Purinoceptor-coupled Cl^- channels in mouse heart: a novel, alternative pathway for CFTR regulation

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1. P_2-purinoceptors couple extracellular ATP to the activation of a Cl^- current ($I_{Cl,ATP}$) in heart. We studied the molecular mechanism and intracellular signalling pathways of $I_{Cl,ATP}$ activation in mouse heart.

2. Extracellular adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS; 100 μM) activated $I_{Cl,ATP}$ in both atrial and ventricular myocytes. A specific PKC inhibitor, bisindolylmaleimide blocked the effect of ATPγS while a PKC activator, phorbol 12,13-dibutyrate (PDBu) activated a current with identical properties to $I_{Cl,ATP}$. Maximal activation of $I_{Cl,ATP}$ by ATPγS or PDBu occluded further modulation by the other agonist, suggesting that they may activate the same population of Cl^- channels.

3. Isoprenaline increased $I_{Cl,ATP}$ pre-activated by ATPγS or PDBu, while isoprenaline or forskolin alone failed to activate any Cl^- current in these myocytes. Adenosine 3′,5′-cyclic monophosphothionate, a PKA inhibitor, prevented ATPγS or PDBu activation of $I_{Cl,ATP}$. Thus, $I_{Cl,ATP}$ is regulated by dual intracellular phosphorylation pathways involving both PKA and PKC in a synergistic manner similar to cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels.

4. Glibenclamide (50 μM) significantly blocked $I_{Cl,ATP}$ activated by ATPγS or by the CFTR channel activator, levamisole.

5. The slope conductance of the unitary $I_{Cl,ATP}$ in cell-attached patches was $11{\cdot}8 \pm 0{\cdot}3$ pS, resembling the known properties of CFTR Cl^- channels in cardiac myocytes.

6. The reverse transcription polymerase chain reaction and Northern blot analysis revealed CFTR mRNA expression in mouse heart.

7. We conclude that $I_{Cl,ATP}$ in mouse heart is due to activation of CFTR Cl^- channels through a novel intracellular signalling pathway involving purinergic activation of PKC and PKA.

The cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan *et al.* 1989) encodes a chloride (Cl^-) channel (Sheppard & Welsh, 1999) that is activated by the binding of ATP to its cytoplasmic nucleotide-binding domains (NBDs) and by the phosphorylation of key serine residues in the regulatory (R) domain. Phosphorylation is mediated principally by cyclic AMP (cAMP)-dependent protein kinase A (PKA) and by protein kinase C (PKC) (Jia *et al.* 1997; Gadsby & Nairn, 1999; Yamazaki *et al.* 1999). Mutations of epithelial CFTR lead to dysfunction of the CFTR Cl^- channel in cystic fibrosis (CF) patients, resulting in abnormal epithelial chloride transport (Pilewski & Frizzell, 1999).

In the heart, an isoform (exon 5-) of the epithelial CFTR gene product is responsible for cAMP-PKA-activated Cl^- currents ($I_{Cl,PKA}$) (Levesque *et al.* 1992; Nagel *et al.* 1992; Hart *et al.* 1996) and PKC-activated Cl^- currents ($I_{Cl,PKC}$) as well (Zhang *et al.* 1994; Collier & Hume, 1995; Middleton & Harvey, 1998). While several observations suggest that cardiac CFTR Cl^- current ($I_{Cl,CFTR}$) may modulate resting membrane potential and action potential duration of mammalian cardiac myocytes (Hiraoka *et al.* 1998), the exact physiological and pathophysiological role of this channel in both normal heart and CF heart remain poorly understood (Gadsby *et al.* 1995; Hume *et al.* 2000). Since the cardiac and epithelial CFTR Cl^- channel proteins are nearly identical, it is very important to know whether the cardiac CFTR Cl^- channel is also defective in CF patients. In order to assess whether or not the mouse CFTR knockout model (Snouwaert *et al.* 1992) would be useful for determining the

functional role of CFTR in heart, previous studies have attempted to characterize the properties of CFTR in mouse heart; however, these studies (Levesque & Hume, 1995; Walsh & Wang, 1996) failed to detect $I_{Cl,PKA}$ in normal control mouse atrial and ventricular myocytes, raising doubts about whether $I_{Cl,CFTR}$ is functionally expressed in mouse heart, and seemed to obviate the potential use of the CFTR knockout mouse model to assess the physiological and pathophysiological role of CFTR in heart.

In mouse ventricular myocytes, stimulation of purinergic receptors by extracellular nucleotides, including ATP, ADP, and ATPγS, but not adenosine nor AMP, does result in activation of a Cl^- current ($I_{Cl,ATP}$) (Levesque & Hume, 1995) similar to the $I_{Cl,ATP}$ described previously in guinea-pig (Matsuura & Ehara, 1992) and rat (Kaneda *et al.* 1994) ventricular myocytes. In both rat and mouse ventricular myocytes, the activation of $I_{Cl,ATP}$ appeared to be coupled to P_2-purinoceptor stimulation (Kaneda *et al.* 1994; Levesque & Hume, 1995). To date, however, the intracellular signal transduction pathways linking P_2-purinoceptor stimulation to the activation of $I_{Cl,ATP}$ in heart have not been characterized and the identity of the channel responsible for $I_{Cl,ATP}$ is unknown. Although $I_{Cl,ATP}$ represents the least-studied Cl^- channel in heart (Hume *et al.* 2000), many of the known electrophysiological and pharmacological properties of $I_{Cl,ATP}$ resemble those of $I_{Cl,CFTR}$ (Matsuura & Ehara, 1992; Kaneda *et al.* 1994; Levesque & Hume, 1995). Furthermore, in epithelial cells it has been suggested that extracellular ATP may directly activate CFTR Cl^- channels (Cantiello *et al.* 1994; Stutts *et al.* 1995). It seems possible, therefore, that $I_{Cl,ATP}$ and $I_{Cl,CFTR}$ in heart might be generated by the same protein or by proteins molecularly related to CFTR.

In the present study, we used electrophysiological, pharmacological, and molecular biological techniques to further characterize the properties of $I_{Cl,ATP}$ in mouse heart. We first demonstrate that PKA and PKC couple purinergic receptors to the activation of $I_{Cl,ATP}$ through dual synergistic PKC and PKA phosphorylation pathways, in a manner highly homologous to the regulation of CFTR. We then provide evidence that the properties of the unitary conductance responsible for $I_{Cl,ATP}$ in cell-attached patches of mouse ventricular myocytes are nearly identical to the known properties of cardiac unitary CFTR Cl^- channels. Finally, we present evidence using the reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis confirming the expression of the mouse CFTR homologue (Tata *et al.* 1991; Yorifuji *et al.* 1991) in normal mouse atrium and ventricle. These results provide evidence that purinergic receptor stimulation in mouse heart is coupled to the activation of CFTR by dual synergetic phosphorylation pathways involving both PKC and PKA, and support the potential usefulness of the CFTR knockout mouse for future functional studies of CFTR in heart.

METHODS

Preparation of single cardiac cells

Single atrial and ventricular myocytes from mouse hearts were isolated using an enzymatic dispersion technique as originally described (Levesque & Hume, 1995). Briefly, the technique involves rapidly removing the heart from mice (C-57/black inbred, male, 20 g) killed by cervical dislocation, in accordance with national guidelines, and perfusing the heart, using a modified Langendorff technique, with a physiological saline solution (PSS) warmed to 37 °C. PSS contained (mM): 126 NaCl, 22 dextrose, 4·4 KCl, 5·0 $MgCl_2$, 1·5 $CaCl_2$, 12 Hepes, 20 taurine, 5 creatine, 5 sodium pyruvate, and 1 $NaHPO_4$. The heart was perfused with PSS until free of blood and then with a nominally Ca^{2+}-free PSS until the heart ceased to beat, and finally with the Ca^{2+}-free solution containing 0·04% collagenase (CLSII, Sigma) and 1·0% bovine serum albumin (BSA) for 20–30 min. The atria and ventricles were removed and further dissected into small pieces, and cell dissociation was achieved by gentle mechanical agitation. Only rod-shaped myocytes with clear cross-striations and no blebs under isotonic conditions were used for electrophysiology and molecular biology studies.

Electrophysiological recordings

The tight-seal whole-cell patch-clamp technique was used to record whole-cell currents in isolated mouse atrial and ventricular myocytes and the cell-attached configuration of the patch-clamp technique was employed to record single Cl^- channel currents from mouse ventricular myocytes (Hamill *et al.* 1981). Recording pipettes were prepared from borosilicate glass electrodes (1·5 mm o.d.) with tip resistance of 1–2 MΩ when filled with pipette solution. A bridge (3 M KCl in agar salt) between the bath and an Ag–AgCl reference electrode immersed in pipette solution was used to minimize changes in liquid junction potential, and junction potentials were zeroed before establishing a membrane seal. Voltage-clamp recordings were obtained using a Dagan 3900A or an Axopatch 200B patch-clamp amplifier (Axon Instruments). Single-channel currents were recorded at a gain of 500 mV pA^{-1}, low-pass filtered with an eight-pole Bessel filter at 1 kHz, and digitized (Digidata 1200, Axon Instruments Inc.) and stored on the hard disk of an IBM PC/AT-compatible computer. Whole-cell currents were filtered at a frequency of 2 kHz and pCLAMP 6.0 or 7.0 from Axon Instruments (Clampex, Clampfit, Fetchex, Fetchan and Pstat) were used to control voltage-clamp protocols, and perform data acquisition and analysis. Voltage-clamp pulses were generated by a 12-bit digital-to-analog (D/A) convertor. To obtain whole-cell current–voltage (I–V) relations, cells were clamped from a holding potential of 0 mV to a series of test potentials from −80 to +80 mV for 400 ms in +20 mV increments at an interval of 10 s (as shown in Fig. 1). To obtain single-channel I–V relations, the membrane was clamped from a holding potential of 0 mV relative to the resting potential (RP) to a series of test potentials for 2 s at a time. The voltage of all cell-attached single-channel voltage clamps in this manuscript is expressed as RP + V, where RP is the cell membrane resting potential and V is the transpatch voltage step applied by the amplifier as would be measured at the intracellular side of the patch membrane. Hyperpolarizing and depolarizing pulses were imposed at 0·1 Hz in +20 mV increments between RP − 60 mV and RP + 120 mV. All command voltages and single-channel currents are displayed as they would be measured at the intracellular side of the membrane. Single-channel events at various voltages were manually reviewed, and the open probability of a number of channels (NP_o) at any one potential was calculated as

described previously (Collier & Hume, 1995; Duan *et al.* 1997*a*). The cell-attached patch configuration was checked at the end of each experiment by rupturing the patch to confirm passage from the cell-attached to the whole-cell configuration. Intracellular potentials of myocytes were immediately measured. The average intracellular potential was $-83{\cdot}5 \pm 5{\cdot}2$ mV ($n = 7$). All experiments were performed at room temperature (22–24 °C).

