of 20 μl of 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂. The mixture was incubated for 10 minutes
at room temperature [to anneal oligo(dT)] and then 120 minutes at 42°C (for first-strand synthesis).

**Polymerase Chain Reaction, Subcloning, and Sequencing**

Oligonucleotide primers (25 pmol) were added to the first-strand synthesis mixture with the following: 2.5 units *Thermus aquaticus* (Taq) polymerase, 7.5 μl of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1 mM MgCl₂, and 0.01% gelatin and water to bring the final volume to 100 μl. The reaction mixture was overlaid with 90 μl mineral oil. The mixture was then incubated in a thermal cycler (Cetus) with the following amplification profile: two cycles at 94°C for 1 minute, 37°C for 3 minutes, 72°C for 15 seconds; then 33 cycles with the profile 94°C for 1 minute, 55°C for 2 minutes, 72°C for 15 seconds with an extra 5 seconds at 72°C added in each cycle. The oligonucleotide sequences used were: 5’ATTGGATC-CAATGTGATGACGCCCTTC (sense primer) and 5’ATTGAACTAGTTACAGACAGCT (anti-sense primer). These sequences border the CF locus and have been used to detect CFTR expression in other tissues and cell lines.⁹ The 5’ ends of the primers contain EcoRI/BamHI restriction enzyme sites (in bold) to facilitate subcloning of the polymerase chain reaction (PCR) amplification products. The three “extra” bases at the 5’ end of the primers protect the restriction sites against partial degradation by the Taq polymerase. The amplified DNA was analyzed with agarose gel electrophoresis in 3% agarose gels and stained with ethidium bromide to determine the approximate molecular weight of the amplified products. The DNA was excised from the agarose gel, purified, and subcloned into an appropriately digested pGEM (Promega, Madison, Wis.) vector. Plasmids that contain inserts were sequenced by the dideoxy method.²⁰

**Northern Blot Analysis**

The DNA fragment representing the PCR amplification product from rabbit heart was excised from 3% agarose gels, purified, and labeled with phosphorus-32 using random hexanucleotide priming.²¹ RNA was size fractionated on 1.0% agarose/formaldehyde gels and transferred to immobilon filters (Millipore, Bedford, Mass.).²² Filters were baked and prehybridized in 50% formamide overnight at 42°C. Hybridization to ³²P-labeled probes was performed under the same conditions. The filters were washed at high stringency (3 times in 2× standard saline citrate buffer [SSC] [0.3 M NaCl/0.03 M sodium citrate, pH 7.0, with 0.1% sodium dodecyl sulfate (SDS)] at room temperature for 5 minutes each and then twice for 30 minutes each in 0.2× SSC/0.1% SDS at 65°C) to assure specificity of labeling. Autoradiography was performed at ~80°C with intensifying screens.

**Preparation and Injection of Oocytes**

Adult *Xenopus laevis* were anesthetized by immersion in a solution of tricaine methanesulfonate (1 g/l), and oocytes were surgically removed. The oocytes were prepared for injection by teasing apart the ovarian lobes, rinsing several times, and then incubating them at room temperature in Ca²⁺-free OR2 solution (mM: NaCl 82.5, KCl 2.5, MgCl₂ 1, HEPES 5, pH 7.5) containing 1 ml collagenase (Sigma Type 1A). Individual oocytes become separated within 2–3 hours with gentle shaking. Stage V and VI oocytes were collected under a dissecting microscope, rinsed in the OR2 solution, and then stored in ND 96 solution (mM: NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, pH 7.5) containing 2.5 mM sodium pyruvate and gentamicin (100 mg/100 ml) for 1–2 hours before injection. Injection pipettes were pulled on a horizontal pipette puller (Sutter P-87, San Rafael, Calif.) and broken back under microscopic observation until the tips were about 20 μm in diameter. The pipettes were then baked at >150°C for 3–4 hours to destroy RNAses. For injection, the pipettes were mounted in a Drummond Nanoject autoinjector (Drummond Scientific, Broomall, Pa.) that in turn was mounted on a micromanipulator. Typically, individual oocytes were injected with 46 nl poly A⁺ mRNA (1 mg/1 ml). Oocytes were then stored in separate wells of 48-well culture plates in ND 96 plus pyruvate and gentamicin at 18°C until electrophysiological assay, usually 3–4 days later. Only about one third of all frogs tested gave oocytes capable of expressing an exogenous cAMP-activated conductance. The cAMP-activated conductance was detected in approximately 20% of injected oocytes taken from designated donor frogs. These expression rates are close to those reported in other studies in which expression of exogenous channel proteins was assayed after injecting *Xenopus* oocytes with total mRNA.²³,²⁴ Successful expression was critically dependent on oocyte viability and the quality of the tissue from which the mRNA was isolated.

