P2Y purinergic receptor regulation of CFTR chloride channels in mouse cardiac myocytes

Shintaro Yamamoto-Mizuma, Ge-Xin Wang and Joseph R. Hume

Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada School of Medicine, Reno, NV, USA

The intracellular signalling pathways and molecular mechanisms responsible for P2-purinoceptor-mediated chloride (Cl^-) currents ($I_{Cl,ATP}$) were studied in mouse ventricular myocytes. In standard NaCl-containing extracellular solutions, extracellular ATP (100 μM) activated two different currents, $I_{Cl,ATP}$ with a linear *I–V* relationship in symmetrical Cl^- solutions, and an inwardly rectifying cation conductance (cationic I_{ATP}). Cationic I_{ATP} was selectively inhibited by Gd^{3+} and Zn^{2+}, or by replacement of extracellular NaCl by NMDG; $I_{Cl,ATP}$ was Cl^- selective, and inhibited by replacement of extracellular Cl^- by Asp^-; both currents were prevented by suramin or DIDS pretreatment. In GTPγS-loaded cells, $I_{Cl,ATP}$ was irreversibly activated by ATP, but cationic I_{ATP} was still regulated reversibly. GDPβS prevented activation of the $I_{Cl,ATP}$, even though pertussis toxin pretreatment did not modulate $I_{Cl,ATP}$. These results suggest that activation of $I_{Cl,ATP}$ occurs via a G-protein coupled P2Y purinergic receptor. The $I_{Cl,ATP}$ persistently activated by GTPγS, was inhibited by glibenclamide but not by DIDS, thus exhibiting known pharmacological properties of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels. In ventricular cells of *cftr*$^{-/-}$ mice, extracellular ATP activated cationic I_{ATP}, but failed to activate any detectable $I_{Cl,ATP}$. These results provide compelling evidence that activation of CFTR Cl^- channels in mouse heart are coupled to G-protein coupled P2Y purinergic receptors.

(Received 16 December 2003; accepted after revision 13 February 2004; first published online 20 February 2004)
Corresponding author J. R. Hume: Department of Pharmacology/318, University of Nevada School of Medicine, Reno, NV 89557-0046, USA. Email: joeh@med.unr.edu

Adenine nucleotides are continually present in variable amounts in the extracellular space of the heart, being normally released as a cotransmitter from sympathetic perivascular nerves (for review see Vassort, 2001). Extracellular ATP mainly acts on cell surface receptors of the P2X and P2Y subtypes, which are much more sensitive to ATP and ADP than to AMP and adenosine, and genes encoding seven ionotropic P2X nucleotide receptor subtypes and eight G-protein coupled P2Y nucleotide receptor subtypes have been identified in human and other mammalian tissues (Fredholm *et al.* 1994, 1997; Inscho, 2001; Vassort, 2001; North, 2002; Dubyak, 2003; Lee & O'Grady, 2003). In contrast to P2Y receptors, P2X receptors are ligand-gated ion channels permeable to small monovalent cations (Inscho, 2001; North, 2002). Based on their functional coupling to particular G-proteins and effector proteins, P2Y receptors can be broadly subdivided into five G_q-coupled subtypes ($P2Y_{1–4}$, $P2Y_6$, $P2Y_{11}$) which can stimulate a variety of pathways including protein kinase C (PKC), and three G_i-coupled subtypes ($P2Y_{12–14}$). Expression of several G_q-coupled subtypes have been reported in several mammalian species of cardiac myocytes (Vassort, 2001). It has been observed in neonatal and adult cardiac cells that extracellular ATP increases PKC activity through both ε- and δ-PKC, two Ca^{2+}-insensitive PKC isoforms (Puceat *et al.* 1994; Vassort, 2001). In addition, the activation of P2-purinergic receptors in the heart has been shown to elevate cyclic AMP (cAMP) due to activation of a specific isoform (V) of adenylyl cyclase that may be different from the isoform activated by β-adrenergic receptor stimulation (Puceat *et al.* 1998).

In heart, stimulation of purinergic receptors is known to activate non-selective cation currents (I_{ATP}) as well as chloride (Cl^-)-dependent currents ($I_{Cl,ATP}$) (Vassort, 2001). Extracellular nucleotides, including ATP, ADP, and ATPγS, but not adenosine nor AMP in mouse ventricular myocytes (Levesque & Hume, 1995), activated $I_{Cl,ATP}$ similar to the $I_{Cl,ATP}$ previously described in guinea-pig atrial (Matsuura & Ehara, 1992) and rat myocytes (Kaneda *et al.* 1994). In both rat and mouse ventricular myocytes, the activation of $I_{Cl,ATP}$ appeared to be coupled to P2-purinoceptor stimulation. In a subsequent report

 DOI: 10.1113/jphysiol.2003.059881

(Duan *et al.* 1999) it was suggested that $I_{Cl,ATP}$ in mouse heart may be due to activation of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels through a novel intracellular signalling pathway involving purinergic activation of PKC and protein kinase A (PKA), since the CFTR gene encodes a Cl^- channel activated by both PKA and PKC (for reviews see Gadsby & Nairn, 1999; Jentsch *et al.* 2002). Although several cAMP activators (isoproterenol, forskolin and isobutyl methylxanthine) alone were unable to activate any detectable $I_{Cl.PKA}$ in mouse ventricular myocytes, RT-PCR clearly confirmed expression of the mouse homologue of CFTR in heart, and P2-purinergic activation of the current was prevented by inhibition of either endogenous PKC or PKA activity (Duan *et al.* 1999). The possibility that $I_{Cl,ATP}$ in mouse heart might be due to activation of CFTR Cl^- channels was also supported by strong similarities in the electrophysiological properties of $I_{Cl,ATP}$ and CFTR Cl^- currents ($I_{Cl,CFTR}$), and the finding that constitutive PKC phosphorylation may be essential for acute activation of CFTR by PKA (Jia *et al.* 1997). More recently, in *Xenopus* oocytes coexpressing the $P2Y_6$ receptor together with CFTR, it has been reported that extracellular nucleotides activated both Ca^{2+}-activated Cl^- currents and CFTR Cl^- currents (Kottgen *et al.* 2003). 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 transgenic approaches to further characterize the properties of cationic I_{ATP} and $I_{Cl,ATP}$ in mouse heart. We first demonstrate that extracellular ATP activation of cationic I_{ATP} involves G-protein uncoupled purinergic receptors, whereas ATP activation of $I_{Cl,ATP}$ involves a G-protein signalling pathway mediated by the P2Y purinergic receptor subtype. We then confirm that the pharmacological properties of $I_{Cl,ATP}$ are similar to the known properties of cardiac $I_{Cl,CFTR}$, including sensitivity to block by glibenclamide and insensitivity to DIDS. Finally, we directly test the hypothesis that $I_{Cl,ATP}$ may be mediated by cardiac CFTR Cl^- channels by examining membrane currents activated by extracellular ATP in ventricular cells isolated from *cftr*$^{-/-}$ mouse.