Solutions and drugs

Bath and pipette solutions were chosen to facilitate Cl^- current recording. Cd^{2+} (0·2 mM), Cs^+ (5 mM), and 4-aminopyridine (2 mM) were present continuously in the bath solution to block Ca^{2+} and K^+ currents, respectively.

The standard extracellular bath solutions used for whole-cell recordings contained (mM): 140 NaCl, 0·8 $MgCl_2$, 1·5 $CaCl_2$, 5 CsCl, 0·2 $CdCl_2$, 10 Hepes, 5·5 glucose; pH 7·4; total $[Cl^-]_o = 150$ mM; 300 mosmol (kg H_2O)$^{-1}$ with mannitol. In experiments where the external Cl^- was to be reduced, Cl^- was replaced by an equimolar concentration (140 mM) of the monovalent anion iodide (I^-) or aspartate (Asp^-). Possible contamination from outward cation currents and non-selective cation currents was prevented by using the large impermeant cation *N*′-methyl-D-glucamine (NMDG) to replace the cations in the pipette solution. The standard intracellular pipette solution used for whole-cell recordings contained (mM): 150 NMDG-Cl, 5 MgATP, 0·1 NaGTP, 5 EGTA, 5 Hepes; pH 7·4; total $[Cl^-]_i = 150$ mM; 290 mosmol (kg H_2O)$^{-1}$ using mannitol. In experiments where the internal Cl^- was to be reduced, Cl^- was replaced by an equimolar concentration of Asp^- (110 mM).

The standard pipette (extracellular) solutions used in cell-attached patch-clamp experiments contained (mM): 150 NMDG-Cl, 5 Hepes, 10 glucose; pH 7·4; total $[Cl^-]_i = 150$ mM. Inward cation currents such as I_{Na}, I_{Ca}, and non-selective cation currents were prevented by using the large impermeant cation NMDG to replace the cations in the pipette solution. The standard bath solutions for cell-attached configuration were the same bath (extracellular) solutions used for whole-cell recordings.

All chemicals, including isoprenaline, and forskolin were obtained from Sigma. Levamisole was purchased from Aldrich and phorbol 12,13-dibutyrate (PDBu), bisindolylmaleimide I-HCl (BIM), and adenosine-3′,5′-cyclic monophosphothioate R_P-isomer (Rp-cAMP) were purchased from Calbiochem and prepared as stock solutions of 1 or 10 mM in dimethyl sulphoxide and added to a known volume of superfusion solutions to produce the desired concentrations.

RNA isolation and cDNA synthesis

Total RNA was isolated from mouse atrial and ventricular tissues using the Trizol reagent (Life Technologies), following the manufacturer's instructions. Tissues from several animals were pooled (50–100 mg) for each RNA preparation. Total RNA was incubated with RNase-free DNase (10 units) for 20 min at room temperature, followed by heat inactivation. One microgram of total RNA was reverse transcribed with Superscript II reverse transcriptase (Life Technologies) in a 20 μl reaction containing 25 ng oligo(dT) (12–18) primer, 500 μM each dNTP, 50 mM Tris-HCl, pH 8·3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT.

Reverse transcription PCR (RT-PCR)

RT-PCR was performed using primers specific for the mouse CFTR gene (Tata *et al.* 1991; GenBank Accession No. M69298). A 392 base pair (bp) region corresponding to nucleotides 1340–1730 was amplified with the forward primer 5′-GGGAGGAGGGATTTG-GGGAA-3′ and the reverse primer 5′-GTGATGTCCTGCTGT-AGTTG-3′. PCR was performed in 25 μl reactions containing *Taq* buffer (50 mM KCl, 10 mM Tris-HCl, 1·5 mM $MgCl_2$, 0·1 % Triton X-100, 250 μM each dNTP, 20 μM each primer, 2·5 μl cDNA and 1 unit *Taq* polymerase (Promega)). Amplifications were performed in a GeneAmp 2400 thermal cycler (Perkin Elmer) for 30 cycles of 94 °C for 30 s; 50 °C for 30 s; 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The RT-PCR products were resolved

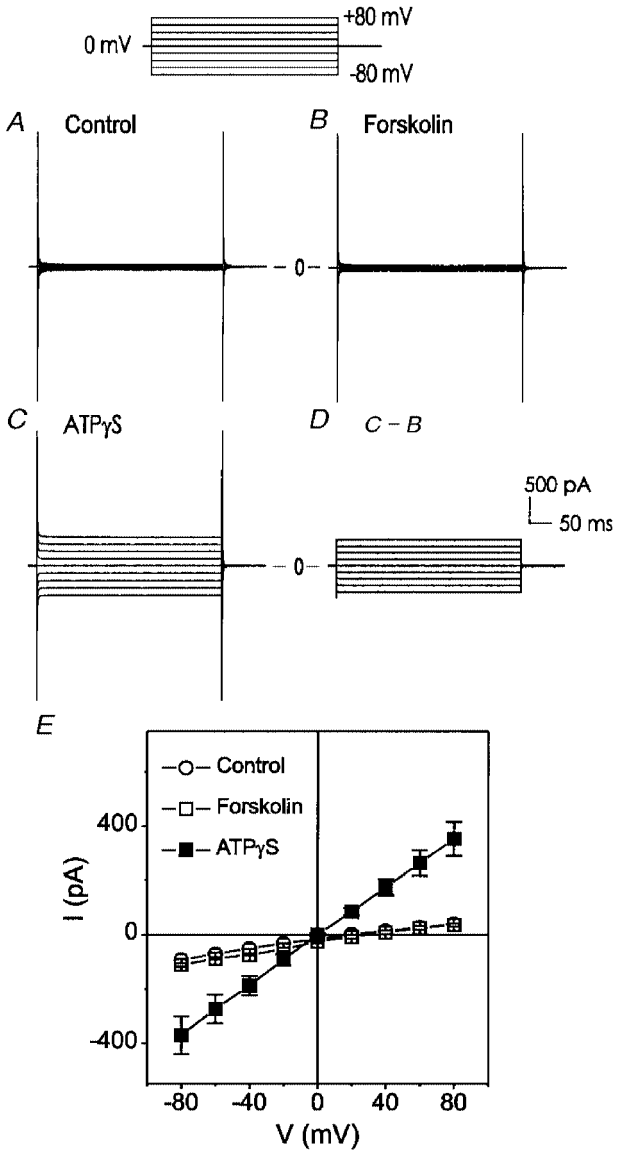


Figure 1. Extracellular ATPγS, but not forskolin, activates a linear membrane conductance with reversal potential near the predicted equilibrium potential of Cl^- under symmetrical 150 mM Cl^-

The voltage-clamp protocol is shown at the top. Cells were held at 0 mV and test potentials were applied from −80 to +80 mV in +20 mV increments at an interval of 10 s. *A*, negligible currents were detected under control conditions. *B*, subsequent exposure of the same cell to forskolin (10 μM) failed to activate any current. *C*, ATPγS (100 μM) caused a significant increase in membrane conductance. *D*, ATPγS (100 μM)-sensitive current. *E*, mean current–voltage (*I–V*) relationships of 6 different cells under control (○), forskolin (□), and ATPγS (100 μM) (■) conditions.

on a 2% agarose gel alongside a molecular weight marker. The murine CFTR 390 bp fragment was gel eluted and subcloned into the TOPO 2.1 T/A vector (Invitrogen). Plasmid DNA was prepared from overnight cultures using the QIAprep Miniprep kit (Qiagen). To confirm the presence of mouse CTFR, both strands of the plasmid DNA were sequenced using the ABI Prism cycle sequencing kit (Perkin Elmer) and analysed on a Genetic Analyser, Model 310 (Perkin Elmer).

Northern blot analysis

To confirm the expression of CFTR mRNA in mouse cardiac tissues, Northern blot analysis was performed as described previously (Duan *et al.* 1997*b*). Briefly, 20 μg of total RNA from atrial and ventricular tissue were size-fractionated on a 1% agarose–formaldehyde gel and transferred to nylon filters. Filters were baked and pre-hybridized in 50% formamide, 5 × SSC, 50 mM sodium phosphate, 5 × Denhardt's solution, sonicated salmon sperm DNA (50 μg ml^{-1}), 0·1% SDS, 10% dextran sulphate, at 42 °C overnight. The 390 bp murine CFTR cDNA probe was radiolabelled with ^{32}P by random priming (Feinberg & Vogelstein, 1983). Hybridization was performed under the same conditions overnight. The filters were washed at high stringency (3 times in 2 × SSC at room temperature for 5 min then twice in 0·2 × SSC/0·1% SDS at 65 °C for 30 min) to assure specificity of labelling. Filters were exposed to film and autoradiography was performed using a phosphoimager (BioRad).