**Voltage-Clamp Recording**

Membrane currents were recorded from oocytes at room temperature using a standard two-microelectrode voltage-clamp system (Dagan TEV-200, Minneapolis, Minn.). Microelectrodes were made from borosilicate glass tubing on a one-stage vertical electrode puller. The microelectrodes were filled with 3 M KCl and had resistances of <10 MΩ. Voltages were reported with reference to the bath. Most Cl⁻ current recordings were made in Ca²⁺-free Ringer solution (mM: NaCl 96, KCl 2, MgCl₂ 2.8, EGTA 1, HEPES 5, pH 7.5) to prevent activation of the endogenous Ca²⁺-activated Cl⁻ channel that might otherwise interfere with efforts to detect expression of exogenous Cl⁻ currents. In some experiments, the dependence of the expressed conductance on Cl⁻ was determined by partially replacing bath NaCl with sodium aspartate. The membrane-permeable cAMP analogue 8-bromo-cAMP (8-Br-cAMP) was included in the external solution to directly elevate intracellular cAMP. Forskolin (1 μM) and 3-isobutyl-1-methylxanthine (100 μM) were used in external solutions in some experiments to activate adenylyl cyclase and inhibit phosphodiesterase activity, respectively. Intracellular cAMP levels were also elevated in some experiments by direct intracellular injections. The method involves connecting a pneumatic pressure ejection system (Picospritzer II, General Valve Corp., East Hanover, N.J.) to the voltage electrode, which contains the chemical to be injected. For these experiments the voltage electrode was filled with (mM): KOH 120, MES 120, MgCl₂ 2, HEPES 10, cAMP 0.1, pH 7.5. The command voltage pulses and data acquisition functions were handled by an IBM-AT-compatible computer and pCLAMP 5.5 software (Axon Instruments, San Rafael, Calif.).
Burlingame, Calif.). Current signals were low pass filtered at 1 kHz through an 8-pole bessel filter (Frequency Devices, Haverhill, Mass.) and digitized on-line at 1 kHz.

Results

The PCR technique was used to determine whether a transcript homologous to CFTR is expressed in heart. Poly A+ mRNA was isolated from freshly dissected rabbit ventricle and converted to single-stranded cDNA using oligo(dT) primers. The cDNA was used as a template for enzymatic amplification primed by oligonucleotides designed to hybridize to both ends of the first nucleotide-binding domain (NBD1) of CFTR (Figure 1A). NBD1 is critical for normal functioning of CFTR since ATP hydrolysis by NBD1 is necessary for channel opening, and the majority of missense mutations that cause cystic fibrosis are found in this ATP-binding domain. The amplification product was analyzed on 3% agarose by electrophoresis. A prominent band was present with a molecular weight of approximately 550 base pair (bp). This size closely corresponds to the expected distance between the primer hybridization sequences. The band was excised from the gel and DNA fragments were purified; these fragments were subcloned and the DNA sequences were determined. The PCR product and human CFTR gene share about 93% sequence homology over the 550 bp cDNA fragment. The deduced amino acid sequence displays only four different residues when compared with human CFTR, corresponding to 98% identity (Figure 1B).