Methods

Cell preparation

The Institutional Animal Use and Care Committee at the University of Nevada approved the use and treatment of all animals used in the experiments described here. Single ventricular myocytes from mouse hearts were isolated using an enzymatic dispersion technique as originally described (Levesque & Hume, 1995; Duan *et al.* 1999). Briefly, *cftr*$^{+/+}$ (wild-type; C-57BL/6J/black inbred, 10–20 weeks, 34 animals; Jackson Laboratory, Bar Harbour, MA, USA) and *cftr*$^{-/-}$ mice (knockout; B6.129P2-cftr^{tmIUnc}, 6.2 ± 0.4 weeks, 15.9 ± 1.6 g, 4 animals; Jackson Laboratory) were anaesthetized with sodium pentobarbitone (0.5 mg 10 g I.P.). The chest was opened, and the heart was rapidly removed and perfused by using a modified Langendorff technique, with a physiological saline solution (PSS; see Solutions and drugs) warmed to 37°C 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 0.07% collagenase (CLSII, Sigma) and 1.0% bovine serum albumin (BSA) for 20–30 min. The ventricles were removed and cell dissociation was achieved by gentle mechanical agitation. After the enzyme treatment, the cells were dissociated in high-K^+, low-Cl^- storage (modified KB) solution (see Solutions and drugs) and stored in a refrigerator (4°C) for later use (within 8 h). Only rod-shaped myocytes with clear cross-striations and no blebs under isotonic conditions were used for electrophysiology studies.

Electrophysiological techniques

The tight-seal whole-cell patch-clamp technique was used to record whole-cell currents in isolated mouse ventricular myocytes. Patch pipettes (1.5 mm o.d. borosilicate glass electrodes) had a tip resistance of 1–3 MΩ when filled with pipette solution. Voltage-clamp recordings were performed using an Axopatch-200A patch-clamp amplifier (Axon Instruments, Union City, CA, USA) and membrane currents were filtered at a frequency of 1 kHz. Data acquisition and command potentials were controlled by pCLAMP 8.1 software (Axon Instruments). A 3 M KCl–agar bridge between the bath and the Ag–AgCl reference electrode was used to minimize changes in liquid junctional potential. When necessary, the current density was calculated by membrane capacitance, which was obtained using pCLAMP 8.1 software. Usually, 5 min was allowed for adequate cell dialysis after membrane rupture before beginning the voltage clamp protocol. All experiments were performed at room temperature.

Solutions and drugs

The PSS for cell preparation contained (mM): 126 NaCl, 10 glucose, 4.4 KCl, 5.0 $MgCl_2$, 1.5 $CaCl_2$, 20 taurine,

5.0 creatine, 5.0 sodium pyruvate, 1.0 Na_2HPO_4, 10 Hepes; pH 7.4 adjust with NaOH; 300 mosmol l^{-1} with mannitol. Ca^{2+}-free PSS was prepared by simply omitting $CaCl_2$ from the PSS. The modified KB (Kraft-Brühe) solution for cell storage contained (mM): 70 potassium glutamate, 20 KCl, 1.0 $MgCl_2$, 10 KH_2PO_4, 10 Taurine, 10 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 glucose, 0.1% albumin, 10 β-hydroxybutyric acid and 10 Hepes; pH 7.2 with KOH (room temperature); 300 mosmol l^{-1} with mannitol. For $I_{Cl,ATP}$ recording, the extracellular and intracellular solutions were chosen to maximize recording of Cl^- currents and reduce possible contamination by cation currents and Ca^{2+}-dependent currents. The standard extracellular solutions contained (mM): 77 NaCl, 0.8 $MgCl_2$, 1.0 $CaCl_2$, 5.0 CsCl, 2.0 $BaCl_2$, 0.2 $CdCl_2$, 5.5 glucose, 10 Hepes, 0.01 nicardipine; pH 7.4 adjusted with NaOH; total $[Cl^-]_o$ = 90 mM; 320 mosmol l^{-1} with mannitol. In some experiments, extracellular NaCl and CsCl were replaced by an equimolar concentration (82 mmol l^{-1}) of sodium aspartate or *N*′-methyl-D-glucamine (NMDG) chloride. The standard intracellular pipette solution contained (mM): 140 NMDG, 90 HCl, 5 MgATP, 0.1 Na_2GTP, 20 EGTA, 10 Hepes; pH 7.3 adjusted with NMDG; total $[Cl^-]_i$ = 90 mmol l^{-1}; 280 mosmol l^{-1} using mannitol. Mannitol was used to adjust osmolarity in these solutions so that the activation of $I_{Cl,vol}$ by hypotonic solutions could be easily tested in control experiments in cells from *cftr*$^{-/-}$ mice. In some experiments studying intracellular guanosine 5′-[γ-thio]triphosphate (GTPγS; Sigma, St Louis, MO, USA) or guanosine 5′-[β-thio]diphosphate (GDPβS; Sigma), 0.1 mM Na_2GTP was replaced by 0.1 mM GTPγS or 1.0 mM GDPβS. The cells were preincubated for 2 h at room temperature in the modified KB solution (pH 7.4 at room temperature) containing 5.0 μg ml^{-1} pertussis toxin (PTX; Calbiochem, San Diego, CA, USA) in some experiments. The osmolarity of solutions was measured using a freezing point depression osmometer (model 3300; Advanced Instruments Inc., Norwood, MA, USA). Chemicals, including glibenclamide (Sigma), 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS; Sigma), phorbol 12,13-dibutyrate (PDBu; Sigma) and suramin (Sigma) were prepared as stock solutions in dimethyl sulphoxide (DMSO) and added to a known volume of superfusion solutions to produce the desired concentrations. Final concentration of DMSO was between 0.001 and 0.1% in solutions, which by itself had no effect on measured currents.

Data analysis

The straight line in the reversal potential–log $[Cl^-]_o$ relationships (Fig. 2*D*), was fitted to the data according to a regression analysis with the least-squares method. Concentration–response curves (Fig. 2*E*) to extracellular ATP were analysed by using a Hill equation:

$$\text{Response} = 1/\{1 + (EC_{50}/[A]^{n_H})\},$$

where [A] is the agonist concentration, EC_{50} is the agonist concentration to achieve 50% of the maximum response and n_H is the Hill coefficient. Currents were normalized to the maximum difference current in each tested cell. The blocking effects of some inhibitors were calculated from the equation:

$$\text{Inhibition} = (I_{max} - I_{drug})/(I_{max} - I_{control}),$$

where I_{max} is the fully activated amplitudes of ATP-induced current, I_{drug} is the minimal amplitudes of ATP-induced current after application of the inhibitors and $I_{control}$ is the basal current amplitudes in control solutions before exposure to ATP.