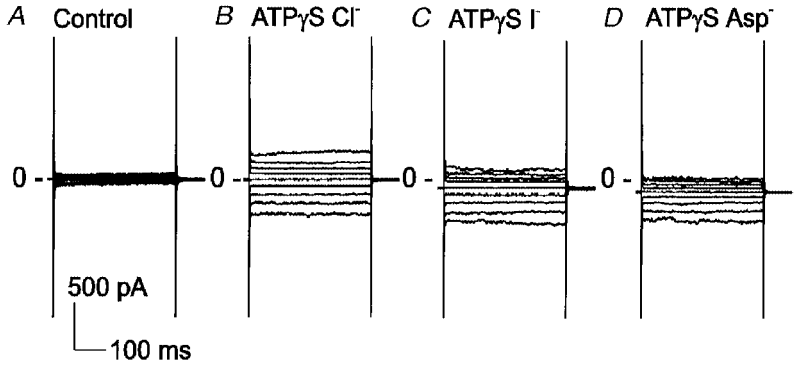
Data analysis

All results are expressed as means ± S.E.M. Statistical comparisons were performed either by analysis of variance (ANOVA) with Scheffé contrasts for group data, or by Student's t test when only two groups were compared. A two-tailed probability of $< 5\%$ is taken to indicate statistical significance.

RESULTS

Anion-selectivity of ATPγS-activated $I_{Cl,ATP}$ in mouse heart

In agreement with an earlier study (Levesque & Hume, 1995), the cAMP activator forskolin (10 μM) failed to activate any current in mouse ventricular myocytes;



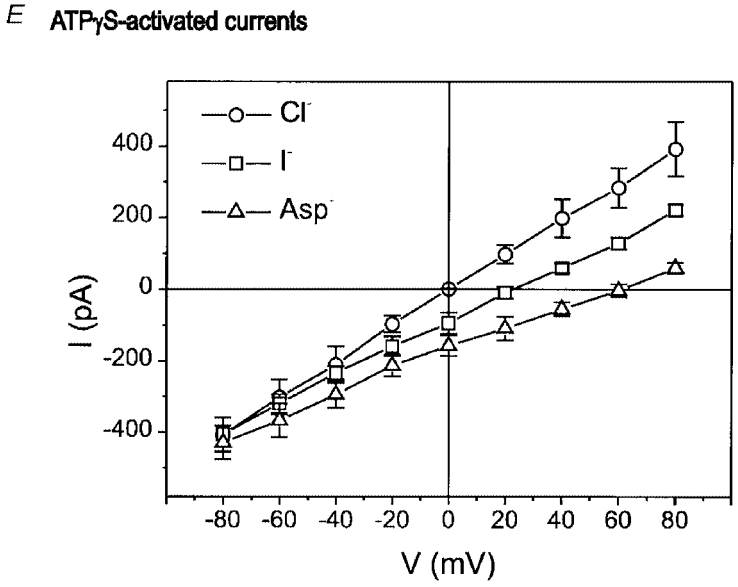


Figure 2. Anion selectivity of $I_{Cl,ATP}$ in mouse ventricular myocytes

A–D, representative whole-cell currents recorded in the absence (*A*, control) and presence of ATPγS (100 μM). After $I_{Cl,ATP}$ was activated by ATPγS in high extracellular Cl^- ($[Cl^-]_o = 150$ mM) bath solution (*B*), $[Cl^-]_o$ was reduced to 10 mM ($E_{Cl} = +69{\cdot}6$ mV) by equimolar (140 mM) replacement of Cl^- with I^- (*C*) or Asp^- (*D*). *E*, mean *I–V* relations of ATPγS-activated whole-cell currents before (○) and after $[Cl^-]_o$ was substituted by I^- (□) or Asp^- (△). I^- and Asp^- substitution of Cl^- shifted the reversal potential of $I_{Cl,ATP}$ from 1·4 ± 1·4 mV to 23·5 ± 1·6 mV and 58·3 ± 3·0 mV ($n = 3$), respectively.

however, application of a poorly hydrolysable ATP analogue, ATPγS (100 μM), caused activation of a significant membrane conductance in 6 of 17 (35·2%) of these cells. This time-independent conductance reversed completely upon washout of ATPγS. The ATPγS-activated current had a linear current–voltage (I–V) relationship and a reversal potential of $-2{\cdot}3 \pm 1{\cdot}2$ mV ($n = 6$), which was very close to the predicted equilibrium potential of Cl^- ($E_{Cl} = 0$ mV) with symmetrical 150 mM Cl^- (Fig. 1E). A similar $I_{Cl,ATP}$ was also observed in 3 of 11 (27·3%) mouse atrial myocytes (data not shown).

We further examined the Cl^- dependence of the current by replacing the $[Cl^-]_o$ with an equimolar concentration of I^- or Asp^-. As shown in Fig. 2, reduction of $[Cl^-]_o$ caused a decrease in the amplitude of outward currents and shifted the reversal potential (V_{rev}) to more positive potentials. I^- and Asp^- substitution of $[Cl^-]_o$ shifted the V_{rev} of $I_{Cl,ATP}$ from $1{\cdot}4 \pm 1{\cdot}4$ mV to $23{\cdot}5 \pm 1{\cdot}6$ mV and $58{\cdot}3 \pm 3{\cdot}0$ mV, respectively. The permeability ratios (permeability ratio of ion X with respect to Cl^-, P_x/P_{Cl}) were then calculated from the shifts of V_{rev} using the modified Goldman-Hodgkin-Katz equation (Hille, 1971). The $I_{Cl,ATP}$ channel had a P_I/P_{Cl} of $0{\cdot}46 \pm 0{\cdot}06$ ($n = 3$) and a P_{Asp}/P_{Cl} of $0{\cdot}13 \pm 0{\cdot}05$ ($n = 3$). These results indicate that the ATPγS-activated current is indeed a Cl^- current with a relative permeability of $Cl^- > I^- \gg Asp^-$.

$I_{Cl,ATP}$ requires activation of endogenous PKC

In the presence of BIM (100 nM), a PKC inhibitor, ATPγS failed to activate any membrane conductance in mouse ventricular cells (Fig. 3B and D). After removal of BIM from superfusate, ATPγS was then able to activate a current in these cells (Fig. 3C and E). Figure 3F shows the mean I–V curves of whole-cell currents from five different cells under control (○), ATPγS + BIM (□), and ATPγS alone (■). These results suggest that activation of $I_{Cl,ATP}$ by purinergic receptors may be mediated by the activation of endogenous PKC. Therefore, we tested whether activation of PKC by phorbol esters might also cause activation of a similar Cl^- conductance.

As shown in Fig. 4, PDBu (100 nM) activated a current with properties (time independence, a linear I–V relationship in symmetrical Cl^-) similar to those of $I_{Cl,ATP}$ (Fig. 4B, D and F). Subsequent addition of ATPγS in the presence of PDBu failed to further increase the membrane conductance, suggesting that PDBu and ATPγS may activate the same channel. Similar results were observed from four different cells and the mean I–V curves from these cells are shown in Fig. 4F. In symmetrical Cl^- gradient ($[Cl^-]_o/[Cl^-]_i$ = 150 mM/150 mM, $E_{Cl} = 0$ mV), the PDBu-activated currents were linear and reversed at $-3{\cdot}3 \pm 2{\cdot}7$ mV ($n = 4$), close to the predicted value of E_{Cl} (~0 mV).

The Cl^- dependence of the PDBu-activated current was further examined using different intracellular Cl^- concentrations ($[Cl^-]_i$). As shown in Fig. 5, when $[Cl^-]_i$ was partially substituted with equimolar Asp^- (110 mM; $[Cl^-]_i = 40$ mM), PDBu reversibly activated a time-independent outwardly rectifying current with a reversal potential ($-34{\cdot}6 \pm 1{\cdot}2$ mV, $n = 4$) very close to the predicted E_{Cl} (−33·9 mV). These results strongly suggest that PDBu-activated current in mouse ventricular myocytes is a Cl^--dependent current and that the activation of $I_{Cl,ATP}$ by ATPγS may be due to purinoceptor-coupled activation of endogenous PKC.