The PCR amplification product from heart, having a high degree of homology to NBD1, was used as a probe in Northern blot analysis to test for expression in pancreas and in cardiac muscle from several species. Pancreas was used in the analysis because CFTR is expressed quite strongly in this tissue, and we wanted to compare hybridization bands resulting from our heart probe to the CFTR signal. Poly A+ mRNA was prepared from freshly dissected dog pancreas, human atrium, and guinea pig and rabbit ventricle. Autoradiograms resulting from the hybridizations are shown in Figure 2. The PCR product hybridized to a transcript of about 6.5 kb in dog pancreas and rabbit, guinea pig, and human heart. A CFTR-specific probe used in a previous study26 hybridized to transcripts of 6.5 kb in several different tissues affected in cystic fibrosis, including airway epithelia and pancreas. The results of our Northern analysis suggest that the heart and CFTR cAMP-activated Cl− channel transcripts are the same size since our heart probe hybridized to 6.5 kb transcripts in heart and pancreas.

Xenopus oocytes were used to functionally express the cAMP-regulated Cl− conductance from mammalian heart and pancreas. Oocytes were injected with 46 nl poly A+ mRNA (1 mg/ml) extracted from rabbit and guinea pig ventricle or dog pancreas. The mRNA from heart or pancreas that was used for Northern analysis was also used for injecting oocytes, ensuring that the proteins giving rise to the hybridization bands were injected into oocytes. The two-microelectrode voltage-clamp technique was used to assay oocytes for expression of an exogenous cAMP-activated Cl− conductance. Native oocyte membranes contain a transient Cl− conductance that is activated by Ca2+ influx through voltage-dependent channels. Thus, all current recordings

![Figure 1. Panel A: Diagram representing the membrane topology of the cystic fibrosis transmembrane regulator (CFTR) Cl− channel. N, amino terminal end; C, carboxy terminal end. Regulatory (R) and nucleotide-binding domains (NBD1, NBD2) are cytoplasmically oriented. Shaded region represents the domain amplified by polymerase chain reaction (PCR). mRNA was isolated from samples of rabbit ventricle and a complementary strand of DNA synthesized. mRNA was degraded and PCR primers allowed to anneal to the first-strand cDNA. PCR was then performed and products of expected size cloned. Panel B: Amino acid sequence of heart Cl− channel: Comparison of amino acid sequence of NBD1 of human CFTR with rabbit heart Cl− channel PCR product. Arrows indicate the position and direction of PCR primer sequences.](http://circres.ahajournals.org/lookup/doi/10.1161/01.CIR.71.4.1004)
were made in Ca\textsuperscript{2+}-free external solutions to prevent activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels that might otherwise interfere with efforts to detect expression of exogenous Cl\textsuperscript{−} currents. Figure 3 shows results obtained from an oocyte that was injected with rabbit ventricle mRNA 3 days before voltage clamping. Currents elicited under control conditions (100 mM Cl\textsuperscript{−}\textsubscript{out}; zero Ca\textsuperscript{2+}) during 500-msec steps from −120 mV to 60 mV in 30-mV increments from a holding potential of −60 mV are shown in Figure 3A. The endogenous Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} conductance was nearly undetectable in the absence of external Ca\textsuperscript{2+}. The same voltage protocol elicited time-independent currents after incubating the oocyte for 35 minutes after addition of 100 μM 8-Br-cAMP to the bath (Figure 3B). This conductance was never observed in noninjected control oocytes (n=12). The prolonged time course of activation of the conductance by bath application of 8-Br-cAMP is presumably due to the slow permeation of this compound across the oocyte membrane.\textsuperscript{23} Replacing 80% of bath Cl\textsuperscript{−} with aspartate markedly attenuated the cAMP-activated currents (Figure 3C), suggesting that the conductance is anion selective. The current–voltage curve of the cAMP-induced currents (Figure 3D) was outwardly rectifying, and the reversal potential was near −36 mV, which is close to the predicted value for Cl\textsuperscript{−} assuming an intra-oocyte Cl\textsuperscript{−} concentration of around 25–30 mM.\textsuperscript{25} The outward rectification may be explained by a low intra-oocyte Cl\textsuperscript{−} concentration compared with that of the external solution. In other injected oocytes, the degree of outward rectification was less pronounced. This variability is consistent with results from studies where CFTR\textsuperscript{11} or other epithelial\textsuperscript{29} Cl\textsuperscript{−} channels have been expressed in oocytes. Perhaps this is due to leakage of Cl\textsuperscript{−} from the intracellular microelectrodes (3 M KCl) and subsequently increased Cl\textsuperscript{−} levels in some oocytes. This would decrease the Cl\textsuperscript{−} gradient and cause a more linear current–voltage relation. In the experiment shown in Figure 3D, substitution of aspartate for 80% of the external Cl\textsuperscript{−} shifted the reversal potential to about −4 mV. In six oocytes the cAMP-activated conductance exhibited a mean reversal potential of −34±7 mV with external Cl\textsuperscript{−} of 101 mM and a mean reversal potential of −1±2 mV with external Cl\textsuperscript{−} of 20 mM. This corresponds to approximately a 42.5-mV shift per 10-fold change in external Cl\textsuperscript{−}. Given that we can only assume an internal Cl\textsuperscript{−} concentration of 25–30 mM,\textsuperscript{25} this shift in reversal potential indicates significant Cl\textsuperscript{−} permeability of the expressed channel. A cAMP-activated Cl\textsuperscript{−} conductance was also observed in oocytes injected with mRNA from guinea pig ventricle (n=4) (not shown).