Data are expressed as means ± S.E.M.; *n* indicates the number of cells. Statistical analysis was performed using paired or unpaired Student's *t* test where appropriate. Differences were considered to be significant with a two-tailed probability (*P*) of < 0.05.

Results

Extracellular ATP-induced currents in mouse ventricular myocytes

Under our experimental conditions with selected extracellular and intracellular solutions (see Methods), most cationic and exchange currents were inhibited and the standard intracellular solution contained 20 mM EGTA to minimize contamination by Ca^{2+}-activated currents. Figure 1*A* shows the time course of extracellular ATP-induced whole-cell currents at +80 mV (filled circles) and −80 mV (open circles) in single ventricular cells isolated from mice. Only small whole-cell leak currents were observed in ventricular cells in the presence of standard extracellular solutions. Current amplitudes began to rapidly increase following a delay of ∼1 min after extracellular application of 100 μM ATP, and reached a steady state level within ∼2 min. Replacement of extracellular NaCl by NMDG-Cl was used to eliminate any ATP-induced non-selective cation current (I_{ATP}). After currents reached steady-state, subsequent replacement of extracellular NaCl by NMDG-Cl solution reversibly

reduced the magnitude of inward currents ($63.8 \pm 3.3\%$ of control at -100 mV, $n = 6$) while having little effect on the magnitude of outward currents ($94.5 \pm 4.2\%$ of control at $+100$ mV, $n = 6$). Figure 1*B* shows examples of raw currents recorded in control (left), ATP with NaCl (middle), and ATP with NMDG-Cl (right) solutions. These currents were obtained at the time points *a*, *b* and *c* in Fig. 1*A*. Difference current–voltage (*I–V*) relationships are shown in Fig. 1*C* ($n = 6$). Extracellular Na^+-dependent ATP-induced currents (*b* – *c*) exhibited inward rectification (0.02 ± 0.46 and -1.49 ± 0.65 pA pF^{-1} at $+80$ mV and -80 mV, respectively), and a mean reversal potential (E_{rev}) of 63.4 ± 16.5 mV, consistent with activation of a non-selective cation current, I_{ATP}. Furthermore, the extracellular Na^+-dependent ATP-induced inward currents were inhibited by 100 μM Gd^{3+} and 2 mM Zn^{2+} (inhibition = 22.8 ± 5.9 ($n = 5$) and $53.9 \pm 2.0\%$ ($n = 3$), respectively, at -100 mV), known blockers of non-selective cation channels (Nilius & Droogmans, 2001).

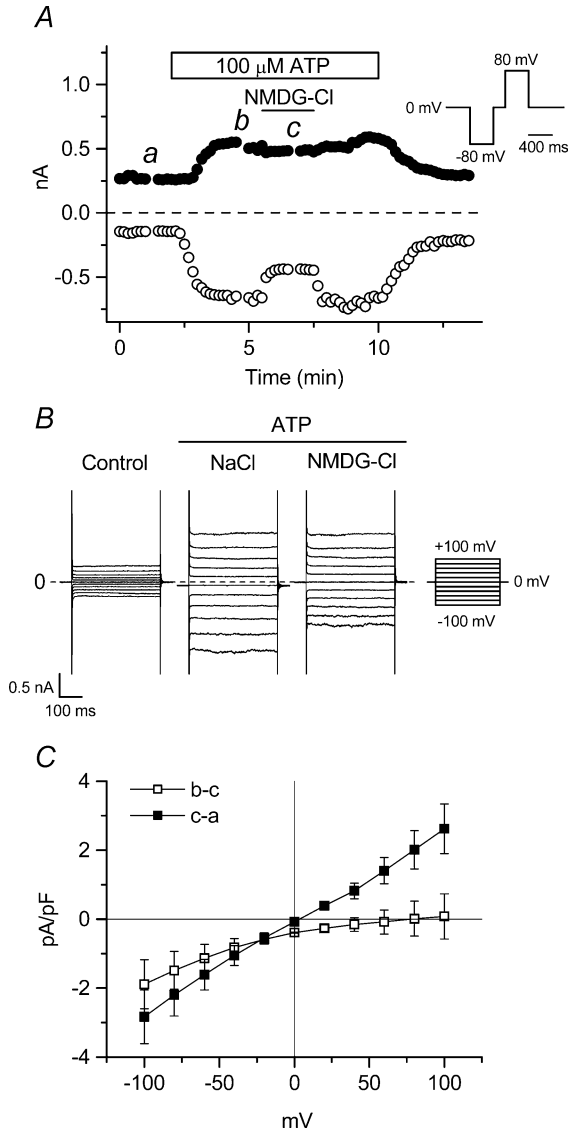

Figure 1. Cation sensitivity of extracellular ATP-induced membrane currents in mouse ventricular myocytes
A, time course of extracellular ATP-induced whole-cell currents at $+80$ mV (●) and -80 mV (○) in single mouse ventricular cells exposed to standard extracellular solution during application of 100 μM ATP to the bath. $[Na^+]_o$ and $[Cs^+]_o$ were replaced with equimolar $[NMDG^+]_o$ during the period indicated by the bar. Here and in subsequent similar figures, the pulse protocol is the same as shown in the inset of *A*. *B*, whole-cell current recordings induced by applying 400 ms voltage-clamp steps to membrane potentials between -100 mV and $+100$ mV in $+20$ mV steps from a holding potential of 0 mV every 2 s. In subsequent similar figures, the step-pulse protocol is the same as shown in the inset of *B*. Currents were recorded at the time points indicated in *A*. *C*, mean *I–V* relationships of $[NMDG^+]_o$-sensitive (*b* – *c*) and -insensitive (*c* – *a*) currents in 6 different cells.

In contrast to I_{ATP}, the Na^+-independent ATP-induced currents (*c* – *a*) were almost linear (2.02 ± 0.47 and -2.25 ± 0.44 pA pF^{-1}, at $+80$ mV and -80 mV, respectively), with a mean reversal potential (E_{rev}: 3.0 ± 2.1 mV) close to the estimated Cl^- equilibrium potential ($E_{Cl} = 0$ mV with symmetrical Cl^- solutions). These properties of the Na^+-independent currents are characteristic of ATP-activated Cl^- currents, $I_{Cl,ATP}$, previously characterized in mouse cardiac myocytes (Levesque & Hume, 1995; Duan *et al.* 1999). Data presented in Fig. 2 further confirm the anionic nature of these currents.