Isoprenaline enhances $I_{Cl,ATP}$ pre-activated by ATPγS or PDBu

The anion selectivity, I–V relations and rectification properties of $I_{Cl,ATP}$ activated by either ATPγS or PDBu in mouse heart (also see Levesque & Hume, 1995) strongly resemble those of cardiac and epithelial $I_{Cl,CFTR}$ in other species (Anderson *et al.* 1991; Gadsby *et al.* 1995). It has been reported that both PKC and PKA activate $I_{Cl,CFTR}$ in

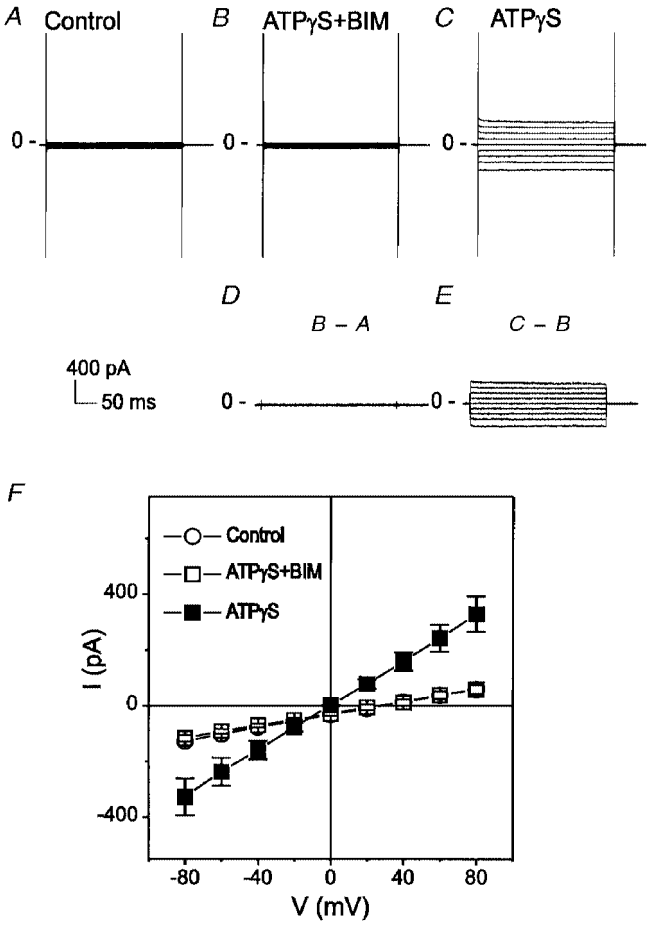


Figure 3. Inhibition of endogenous protein kinase C by BIM prevented the activation of $I_{Cl,ATP}$ by ATPγS

A, whole-cell current under control conditions. B, when BIM (100 nM) was applied together with ATPγS, no increase in the membrane current was observed. C, ATPγS was able to activate $I_{Cl,ATP}$ after removal of BIM from superfusate. D, difference current between B and A. E, difference current between C and B. F, mean I–V curves from 5 different cells under control (○), ATPγS + BIM (□), and ATPγS alone (■) conditions. Only cells which exhibited a distinct $I_{Cl,ATP}$ following washout of BIM were included in this analysis.

heart and their effects are additive (Zhang *et al.* 1994; Collier & Hume, 1995; Yamazaki *et al.* 1999). It is very interesting that Jia *et al.* have recently found that PKA-mediated phosphorylation alone is not a sufficient stimulus to open the CFTR Cl^- channel in the presence of MgATP; initial, constitutive PKC phosphorylation may be required for acute activation of the CFTR channel by PKA (Jia *et al.* 1997). It is possible, therefore, that a low level of basal PKC activity in mouse cardiac myocytes is responsible for the failure of acute elevation of intracellular cAMP to activate Cl^- currents in these cells (Levesque & Hume, 1995; cf. Fig. 1). If this is true, then the $I_{Cl,ATP}$ pre-activated by ATPγS or PDBu should be further enhanced by subsequent application of cAMP stimulators. To test this hypothesis, we examined the effects of isoprenaline on $I_{Cl,ATP}$ pre-activated by ATPγS or PDBu. As shown in Fig. 6, while isoprenaline alone failed to activate any current in ventricular myocytes

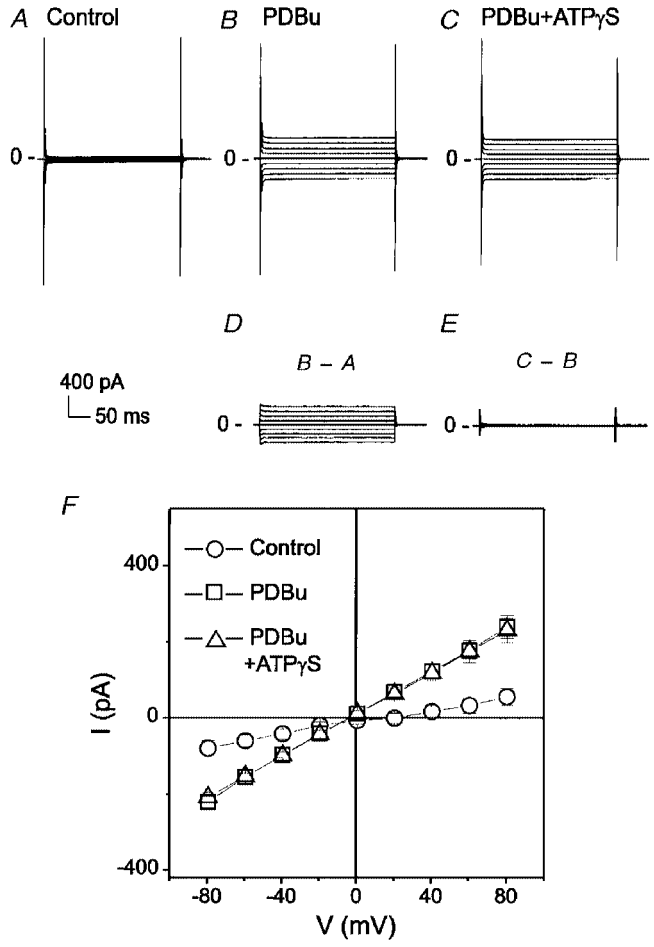


Figure 4. PDBu activates a Cl^- current similar to $I_{Cl,ATP}$

A, whole-cell recording under control conditions. *B*, PDBu (100 nM)-activated currents. *C*, subsequent addition of ATPγS in the presence of PDBu failed to further increase membrane conductance. *D*, PDBu-sensitive current. *E*, difference current between *C* and *B*. *F* shows mean *I–V* curves from 4 different cells under control (○), PDBu (100 nM) (□) and PDBu + ATPγS (100 μM) (△) conditions.

(*A* and *B*), addition of isoprenaline (1 μM) caused a further significant increase in the ATPγS-activated $I_{Cl,ATP}$ in these myocytes (*D*). Removal of ATPγS only partially reduced $I_{Cl,ATP}$ (*E*). Further removal of isoprenaline completely reduced the current (*F*). Similar results were obtained from four different cells under identical conditions and the mean *I–V* curves from these cells are shown in Fig. 6*G*.

Similarly, as shown in Fig. 7, the PDBu pre-activated $I_{Cl,ATP}$ in mouse ventricular myocytes was also enhanced by isoprenaline in mouse ventricular myocytes. These results suggest that activation of PKC by ATPγS or PDBu may be required for the modulation of $I_{Cl,ATP}$ by intracellular cAMP-PKA.

Activation of $I_{Cl,ATP}$ is dependent upon endogenous PKA

The above results indicate that extracellular ATP activates $I_{Cl,ATP}$ through purinoceptor-coupled activation of PKC, which may permit synergistic activation of $I_{Cl,ATP}$ by PKA phosphorylation. It has recently been reported that ATPγS may also stimulate intracellular cAMP through purinoceptor-coupled activation of adenylyl cyclase V (Puceat *et al.* 1998). On the other hand, Middleton & Harvey reported that in guinea-pig ventricular myocytes phorbol esters alone failed to elicit CFTR current although they were able to potentiate subsequent CFTR channel activation by PKA (Middleton & Harvey, 1998). Therefore, we next asked the question whether purinoceptor-activated PKA is also involved in the process of $I_{Cl,ATP}$ activation. We initially observed that the phosphatase inhibitor, calyculin A (10 nM), was able to increase the current amplitude of $I_{Cl,ATP}$ pre-activated by

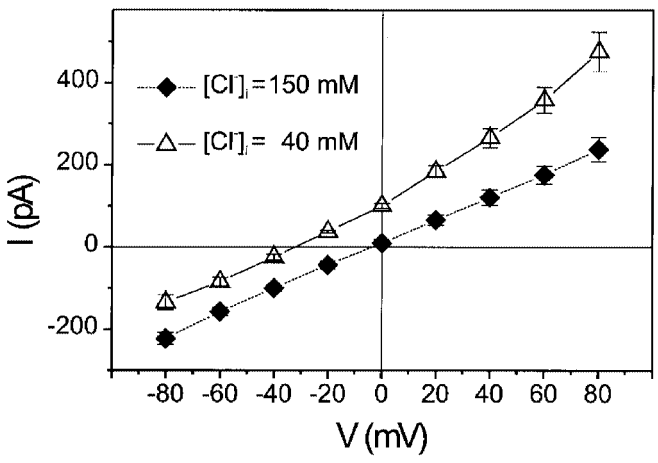


Figure 5. Cl^- sensitivity of PDBu-activated whole-cell currents in mouse ventricular myocytes

Mean *I–V* curves of PDBu-activated currents in asymmetrical (△, $n = 4$), and symmetrical Cl^- gradient (◆, $n = 4$). With an asymmetrical Cl^- gradient ($[Cl^-]_o/[Cl^-]_i = 150$ mM/40 mM, $E_{Cl} = -33{\cdot}9$ mV), PDBu-activated currents were outwardly rectifying and reversed at $-34{\cdot}6 \pm 1{\cdot}2$ mV ($n = 4$). With a symmetrical Cl^- gradient ($[Cl^-]_o/[Cl^-]_i = 150$ mM/150 mM, $E_{Cl} = 0$ mV), PDBu-activated currents were linear and reversed at $-3{\cdot}3 \pm 2{\cdot}7$ mV ($n = 4$).

ATPγS or PDBu (data not shown). We next examined the effects of a specific inhibitor of PKA, Rp-cAMP, on $I_{Cl,ATP}$ pre-activated by ATPγS. Figure 8*A* shows the time course of changes in whole-cell currents at +80 mV and −80 mV monitored continuously when the cell was consecutively exposed to control solution for 10 min, ATPγS (100 μM) until the changes in current amplitudes reached steady state (~15 min), and then ATPγS + Rp-cAMP (100 μM) until the changes in currents reached steady state. Rp-cAMP eventually abolished the ATPγS-activated current. Similar results were observed in three other cells under the same conditions and Rp-cAMP caused a 78·3 ± 6·8% inhibition of $I_{Cl,ATP}$ after application of the drug for 10 min. Furthermore, in cells ($n = 9$) exposed to Rp-cAMP (100 μM), ATPγS (100 μM) failed to activate $I_{Cl,ATP}$ (Fig. 8*B*). Rp-cAMP also prevented the activation of $I_{Cl,ATP}$ by PDBu (data not shown), suggesting that PKA is involved in the activation of $I_{Cl,ATP}$ by ATPγS or PDBu.