Our heart Cl\textsuperscript{−} channel probe hybridized strongly to a 6.5 kb transcript from pancreas mRNA on the Northern blot, as did a probe for CFTR having a nearly identical amino acid sequence in a previous study.\textsuperscript{28} In light of these findings, we used mRNA from pancreas, a tissue that expresses CFTR, as a positive control. Thus,
oocytes injected with mRNA from dog pancreas were assayed for the presence of a cAMP-activated Cl⁻ conductance similar to that observed after injection of heart mRNA or CFTR cRNA. 11 Oocytes were injected with approximately 50 ng dog pancreas poly A⁺ mRNA 4 days before voltage clamping. The bath solutions and voltage protocols used to elicit currents were identical to those used in the experiments with heart mRNA. Injection (see "Materials and Methods") of 100 µM cAMP activated a time-independent conductance (n=5) that was not detectable in noninjected control oocytes (n=10) (Figure 4). Similar currents were observed in other mRNA-injected oocytes after injection of 10 µM forskolin and 100 µM 3-isobutyl-1-methylxanthine to activate adenylate cyclase and inhibit phosphodiesterase activity, respectively. The conductance was the same as that observed in oocytes injected with mRNA from rabbit or guinea pig heart in that it was activated by cAMP (Figures 4A and 4B), exhibited no significant activation or inactivation during 500-msec test pulses (Figure 4B), was outwardly rectifying (Figure 4D), and was anion selective (Figure 4C). Reducing external Cl⁻ to 20 mM with aspartate substitution shifted the reversal potential from a mean of -35±8 mV to 0.5±3 mV (n=5). This corresponds to about a 45-mV shift per 10-fold change in external Cl⁻. Thus, the cAMP-activated Cl⁻ conductance was similar in oocytes injected with mRNA from pancreas, a tissue known to express CFTR, to that in heart mRNA-injected oocytes.