Figure 2*A* shows the time course of the Na^+-independent $I_{Cl,ATP}$ at $+80$ mV (filled circles) and -80 mV (open circles) before (NMDG-Cl) and after replacement of extracellular Cl^- (NMDG-aspartate). Figure 2*B* shows examples of raw currents recorded at the time points *b* and *c* in Fig. 2*A*, and Fig. 2*C* shows *I–V* relationships of currents obtained at the time points *a*, *b* and *c* in Fig. 2*A*. Currents activated by extracellular ATP had almost linear *I–V* relationships under NMDG-Cl conditions, as shown in Fig. 2*C*. Reduction of extracellular $[Cl^-]$ from 90 to 10 mM during ATP application, by replacing 80 mM NMDG-Cl with equimolar NMDG-aspartate, led to a positive shift of E_{rev}, accompanied by a marked decrease in outward current as shown in Fig. 2*A*, *B* and *C*. Figure 2*D* summarizes the changes of E_{rev} in the whole-cell currents at three different $[Cl^-]_o$ levels, performed using the same ventricular myocytes ($n = 4$). The reversal potential–log $[Cl^-]_o$ relation had a slope of 45.4 mV per 10-fold change in $[Cl^-]_o$, indicating that Cl^- ions are the main charge carrier of the ATP-induced current component under NMDG-Cl conditions, as has been observed previously in guinea-pig atrial (Matsuura

& Ehara, 1992) and rat ventricular (Kaneda *et al.* 1994) cells.

Next, to define further the ATP dependence of $I_{Cl,ATP}$, we determined the agonist concentration to achieve a 50% maximal response (EC_{50}) and calculated the Hill coefficient (n_H) for extracellular ATP (see Methods). Figure 2*E* shows the response–concentration relationship obtained at +100 mV with NMDG-Cl solutions (n = 4). This relationship was well fitted by a Hill equation, and extracellular ATP increased the Cl^- current with an estimated EC_{50} value of $1.17 \pm 0.44\ \mu$M and n_H of 0.98 ± 0.33, suggesting that the binding of 1 ATP molecule to P2-receptors activates $I_{Cl,ATP}$ in mouse ventricular cells. This EC_{50} value is similar to that observed previously for P2-purinergic receptor stimulation of $I_{Cl,ATP}$ in rat ventricular cells (Kaneda *et al.* 1994), and I_K in guinea-pig atrial (Matsuura *et al.* 1996) and ventricular (Matsubayashi *et al.* 1999) cells.

Involvement of PTX-insensitive G protein in activation of $I_{Cl,ATP}$

P2 purinergic receptors are divided into two groups: ligand-activated ion channels (P2X receptors) and GTP-binding protein (G protein) coupled receptors (P2Y receptors) (for reviews see Fredholm *et al.* 1994, 1997; North, 2002; Dubyak, 2003; Lee & O'Grady, 2003). To classify the P2-purinergic receptor involved in I_{ATP}, we examined the effects of intracellular dialysis of 100 μM GTPγS, a non-hydrolysable GTP analogue. Several previous reports have showed that intracellular GTPγS itself does not induce cardiac whole-cell currents under conditions similar to those used here (Hwang *et al.* 1992; Iyadomi *et al.* 1995; Hool *et al.* 1997). In contrast, it has been reported that intracellular dialysis of GTPγS in human endothelial cells transiently activates a Cl^- current (Nilius *et al.* 1994). Therefore, we first confirmed that intracellular GTPγS dialysis failed to activate Cl^- currents under basal conditions in mouse ventricular myocytes (n= 3, data not shown). We then tested whether intracellular GTPγS dialysis would alter the purinergic activation of I_{ATP} or $I_{Cl,ATP}$.

Figure 3*A* shows that, in GTPγS-loaded cells with standard extracellular (NaCl) solutions, the outward currents were irreversibly activated by brief exposure to 100 μM ATP, but the inward currents were partially decreased following brief exposure to ATP, as previously found in control cells (Fig. 1*A*). The different *I–V* relationships derived by using currents obtained at the time points *a*, *b* and *c* in Fig. 3*A* are shown in Fig. 3*B*. Intracellular GTPγS-dependent $I_{Cl,ATP}$ ($c - a$) was almost linear with a reversal potential (3.6 mV) close to the estimated Cl^- equilibrium potential (E_{Cl} = 0 mV with symmetrical Cl^- solutions). In contrast, the previously identified Na^+-dependent I_{ATP} ($b - c$), which exhibited inward rectification and a positive reversal potential