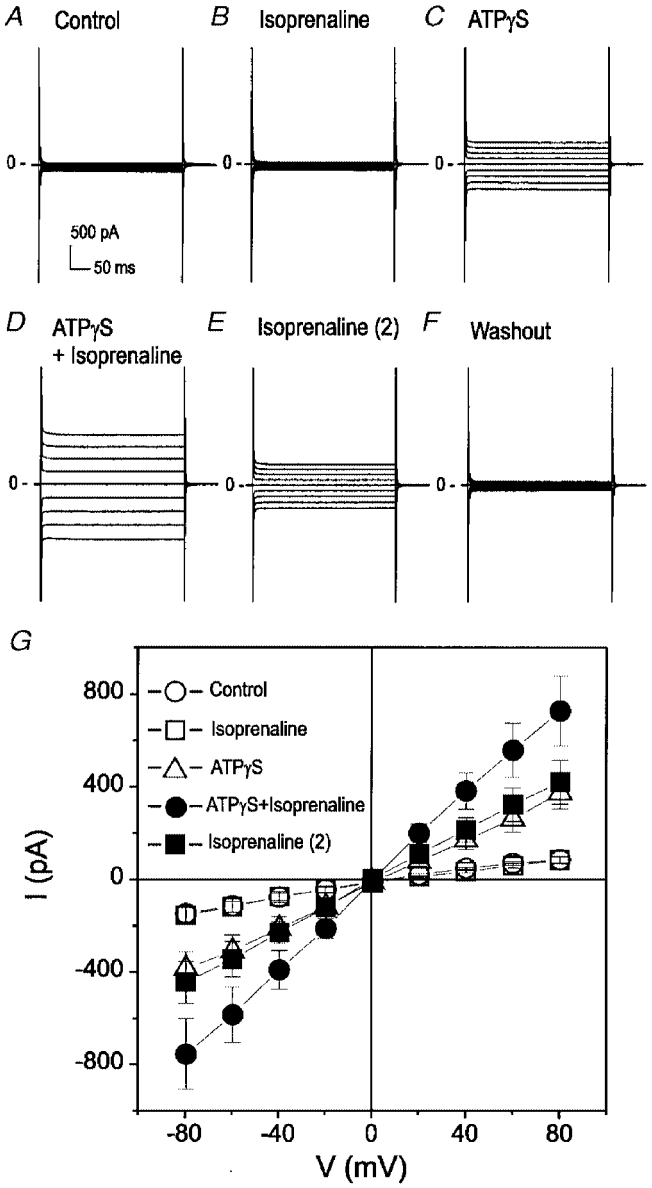


Figure 6. Synergistic activation of $I_{Cl,ATP}$ by ATPγS and isoprenaline in mouse ventricular myocytes

A, whole-cell recording under control conditions. *B*, isoprenaline alone failed to activate $I_{Cl,ATP}$. *C*, ATPγS (100 μM) activated $I_{Cl,ATP}$. *D*, subsequent addition of isoprenaline (1 μM) in the presence of ATPγS caused a further increase in the membrane conductance. *E*, removal of ATPγS partially reduced $I_{Cl,ATP}$. *F*, further removal of isoprenaline completely reduced the current. *G*, mean *I–V* curves from 4 different cells under control (○), isoprenaline (1 μM) (□), ATPγS (100 μM) (△), isoprenaline + ATPγS (●), and isoprenaline alone (■) conditions.

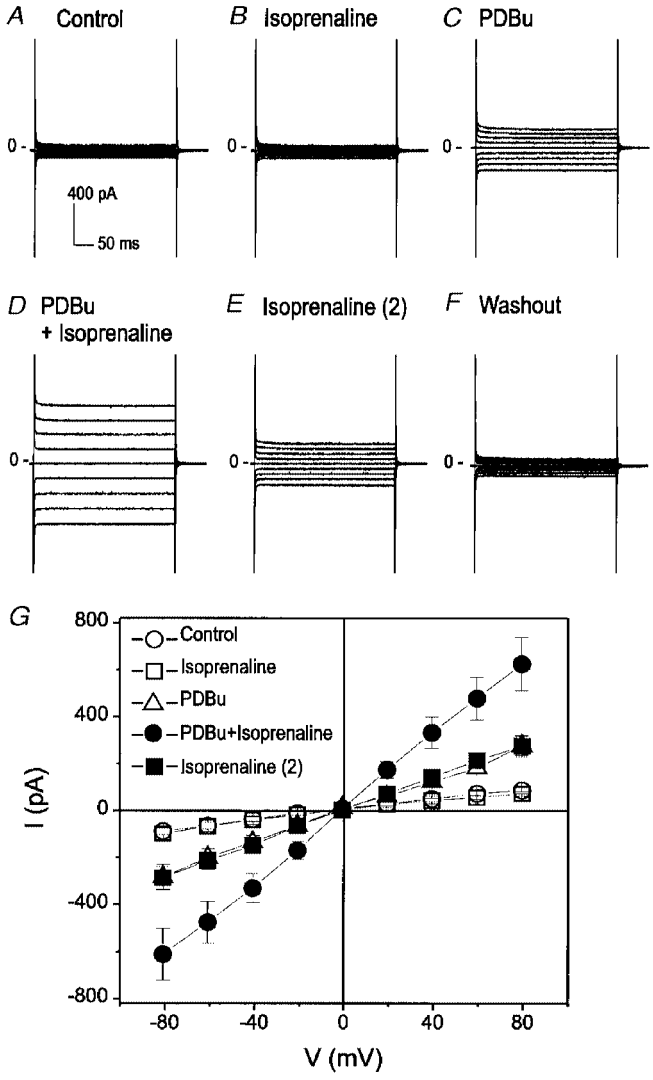


Figure 7. Synergistic activation of $I_{Cl,ATP}$ by PDBu and isoprenaline in mouse ventricular myocytes

A, whole-cell recording under control conditions. *B*, isoprenaline alone failed to activate $I_{Cl,ATP}$. *C*, PDBu (100 nM) activated $I_{Cl,ATP}$. *D*, subsequent addition of isoprenaline (1 μM) in the presence of PDBu caused a further increase in the membrane conductance. *E*, removal of PDBu partially reduced $I_{Cl,ATP}$. *F*, further removal of isoprenaline completely reduced the current. *G*, mean *I–V* curves from 3 different cells under control (○), isoprenaline (1 μM) (□), PDBu (100 nM) (△), isoprenaline + PDBu (●), and isoprenaline alone (■) conditions.

Glibenclamide blocks $I_{Cl,ATP}$ activated by ATPγS and levamisole

Glibenclamide, a K_{ATP} channel inhibitor, is also an effective blocker of both epithelial and cardiac CFTR Cl^- channels (Yamazaki & Hume, 1997; Sheppard & Robinson, 1997). As shown in Fig. 9*A*, 50 μM glibenclamide blocked $I_{Cl,ATP}$ activated by 100 μM ATPγS. The inhibition was not voltage dependent (69·8 ± 3·4% inhibition at −80 mV and 71·8 ± 3·2% inhibition at +80 mV, $n = 3$, P = n.s.).

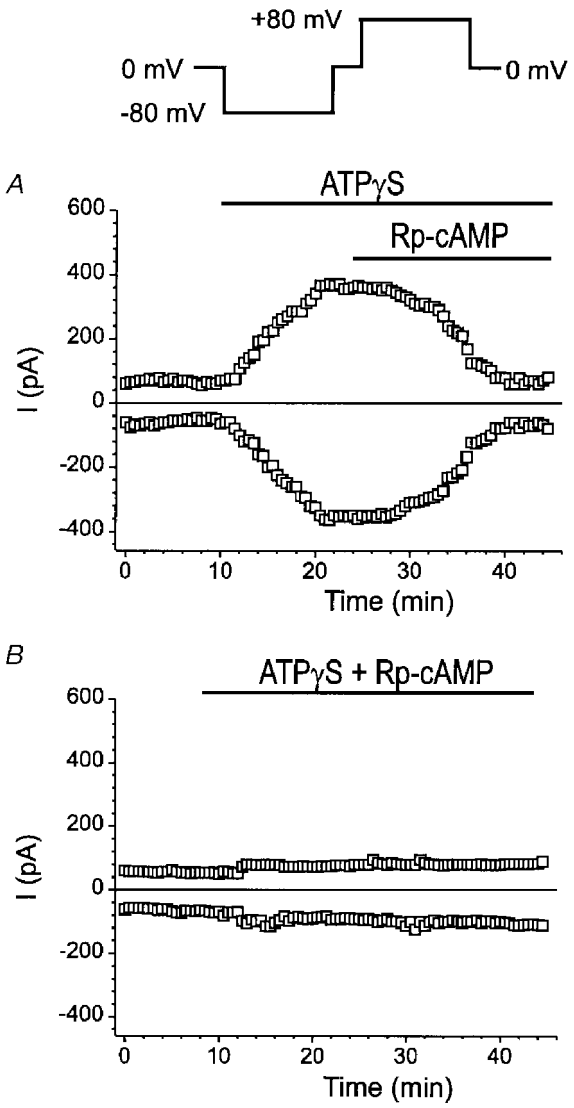


Figure 8. Activation of $I_{Cl,ATP}$ by ATPγS is prevented by inhibition of endogenous PKA by Rp-cAMP in mouse ventricular myocytes

A, time course of changes in whole-cell currents at +80 mV and −80 mV continuously monitored (voltage-clamp protocol is shown on the top) when the cell was consecutively exposed to control solution for 10 min, ATPγS (100 μM) until the changes in current amplitudes reached steady state (~15 min), and ATPγS + Rp-cAMP (100 μM) until the changes in currents reached steady state. Rp-cAMP eventually abolished the activation of the current even in the presence of ATPγS. *B*, in the presence of Rp-cAMP (100 μM) ATPγS (100 μM) failed to activate $I_{Cl,ATP}$. Same protocol as in *A* was used to monitor the time course of whole-cell currents.