Figure 4. Effects of cyclic AMP (cAMP) and external Cl⁻ on membrane currents in oocytes injected with mRNA from dog pancreas. Currents were recorded 72 hours after injecting oocytes with 46 nl poly A⁺ mRNA (1 mg/1 ml) extracted from dog pancreas. Panel A: Zero-Ca²⁺ bath solution inhibited endogenous Ca²⁺-activated Cl⁻ currents during 500-msec voltage-clamp steps from -120 mV to 60 mV in 30-mV increments from a holding potential of -60 mV. Panel B: Same voltage protocol elicited time-independent currents 3-4 minutes after injecting the oocytes with 100 µM cAMP. The cAMP-activated conductance was not observed in noninjected control oocytes. Panel C: Partially substituting external Cl⁻ with aspartate attenuated the cAMP-activated currents. Panel D: Reducing external Cl⁻ shifted the current-voltage relation of the cAMP-induced currents. Solutions and data acquisition parameters are described in "Materials and Methods." Similar results were observed in four other oocytes.

Discussion

The results of the present study provide strong molecular and electrophysiological evidence supporting the hypothesis that the cAMP-activated Cl⁻ channel from heart is the same as the CFTR Cl⁻ channel. The high degree of homology in the deduced amino acid sequence of our heart amplification product and the NBD1 of CFTR provides evidence that the channels are very similar or identical. The results of the Northern analysis also indicate that CFTR is the molecular equivalent of the heart Cl⁻ conductance since the Cl⁻ channel probe from heart hybridized to transcripts of identical size in both heart and pancreas. CFTR-specific probes28 hybridize to a transcript of identical size in pancreas and other epithelial tissues. This suggests that transcripts encoding the cAMP-activated Cl⁻ channels in heart are the same size (6.5 kb) as the CFTR Cl⁻ channels. Also, given the high homology of the heart amplification product to CFTR and the stringent hybridization and washing conditions, it is very unlikely that the heart probe cross-hybridized to transcripts corresponding to nucleotide-binding domains of other (non-CFTR) transporters or regulatory proteins. The ATP-binding domain of CFTR shares only about 30% sequence homology to the ATP-binding domains of a large family of transport proteins.29 In fact, this region is considered a principal distinguishing feature of this family of transport proteins. The 98% amino acid sequence identity shared by our amplification product from heart and the
ATP-binding domain (NBD1) of CFTR indicates that these Cl⁻ transporters are the same protein. Determination of the structure of the heart CFTR message awaits the isolation of a full-length clone.

The Cl⁻ conductance measured in oocytes injected with mRNA from dog pancreas and rabbit and guinea pig heart closely resembles whole cell currents elicited by cAMP in freshly dissociated myocytes from rabbit and guinea pig ventricle in terms of time independence, Cl⁻ selectivity, activation by cAMP, and outward rectification. The dependence of activation of the exogenous Cl⁻ conductance in oocytes on cAMP and the Ca²⁺ and voltage independence of the current suggest that the conductance is unlikely to be due to Cl⁻ movement through exogenously expressed cardiac sarcoplasmic reticum anion channels. With the identification of the structural equivalent for the cAMP-dependent Cl⁻ conductance in heart, we now have a molecular understanding with which to develop and use probes (cDNAs or antibodies) to determine such parameters as the molecular distribution and developmental expression pattern of this physiologically important cardiac protein. In addition, future studies should reveal whether functional alterations of cardiac CFTR Cl⁻ channels occur in patients with cystic fibrosis.

Acknowledgments

We thank Mark Horner for his technical assistance with oocyte injections and Dr. T. Chapman and Associates at Reno Heart Surgeons for assistance in obtaining human atrial tissue.

References


Levesque et al. CFTR Cl⁻ Channels in Heart 1007

Downloaded from http://circres.ahajournals.org/ by guest on April 1, 2017
Expression of cystic fibrosis transmembrane regulator Cl- channels in heart.
P C Levesque, P J Hart, J R Hume, J L Kenyon and B Horowitz

Circ Res. 1992;71:1002-1007
doi: 10.1161/01.RES.71.4.1002

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/71/4/1002