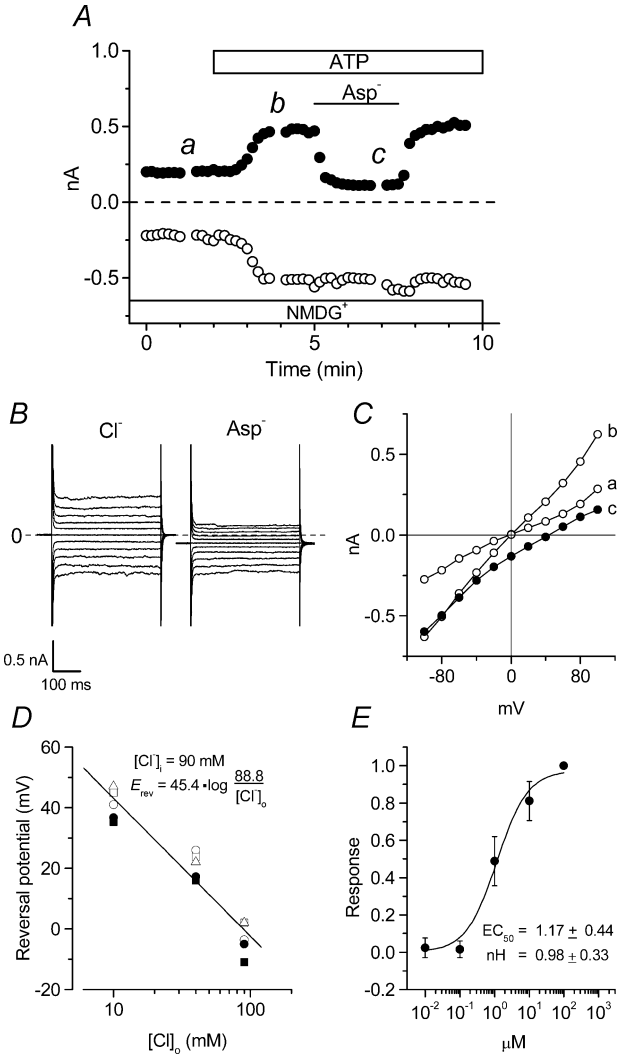

Figure 2. Anion sensitivity of extracellular ATP-induced membrane currents in mouse ventricular myocytes
A, time course of extracellular ATP-induced whole-cell currents in NMDG-Cl solutions during application of 100 μM ATP to the bath. $[Cl^-]_o$ was replaced to equimolar $[Asp^-]_o$ during the period indicated by the bar. *B*, whole-cell current recordings at the time points *b* (Cl^-) and *c* (Asp^-) in *A*. *C*, *I–V* relationships of currents recorded at the time points indicated in *A*. *D*, reversal potential–log $[Cl^-]_o$ relationships of whole-cell currents in the presence of extracellular ATP in 4 different cells in which $[Cl^-]_o$ was changed from 90 to 10 mM in [$NMDG^+$]-rich conditions. *D*, dose–response relationships of anion-sensitive [$NMDG^+$ solutions] extracellular ATP-induced (difference) currents in 4 different cells. Responses were normalized to the maximum current density obtained at 100 μM ATP. The EC_{50} and n_H coefficient correspond to the fitted curve.

(22.6 mV), appeared to be insensitive to intracellular GTPγS, as it reversed following washout of ATP.

To further clarify the GTP dependence of $I_{Cl,ATP}$, we measured current densities of $I_{Cl,ATP}$ (at +100 mV) in control, GTPγS-dialysed, GDPβS (non-hydrolysable GDP analogue)-dialysed, and pertussis toxin (PTX; $G_{i/o}$ protein inhibitor)-pretreated cells. Figure 3*C* compares $I_{Cl,ATP}$ densities obtained under these four experimental conditions in a number of myocytes. The mean outward current density of $I_{Cl,ATP}$ in control was 2.95 ± 0.35 pA pF^{-1} ($n = 33$), whereas mean outward current density of $I_{Cl,ATP}$ in GTPγS-loaded cells and PTX-pretreated cells was 4.37 ± 0.36 ($n = 4$) and 2.91 ± 0.71 pA pF^{-1} ($n = 4$), respectively. In contrast, extracellular ATP induced very little activation of $I_{Cl,ATP}$ in GDPβ-loaded cells (0.25 ± 0.50 pA pF^{-1} ($n = 4$), $P < 0.05$ *versus* control). The cationic I_{ATP} had no sensitivity to these GTP analogues (data not shown). These results suggest that cationic I_{ATP} is regulated by a G protein independent signalling pathway, whereas a G protein-coupled receptor (P2Y purinergic receptor coupled to a PTX-insensitive G protein pathway) plays a crucial role in the activation of $I_{Cl,ATP}$.

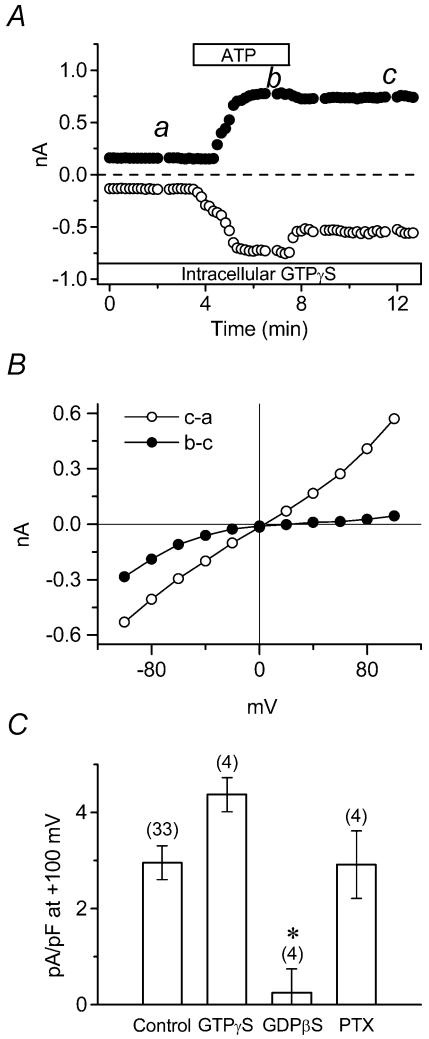

Figure 3. Role of G-protein coupled purinergic receptors in activation of $I_{Cl,ATP}$

A, time course of extracellular ATP-induced whole-cell currents in standard extracellular solution during application of 100 μM ATP to the bath. The tested cell was dialysed with 0.1 mM GTPγS, and was exposed to ATP as indicated by the bar. *B*, *I–V* relationships of ATP-induced currents (*b* – *a*) and the persistently activated $I_{Cl,ATP}$ (*c* – *a*). Whole-cell currents were activated by voltage-clamp pulses as in Fig. 1*B*, at time points *a*, *b* and *c* in *A*. *C*, mean current densities at +100 mV of $I_{Cl,ATP}$ in GTPγS-dialysed ($n = 4$), GDPβS-dialysed ($n = 4$) or PTX-pretreated ($n = 4$) cells. * signifies significantly smaller than control with $P < 0.05$.

Absence of $I_{Cl,ATP}$ in myocytes from *cftr*$^{-/-}$ mice

In a previous report (Duan *et al.* 1999) it was suggested that CFTR may be a molecular candidate responsible for $I_{Cl,ATP}$ in mouse cardiac myocytes, based on: (1) the observation that $I_{Cl,ATP}$ was regulated by a dual intracellular signal phosphorylation pathway involving both protein kinase A (PKA) and protein kinase C (PKC), (2) the observation that $I_{Cl,ATP}$ was inhibited by 50 μM glibenclamide, a concentration known to inhibit CFTR Cl^- currents, but not volume-regulated Cl^- currents ($I_{Cl,vol}$) or Ca^{2+}-activated Cl^- currents ($I_{Cl,Ca}$) (Yamazaki & Hume, 1997), and (3) single channel properties (conductance and rectification) that resembled those of CFTR Cl^- channels in cardiac myocytes (Ehara & Ishihara, 1990). In addition, RT-PCR and Northern blot analysis clearly showed CFTR mRNA expression in mouse atrium and ventricle. To further test this hypothesis, we examined the effects of extracellular ATP in single ventricular cells isolated from *cftr*$^{-/-}$ (CFTR knockout) mice. As shown in Fig. 4*A*, extracellular ATP activated cationic I_{ATP} in NaCl-containing solutions in cells from *cftr*$^{-/-}$ mice; however, activation of $I_{Cl,ATP}$, which was prominent in cells from *cftr*$^{+/+}$ mice (cf. Figure 1), was not detectable. PDBu (100 nM) and isoprenaline (1.0 μM), which previously have been shown to significantly augment the amplitude of $I_{Cl,ATP}$ in mouse cardiac myocytes (Duan *et al.* 1999), alone or in combination with ATP, also failed to activate $I_{Cl,ATP}$ in cells from *cftr*$^{-/-}$ mice (data not shown). Despite the absence of $I_{Cl,ATP}$ in cells from *cftr*$^{-/-}$ mice, exposure of these cells to hypotonic solutions elicited typical changes in $I_{Cl,vol}$ with current densities, kinetics and rectification properties similar to $I_{Cl,vol}$ in cells from normal *cftr*$^{+/+}$ mice (data not shown). In Fig. 4*B*, representative raw currents activated by voltage clamp pulses obtained at the time points *a* and *b* in Fig. 4*A*, and the difference