It was recently shown that phenylimidazothiazole compounds, especially levamisole, are able to activate CFTR Cl^- channels through a cAMP-independent pathway (Becq *et al.* 1996). Therefore we tested whether levamisole also activates a Cl^- current in mouse cardiac myocytes. As shown in Fig. 9*B*, 1 mM levamisole activated a linear time-independent current which was also inhibited by 50 μM glibenclamide in four of eight (50%) mouse ventricular myocytes tested. The glibenclamide inhibition of the levamisole-activated current was identical to the inhibition of $I_{Cl,ATP}$ activated by ATPγS in terms of the voltage-independence and the sensitivity of the channel to the blocker (73·9 ± 2·5% inhibition at −80 mV and 75·5 ± 1·5% inhibition at +80 mV, $n = 4$), suggesting that levamisole may activate the same $I_{Cl,ATP}$ in mouse ventricular myocytes. Although it is not clear at this point whether levamisole activates the current through an inhibition of membrane-associated alkaline phosphatases (Becq *et al.* 1996) or a second target other than phosphatase inhibition (Mun *et al.* 1998).

Single-channel properties of $I_{Cl,ATP}$ in mouse ventricular myocytes

The cell-attached patch-clamp mode was used to record single-channel currents from cells exposed to ATPγS. Pipette (external) solutions contained 150 mM Cl^-. Figure 10*A* shows representative currents recorded (2 min) when the patch was clamped at −40 mV relative to the resting membrane potential (RP − 40 mV) under control conditions and after exposure of the cell to ATPγS (100 μM). Corresponding amplitude histograms are shown on the right of each panel. No channel opening was detected under control conditions (Fig. 10*Aa*). Subsequent exposure of the same cell to ATPγS activated inward currents with an amplitude of ~2·24 pA and an open probability (NP_o) of 0·34 (Fig. 10*Ab*). Similar single-channel activity activated by ATPγS was observed in 5 of 16 (31%) ventricular myocytes. Mean NP_o of the single-channel $I_{Cl,ATP}$ at RP − 40 mV was 0·25 ± 0·12 ($n = 5$).

The *I–V* relationship of ATPγS-activated unitary $I_{Cl,ATP}$ was also determined (Fig. 10*B*). The mean *I–V* curves from four different cells studied under the same conditions were outwardly rectifying with a slope conductance of (RP + 20 mV to RP + 120 mV) 11·8 ± 0·3 pS ($n = 4$) when the pipette (external) solution contained 150 mM NMDG-Cl. The reversal potential was RP + 28·1 ± 2·6 mV ($n = 4$).

CFTR is transcriptionally expressed in both atrium and ventricle of mouse heart

The results described thus far on $I_{Cl,ATP}$ anion-selectivity, rectification, single-channel conductance, PKC- and PKA-dependent activation mechanisms, and levamisole activation and glibenclamide inhibition, suggest that $I_{Cl,ATP}$ and

$I_{Cl,CFTR}$ may be one and the same in mouse heart. Therefore, we performed experiments to verify molecular expression of CFTR in mouse heart.

Figure 11*A* shows an agarose gel depicting a CFTR-specific RT-PCR product generated from RNA derived from mouse atrial and ventricular tissue. The RT-PCR reaction of total RNA prepared from both atrial and ventricular tissue with specific primers designed to amplify a 392-nucleotide product of mouse CFTR (nucleotide positions 1340–1730) confirmed the transcriptional expression of CFTR in both atrium and ventricle. The RT-PCR product was sequenced and determined to be identical to the previously cloned murine CFTR (Tata *et al.* 1991; Yorifuji *et al.* 1991).

Northern blot analysis also indicated that CFTR messenger RNA is expressed in both atrium and ventricle of mouse heart. Figure 11*B* shows hybridization to a transcript of ~7·0 kb, which is similar in size to CFTR mRNA (Riordan *et al.* 1989; Levesque *et al.* 1992; Nagel *et al.* 1992).

DISCUSSION

A previous study from this laboratory demonstrated that extracellular ATP activates $I_{Cl,ATP}$ through stimulation of P_2-receptors in mouse ventricular myocytes (Levesque & Hume, 1995). In the present study, we further examined the intracellular signal transduction mechanisms responsible for purinergic activation of $I_{Cl,ATP}$, identified the single channels responsible for $I_{Cl,ATP}$, and demonstrated transcriptional expression of CFTR in mouse heart. Our data strongly suggest that $I_{Cl,ATP}$ in mouse heart may be attributed to the activation of CFTR Cl^- channels.

In the past decade at least seven different types of Cl^- currents have been described in the heart (Hiraoka *et al.* 1998; Hume *et al.* 2000). These include Cl^- currents activated by intracellular cAMP-PKA ($I_{Cl,PKA}$), PKC ($I_{Cl,PKC}$), Ca^{2+} ($I_{Cl,Ca}$), extracellular ATP ($I_{Cl,ATP}$), cell swelling ($I_{Cl,swell}$), hyperpolarization, and a basally active Cl^- current ($I_{Cl,b}$). Functional and molecular studies indicate

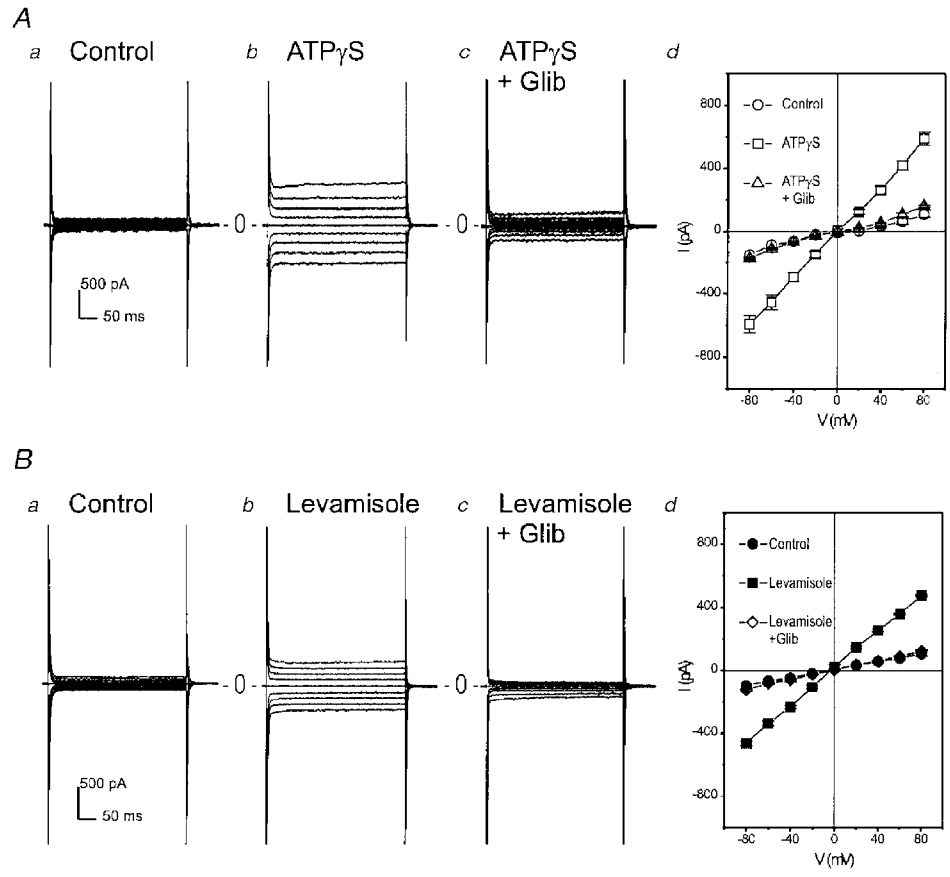


Figure 9. Glibenclamide blocks $I_{Cl,ATP}$ activated by ATPγS and levamisole

A, ATPγS (100 μM)-activated $I_{Cl,ATP}$ (*b*) in mouse ventricular myocytes was significantly inhibited by 50 μM glibenclamide (*c*). Panel *d* shows mean *I–V* curves of whole-cell currents recorded from 3 different myocytes in the absence (○) and presence (□) of ATPγS (100 μM) and in the presence (△) of ATPγS and glibenclamide (50 μM). *B*, levamisole, a reported CFTR Cl^- channel activator, activated a linear time-independent current in mouse ventricular myocytes (*b* and *d*) and this levamisole-activated current was also significantly inhibited by 50 μM glibenclamide (*c* and *d*). Panel *d* shows mean *I–V* curves of whole-cell currents recorded from 4 different myocytes in the absence (●) and presence (■) of levamisole (500 μM) and in the presence (◇) of levamisole and glibenclamide (50 μM).

that $I_{Cl,PKA}$, and $I_{Cl,PKC}$ as well, are encoded by an isoform of CFTR (Levesque *et al.* 1992; Nagel *et al.* 1992; Hart *et al.* 1996; Yamazaki *et al.* 1999), whereas the volume-regulated outwardly rectifying Cl^- channel ($I_{Cl,vol}$), including $I_{Cl,b}$, may be encoded by a member of the ClC Cl^- channel family, ClC-3 (Duan *et al.* 1997*b*, 1999*b*). Another member of the ClC family, ClC-2, may encode the volume-regulated inwardly rectifying Cl^- channel recently found in heart (Furukawa *et al.* 1998; Duan *et al.* 1999*a*). At this point, the molecular identity of the proteins responsible for $I_{Cl,ATP}$ and $I_{Cl,Ca}$ remain unknown (Hume *et al.* 2000).

The electrophysiological properties of $I_{Cl,ATP}$ reported here and by other authors (Matsuura & Ehara, 1992; Kaneda *et al.* 1994; Levesque & Hume, 1995) are indistinguishable from $I_{Cl,CFTR}$ in terms of their anion selectivity, rectification, time independence, and single-channel conductance

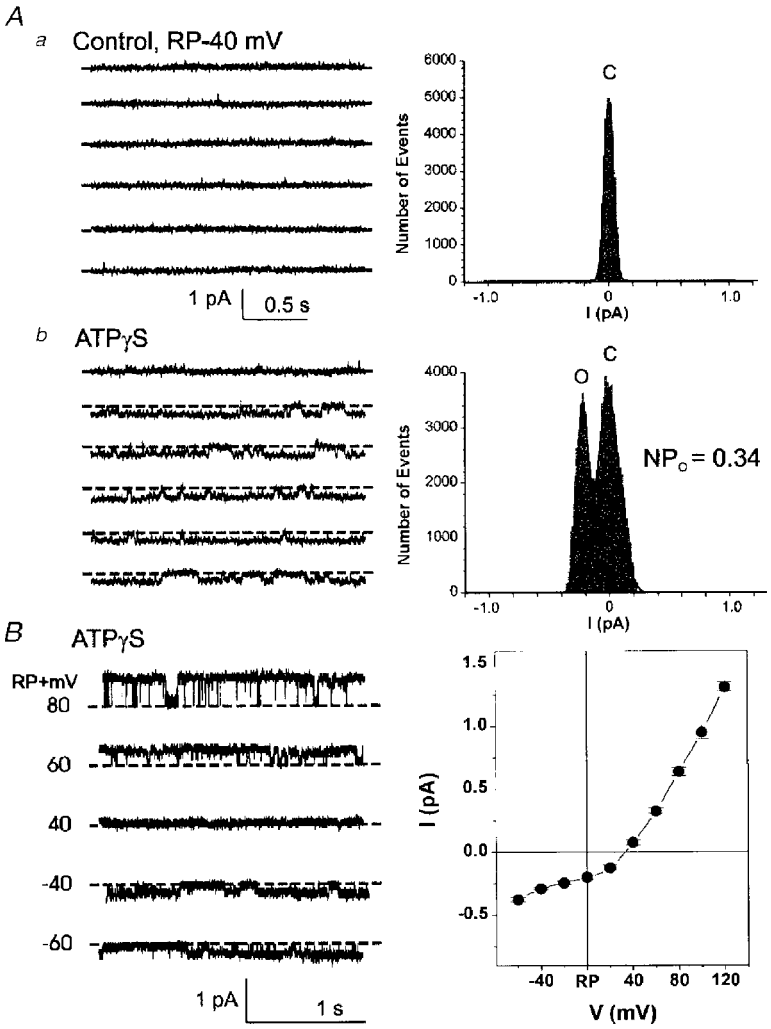


Figure 10. Single-channel properties of extracellular ATPγS-activated Cl^- currents in mouse ventricular myocytes

Cell-attached patch-clamp mode was used to record unitary currents before and after the cells were exposed to ATPγS (100 μM). Pipette (external) solutions contained 150 mM Cl^-. *A*, representative current tracings (2 min recording) when the cell-attached patch was clamped at −40 mV relative to the resting membrane potential (RP − 40 mV) under control conditions (*a*) and in the presence of ATPγS (100 μM) (*b*); displayed currents low-pass filtered at 150 Hz. Corresponding amplitude histogram is shown on the right of each panel. Data were fitted to a Gaussian distribution (Fetchan) and the continuous line indicates the results. *Aa*, no channel opening was detected under control conditions. *Ab*, subsequent exposure of the same cell to ATPγS activated small inward currents with an amplitude of ~2·24 pA and an open probability (NP_o) of 0·34. *B*, current–voltage (*I–V*) relationship of ATPγS-activated unitary Cl^- currents. Representative current tracings from cell-attached patches when the patch was clamped from a holding potential of 0 mV relative to the resting membrane potential (RP) to various potentials from RP − 60 mV to RP + 80 mV for 2 s in increments of +20 mV; displayed currents low-pass filtered at 1 kHz. Mean *I–V* relationship (± S.E.M.) from 4 different cells studied under the same conditions is shown in the right panel. Potentials are expressed as a change from RP as measured from the inside of the cell. The mean reversal potential was RP + 28·1 ± 2·6 mV, and the slope conductance (from RP + 20 mV to RP + 120 mV) was 11·8 ± 0·3 pS when the pipette (external) solution contained 150 mM NMDG-Cl.

(Gadsby *et al.* 1995; Hume *et al.* 2000). Furthermore, in the present study, we found that a specific PKC inhibitor prevented the activation of $I_{Cl,ATP}$, while the phorbol ester, PDBu, activated a Cl^- current identical to that activated by ATPγS, strongly indicating that activation of intracellular PKC may play a major role in the coupling of P_2-receptor stimulation to channel activation. Direct evidence for extracellular ATP-induced increase in PKC activity in cardiac myocytes has been obtained (Legssyer *et al.* 1988; Kunapuli & Daniel, 1998), suggesting that PKC may be a common second messenger for P_2-receptor regulation of Cl^- channels in other species as well (Matsuura & Ehara, 1992; Kaneda *et al.* 1994). It has been well established that PKC and PKA activate CFTR Cl^- channels in heart (Zhang *et al.* 1994; Collier & Hume, 1995; Yamazaki *et al.* 1999) and other tissues (Bajnath *et al.* 1993; Winpenny *et al.* 1995; Jia *et al.* 1997; Lansdell *et al.* 1998). Potentiation of cAMP-PKA activation of CFTR by PKC has been observed in many other cells as well (Bajnath *et al.* 1993; Winpenny *et al.* 1995; Jia *et al.* 1997; Lansdell *et al.* 1998; Gadsby & Nairn, 1999). Therefore, it is not too surprising that purinergic receptor stimulation in mouse cardiac myocytes may be linked to activation of CFTR Cl^- channels through a mechanism involving both PKC and PKA phosphorylation. Future studies are planned to test this hypothesis further using cardiac myocytes isolated from homozygous CFTR knockout mice (which were not readily available from commercial sources for the present experiments).

$I_{Cl,ATP}$ is unlikely to be due to the activation of $I_{Cl,Ca}$, because the electrophysiological properties of $I_{Cl,ATP}$ and $I_{Cl,Ca}$ are very different and ATP and its analogue ATPγS activate $I_{Cl,ATP}$ in the presence of Ca^{2+} channel blockers and high intracellular EGTA (10 mM) or BAPTA (20 mM) (Levesque & Hume, 1995). It is also unlikely that $I_{Cl,ATP}$ is associated with $I_{Cl,vol}$, because $I_{Cl,ATP}$ is activated by extracellular nucleotide and intracellular PKC and PKA while $I_{Cl,vol}$ is inhibited by these manoeuvres (Duan *et al.* 1997*b*; Clemo & Baumgarten, 1998; Duan *et al.* 1999*b*). The properties of $I_{Cl,ATP}$ are also very different from those of $I_{Cl,vol}$: $I_{Cl,ATP}$ has a linear I–V with symmetrical Cl^-, a small single-channel conductance (~11 pS), and an anion selectivity of $Cl^- > I^-$ while $I_{Cl,vol}$ has an outwardly rectifying I–V with symmetrical Cl^-, an intermediate single-channel conductance (~40 pS), and an anion selectivity of $I^- > Cl^-$ (Vandenberg *et al.* 1994; Duan *et al.* 1997*b*).

The pharmacological properties of $I_{Cl,ATP}$ examined are also similar to those of $I_{Cl,CFTR}$. $I_{Cl,ATP}$ was significantly inhibited by 50 μM glibenclamide (72% at +80 mV), consistent with the reported lower IC_{50} value (12·5 μM at +50 mV) for glibenclamide block of cardiac $I_{Cl,CFTR}$, compared to reported IC_{50} values for glibenclamide block of cardiac $I_{Cl,vol}$ and $I_{Cl,Ca}$ (193 μM and 62 μM, respectively; Sheppard & Robinson, 1997). In addition, the CFTR Cl^- channel activator, levamisole, was found to activate macroscopic Cl^- currents similar to $I_{Cl,ATP}$ in mouse myocytes, which were also potently inhibited by 50 μM glibenclamide.