current, are shown. The mean *I–V* relationships (Fig. 4*C*) of the extracellular ATP-sensitive current ($n = 10$ from 4 *cftr*$^{-/-}$ mice) was inwardly rectifying (0.14 ± 0.18 and -1.04 ± 0.26 pA pF^{-1} at +80 and −80 mV, respectively) and the mean E_{rev} was 36.3 ± 7.3 mV. These results indicate that typical cationic I_{ATP} can be recorded in myocytes from *cftr*$^{-/-}$ mice, and the absence of $I_{Cl,ATP}$ in ventricular cells can be attributed to targeted inactivation of *cftr*.

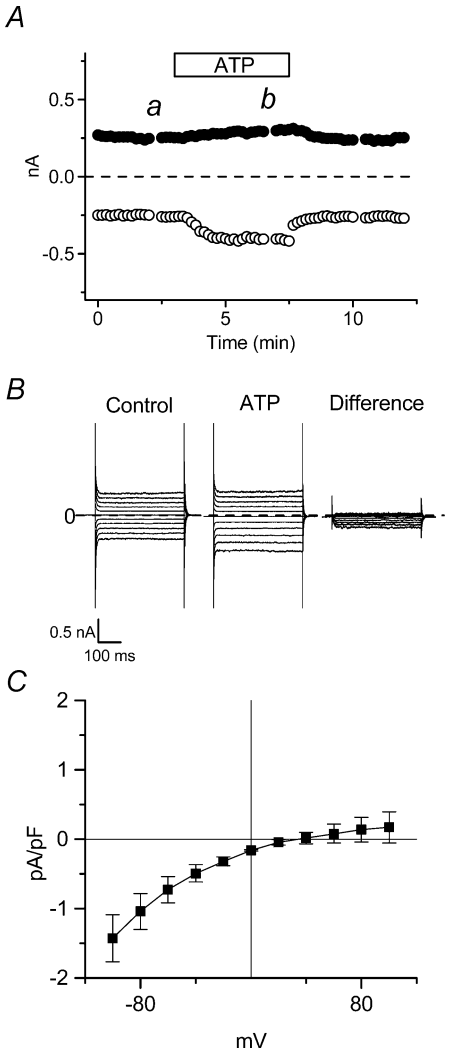

Figure 4. Extracellular ATP-induced membrane currents in ventricular cells from *cftr*$^{-/-}$ mice

A, time course of extracellular ATP-induced whole-cell currents in standard extracellular solution in ventricular cells from *cftr*$^{-/-}$ mice. *B*, whole-cell current recorded using same protocol as in Fig. 1*B* at time points *a* (control) and *b* (ATP) in panel *A*. Difference currents obtained by subtracting currents obtained at time *b* from currents obtained at time *a*. *C*, mean *I–V* relationships of ATP-induced difference currents in 10 cells from 4 *cftr*$^{-/-}$ mice.

Mechanism of block of $I_{Cl,ATP}$ by DIDS

It was previously demonstrated (Kaneda *et al.* 1994) that $I_{Cl,ATP}$ is potently inhibited by DIDS, which is a known inhibitor of several types of Cl^- channels, excluding CFTR Cl^- channels (Vandenberg *et al.* 1994). It is possible that these apparently contradictory effects on $I_{Cl,ATP}$ might be due to the ability of DIDS to directly antagonize P2 purinergic receptors (Hume *et al.* 2000; Vassort, 2001; North, 2002). To directly distinguish between possible inhibitory effects of DIDS on $I_{Cl,ATP}$ from DIDS antagonism of P2 purinergic receptors, we examined the effects of DIDS on $I_{Cl,ATP}$ persistently activated in GTPγS-dialysed cells. As shown in Fig. 5*A*, 100 μM DIDS pre-treatment completely prevented the activation of both cationic I_{ATP} and $I_{Cl,ATP}$ in GTPγS-dialysed cells; both currents were activated in the continued presence of ATP following washout of DIDS. As previously shown in Fig. 3, $I_{Cl,ATP}$ remained persistently activated following washout of ATP, and re-exposure to 100 μM DIDS failed to significantly alter the amplitude of the persistently activated $I_{Cl,ATP}$. This result rules out a direct effect of DIDS on $I_{Cl,ATP}$. Figure 5*A* also demonstrates that the DIDS-insensitive persistently activated $I_{Cl,ATP}$ was nearly completely inhibited by 100 μM glibenclamide. Figure 5*B* illustrates *I–V* relationships of the DIDS-sensitive ($e - f$) and glibenclamide-sensitive ($e - g$) persistently activated $I_{Cl,ATP}$ in GTPγS-dialysed cells. Overall, these results suggest that DIDS inhibition of $I_{Cl,ATP}$ is due to P2 purinergic receptor antagonism, and that the channels mediating $I_{Cl,ATP}$ are DIDS insensitive and glibenclamide sensitive, thus resembling the known properties of CFTR Cl^- channels in cardiac cells (Vandenberg *et al.* 1994; Tominaga *et al.* 1995).

Figure 5*C* summarizes the effects of pretreatment of DIDS (100 μM, $n = 4$) and suramin (100 μM, $n = 4$), a P2 purinergic receptor antagonist, on $I_{Cl,ATP}$ activation by extracellular ATP at +100 mV in control (absence of GTPγS) myocytes. These results show that pretreatment of DIDS or suramin strongly inhibited the activation of $I_{Cl,ATP}$ by extracellular ATP. In addition, the activation of cationic I_{ATP} was also inhibited by DIDS (Fig. 5*A*) or suramin pretreatment (data not shown).