The single-channel conductance and reversal potential of unitary $I_{Cl,ATP}$ recorded in the cell-attached membrane patches from mouse ventricular myocytes (Fig. 10*B*) are very similar to those reported for CFTR Cl^- channels in cell-attached membrane patches activated by either PKA (Ehara & Ishihara, 1990; Sheppard & Welsh, 1999) or PKC (Bajnath *et al.* 1993; Collier & Hume, 1995). Although the NP_o of unitary $I_{Cl,ATP}$ in mouse heart is lower than that reported for CFTR Cl^- channels in guinea-pig heart, it is close to the NP_o of murine epithelial CFTR Cl^- channels

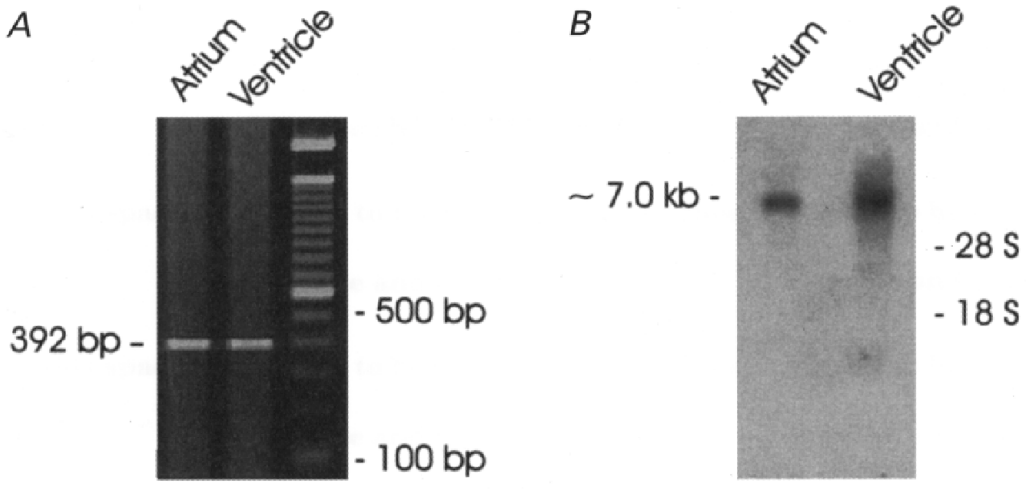


Figure 11. Molecular expression of CFTR in mouse heart

A, agarose gel depicting CFTR-specific RT-PCR product from mouse atrial and ventricular tissue. The CFTR-specific primers amplified a 392-nucleotide product of mouse CFTR (nucleotide positions 1340–1730) which confirmed transcriptional expression of CFTR in both atrial and ventricular cells. *B*, Northern blot analysis of CFTR expression in mouse cardiac tissues. Total RNA from atrial (9 μg) and ventricular (20 μg) tissue was hybridized with a ^{32}P-labelled mouse CFTR probe (392 bp) as described in Methods. The mouse CFTR transcript was detected at ~7·0 kb.

expressed in Chinese hamster ovary cells (Lansdell *et al.* 1998), which were incidentally also found to be activated by PDBu but somewhat refractory to stimulation by activators of human epithelial CFTR Cl^- channels.

We also observed that the activation of $I_{Cl,ATP}$ by ATPγS or phorbol esters enabled a stimulatory effect of isoprenaline on $I_{Cl,ATP}$, indicating that initial PKC phosphorylation of the channel is a prerequisite for activation of $I_{Cl,ATP}$ by cAMP-PKA. This is very similar to the results of a recent study from Jia *et al.* on PKA and PKC regulation of epithelial CFTR Cl^- channel function, in which they found that constitutive PKC phosphorylation is essential for acute activation of CFTR by PKA (Jia *et al.* 1997). They suggest that PKA phosphorylation alone is not a sufficient stimulus to open CFTR chloride channels and initial phosphorylation of the channel by PKC has a permissive role in priming the channel for acute activation by PKA. Furthermore, Liedtke & Cole also found that PKC inhibitors as well as antisense oligonucleotide to PKC-ϵ prevented the stimulatory effects of forskolin + CPT-cAMP or adrenaline (epinephrine) on CFTR-mediated Cl^- secretion (Liedtke & Cole, 1998). Similar synergistic effects of PKA and PKC phosphorylation on cardiac CFTR channels have also been described (Middleton & Harvey, 1998; Yamazaki *et al.* 1999). Differences in basal PKC activity may contribute to the variable cAMP responsiveness of CFTR channels in different cell types. After pretreatment of cells with PKC inhibitors native CFTR channels in cardiac myocytes are almost completely refractory to PKA stimulation by elevation of intracellular cAMP (Middleton & Harvey, 1998). Therefore, purinergic receptor stimulation, which causes an activation of PKC and possibly PKA as well (Puceat *et al.*

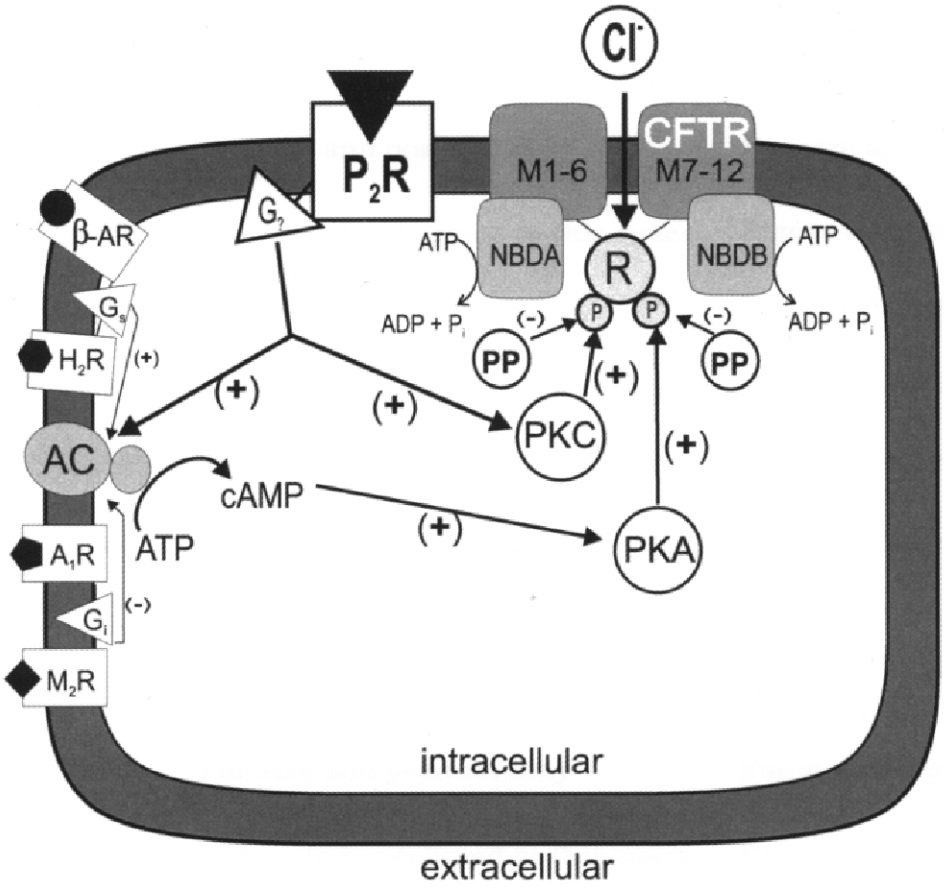


Figure 12. Schematic representation of proposed novel intracellular signal transduction pathway for regulation of CFTR chloride channel function through purinergic receptors

Stimulation of P_2-purinergic receptors (P_2R) increases intracellular PKC and adenylyl cyclase (AC)–cAMP–PKA activities through an unidentified heterotrimeric G protein ($G_?$) (Legssyer *et al.* 1988; Kunapuli & Daniel, 1998; Puceat *et al.* 1998). Initial PKC phosphorylation permits subsequent PKA phosphorylation of the regulatory domain (R) of the CFTR channel (Jia *et al.* 1997), which causes activation of the two nucleotide-binding domains (NBDA and NBDB). ATP hydrolysis at NBDA and NBDB opens the channel (Sheppard & Welsh, 1999; Gadsby & Nairn, 1999). Other reported regulatory pathways for CFTR regulation are included on the left part of the model. Other abbreviations include: M1–6, the predicted CFTR membrane-spanning segments 1–6; M7–12, the predicted CFTR membrane-spanning segments 7–12; PP, phosphatase; β-AR, β-adrenergic receptor; G_s, heterotrimeric stimulatory G protein; H_2R, histamine type 2 receptor; A_1R, adenosine type 1 receptor; G_i, heterotrimeric inhibitory G protein; M_2R, muscarinic type 2 receptor; (+), stimulatory effect; (−), inhibitory effect.

1998), may represent a novel, alternative pathway for CFTR regulation (see Fig. 12). This may explain why stimulation of P_2-purinoceptors is able to activate a Cl^- current in mouse cardiac myocytes, but stimulation of intracellular cAMP by isoprenaline or forskolin alone fails to, even though molecular evidence clearly shows that CFTR mRNA is expressed in these cells. This may also explain why electrophysiological studies on human atrial and ventricular myocytes have failed to detect the activation of $I_{Cl,PKA}$ by isoprenaline or forskolin (Oz & Sorota, 1995) while molecular evidence shows that the CFTR message is indeed expressed in both atrial and ventricular human myocardium (Levesque *et al.* 1992; Warth *et al.* 1996). Since CFTR Cl^- channel function is regulated through a multitude of receptor–signal transduction pathways, assessment of functional expression of CFTR Cl^- channels in heart should not be limited to only testing the cAMP-PKA response of the channel.

It is well known that ATP is an important neurotransmitter released from sympathetic nerves and the concentration of ATP can be significantly increased in the coronary circulation during a variety of normal or diseased states (Gordon, 1986; Kunapuli & Daniel, 1998). Therefore, the finding that $I_{Cl,ATP}$ is due to purinergic regulation of CFTR Cl^- channel function may have significant physiological and pathophysiological importance and potential clinical relevance. Although our study did not specifically address which P_2-purinoceptor subtype is linked to activation of CFTR in mouse heart, the fact that activation of $I_{Cl,ATP}$ was prevented by specific PKA or PKC inhibitors, argues against involvement of P_{2X}-purinoceptors, since these are usually associated with ligand-gated channels (Kunapuli & Daniel, 1998). Finally, our results resurrect consideration of the use of CFTR knockout mice as a potentially useful animal model for future investigations of CFTR function in the heart.

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