Discussion

The major findings of the present investigation include the following: (1) extracellular ATP activates both a cationic I_{ATP} and $I_{Cl,ATP}$ in mouse ventricular myocytes; (2) the activation of cationic I_{ATP} involves a G protein-independent signalling pathway, whereas activation of

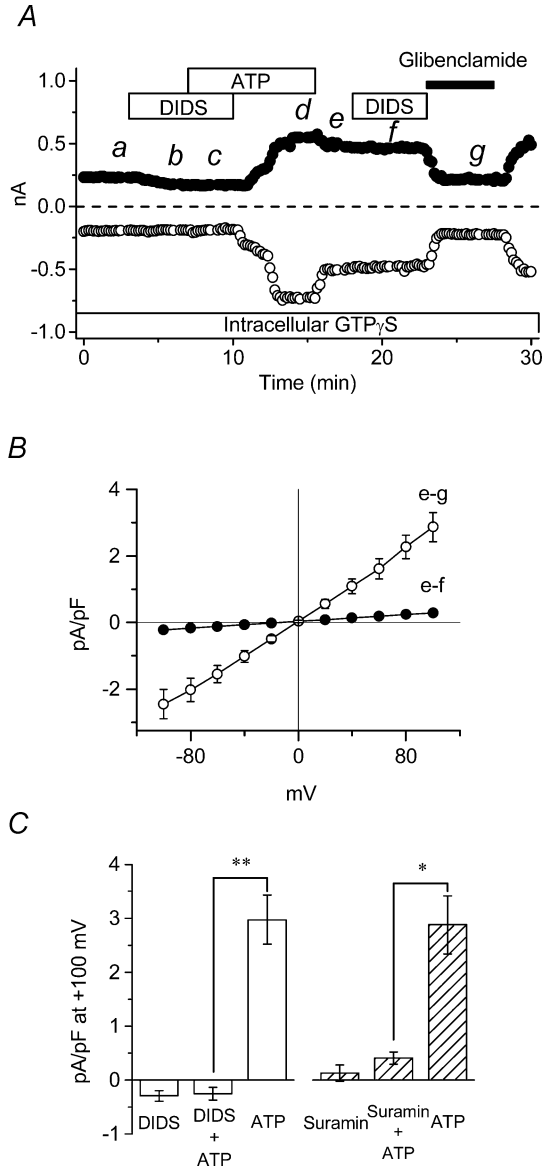

Figure 5. Effects of DIDS and suramin on $I_{Cl,ATP}$ in ventricular cells of wild-type mice

A, time course of extracellular ATP-induced whole-cell currents in standard extracellular solutions in intracellular GTPγS dialysed cell of wild-type mouse. ATP (100 μM), DIDS (100 μM) and glibenclamide (100 μM) were applied during the times indicated by each bar. *B*, mean *I–V* relationships of DIDS- (*e* – *f*) and glibenclamide-sensitive currents (*e* – *g*), which were obtained from whole-cell recordings induced using the same voltage clamp pulse protocol shown in Fig. 1*B*, at time points *e*, *f* and *g* in *A*. *C*, the left side shows the mean ($n = 4$) difference current density at +100 mV in DIDS, DIDS and ATP, and ATP following the removal of DIDS in control (absence of GTPγS) cells. The right side shows the mean ($n = 4$) difference current densities at +100 mV in suramin, suramin and ATP, and ATP following removal of suramin. * and ** signify significantly smaller than ATP alone with $P < 0.05$ and 0.01, respectively.

$I_{Cl,ATP}$ involves a PTX-insensitive G protein signalling pathway; (3) the absence of $I_{Cl,ATP}$ in myocytes from *cftr*$^{-/-}$ mice provides direct evidence that CFTR Cl^- channels are indeed responsible for $I_{Cl,ATP}$ in mouse heart; and (4) the apparent inhibitory effects of the Cl^- channel blocker DIDS on $I_{Cl,ATP}$ can be attributed to antagonism of P2 purinergic receptors, and are not due to direct inhibition of $I_{Cl,ATP}$. Thus the demonstrated DIDS insensitivity and glibenclamide sensitivity of $I_{Cl,ATP}$ are consistent with the known pharmacological properties of CFTR Cl^- channels.

Role of P2Y purinergic receptors in the activation of $I_{Cl,ATP}$

Activation of $I_{Cl,ATP}$ was clearly prevented by suramin, an antagonist of P2 purinergic receptors, showing that binding of extracellular ATP to the P2 purinergic receptor is essential for the activation of $I_{Cl,ATP}$ in mouse ventricular myocytes. $I_{Cl,ATP}$ was also found to be persistently activated by ATP in the GTPγS-dialysed cells, and its activation was abolished in the cells dialysed with GDPβS, suggesting an essential role of G-proteins in the activation of $I_{Cl,ATP}$. In previous reports (Matsuura & Ehara, 1992; Kaneda *et al.* 1994; Levesque & Hume, 1995; Duan *et al.* 1999), the activation of $I_{Cl,ATP}$ appeared to be coupled to P2-purinoceptor stimulation, since $I_{Cl,ATP}$ was activated by ATP, ADP and ATPγS, but not by adenosine or AMP in cardiac cells. However, the type of P2 receptor involved was not characterized in these previous reports. This agonist profile, together with the present demonstration of involvement of a G protein signalling pathway, and inhibition by suramin, provides evidence for an essential role of P2Y receptors for the activation of $I_{Cl,ATP}$ in mouse ventricular myocytes.

Several types of cardiac ion channels are known to be influenced by P2Y purinergic receptor stimulation. Among these, the modulation of L-type Ca^{2+} current (for review see Vassort, 2001) and delayed rectifier K^+ current (Matsuura *et al.* 1996, Matsubayashi *et al.* 1999) has been clearly shown to depend on P2Y purinergic receptors. In contrast, we demonstrated that extracellular ATP-activated cation currents (cationic I_{ATP}) were not influenced by GTPγS and GDPβS although the activation was prevented by pretreatment of suramin and DIDS. In addition, the cationic I_{ATP} was partially inhibited by 100 μM Gd^{3+} and 2 mM Zn^{2+}, inhibitors of non-selective cation channels. These results suggest that the cationic I_{ATP} is most likely regulated by ionotropic P2X receptors, although the remote possibility that a yet to be identified G-protein-independent signalling pathway involving P2Y receptors may play a role cannot be eliminated. In general, it is reported that extracellular ATP elicits a

rapid activating, fast desensitizing inward cation current via P2X receptors in several cardiac cells (Matsuura & Ehara, 1992; Vassort, 2001; North, 2002). However, the cationic I_{ATP} observed in the present study did not appear to exhibit rapid desensitization within a few of minutes of ATP application. A similar long-lasting ATP-induced inward cation current has been shown in rabbit sinoatrial cells (Shoda *et al.* 1997) and rat cardiomyocytes (Ugur & Vassort, 2001). However, the experimental conditions we used were different since bath and pipette solutions were selected to optimize recording of Cl^- currents. In addition, in examining current responses to extracellular ATP, we did not employ a rapid extracellular solution exchange system. These factors therefore prevent us from reaching a definitive conclusion regarding desensitization of the cationic I_{ATP}.

Pharmacological properties of $I_{Cl,ATP}$

In a previous study (Duan *et al.* 1999) it was shown that $I_{Cl,ATP}$ was inhibited by glibenclamide, Rp-cAMP (a specific PKA inhibitor) and bisindolylmaleimide (BIM; a specific PKC inhibitor) in mouse ventricular myocytes. These are all properties characteristic of CFTR Cl^- channels (Gadsby & Nairn, 1999); however, the observed inhibition by DIDS is inconsistent with a role of CFTR channels being responsible for $I_{Cl,ATP}$ (Schultz *et al.* 1999). DIDS also was found to block $I_{Cl,ATP}$ in rat ventricular cells (Kaneda *et al.* 1994). However, since it is known that DIDS and other stilbene derivatives are potent antagonists of P2 purinergic receptors (Vassort, 2001; North, 2002), it is not clear whether the observed inhibition of $I_{Cl,ATP}$ by DIDS is due to a direct inhibitory effect on the channels mediating $I_{Cl,ATP}$ or is due to antagonism of P2 purinergic receptors. In order to distinguish between these possibilities, we examined the effect of DIDS on persistently activated $I_{Cl,ATP}$ in cells dialysed with intracellular GTPγS, since these currents are maintained presumably in the absence of sustained P2 receptor stimulation. Under these conditions we found that DIDS had negligible effects on $I_{Cl,ATP}$, strongly suggesting that the inhibitory effects of DIDS on acutely activated $I_{Cl,ATP}$ are due to P2 purinergic receptor antagonism.

Though it has been reported that stimulation of P2Y purinergic receptors or extracellular ATP itself can lead to an increase in intracellular Ca^{2+} in some cells (Vassort, 2001), thus possibly activating Ca^{2+}-dependent Cl^- channels, the pipette solution used for $I_{Cl,ATP}$ recording in our experiments contained a high concentration (20 mM) of EGTA to chelate intracellular Ca^{2+}. Other types of Cl^- channels which have been shown to be activated by extracellular ATP include volume-regulated Cl^- channels ($I_{Cl,vol}$) (Perez-Samartin *et al.* 2000) and a 'novel' ATP-gated Cl^- channel (Arreola & Melvin, 2003). These are unlikely to be involved in the $I_{Cl,ATP}$ measured in our experiments. With symmetrical Cl^- solutions, it is well known that $I_{Cl,vol}$ has an outwardly rectifying *I–V* relationship (Hume *et al.* 2000; Jentsch *et al.* 2002), whereas $I_{Cl,ATP}$ in mouse ventricular myocytes and $I_{Cl,CFTR}$ exhibit linear *I–V* relationships under these conditions. Moreover, DIDS, is a potent inhibitor of both $I_{Cl,Ca}$ and $I_{Cl,vol}$. The recently described ATP-gated Cl^- channel (Arreola & Melvin, 2003) is insensitive to glibenclamide, and its EC_{50} for ATP is 158 μM. In contrast, as reported here, the EC_{50} of $I_{Cl,ATP}$ for ATP is 1.2 μM, and glibenclamide inhibited the current. These considerations make it highly unlikely that $I_{Cl,ATP}$ in mouse ventricular myocytes is generated by activation of $I_{Cl,Ca}$, $I_{Cl,vol}$ or 'novel' ATP-gated channels.

Absence of $I_{Cl,ATP}$ in cardiac myocytes from *cftr*$^{-/-}$ mice

CFTR is expressed in various mammalian cell types including heart (Hume *et al.* 2000). Analysis of CFTR knockout mouse models has been useful to identify the functional role and potential interactions of CFTR. Several CFTR knockout mouse models have been generated in which the CFTR gene has been disrupted by insertion, duplication, or an in-frame stop codon, and all of these CFTR knockout mice have a defective cAMP-mediated Cl^- conductance in the tissues which have been examined (for review see Devuyst & Guggino, 2002). In previous studies (Levesque & Hume, 1995; Duan *et al.* 1999), the possibility that $I_{Cl,ATP}$ in mouse heart might be due to activation of CFTR Cl^- channels was supported by strong similarities in electrophysiological, biophysical and pharmacological properties of $I_{Cl,ATP}$ and $I_{Cl,CFTR}$. Furthermore, unitary channels associated with $I_{Cl,ATP}$ resembled those CFTR unitary channels in terms of anion selectivity, rectification and conductance, and Northern blot analysis confirmed CFTR mRNA expression in mouse heart. The absence of detectable $I_{Cl,ATP}$ in cardiac myocytes from *cftr*$^{-/-}$ mice demonstrated in the present study provides compelling and conclusive evidence that $I_{Cl,ATP}$ in heart is mediated by CFTR.

While it is possible that extracellular ATP might be converted to adenosine by ectoenzymes, and then activate CFTR channels by increasing cAMP generation, this seems very unlikely in the present experiments because we have previously demonstrated (Levesque & Hume, 1995; Duan *et al.* 1999): (1) that $I_{Cl,ATP}$ can be activated

by extracellular application of ATPγS, an ATP analogue which is resistant to hydrolysis by ectoATPases, and (2) that extracellular application of adenosine alone fails to activate $I_{Cl,ATP}$. Future studies should reveal whether CFTR may be coupled to P2Y purinergic receptor stimulation in other tissues as well.

References

Arreola J & Melvin JE (2003). A novel chloride conductance activated by extracellular ATP in mouse parotid acinar cells. *J Physiol* **547**, 197–208.

Devuyst O & Guggino WB (2002). Chloride channels in the kidney: lessons learned from knockout animals. *Am J Physiol Renal Physiol* **283**, F1176–F1191.

Duan D, Ye L, Britton F, Miller LJ, Yamazaki J, Horowitz B & Hume JR (1999). Purinoceptor-coupled Cl^- channels in mouse heart: a novel, alternative pathway for CFTR regulation. *J Physiol* **521**, 43–56.

Dubyak GR (2003). Knock-out mice reveal tissue-specific roles of P2Y receptor subtypes in different epithelia. *Mol Pharmacol* **63**, 773–776.

Ehara T & Ishihara K (1990). Anion channels activated by adrenaline in cardiac myocytes. *Nature* **347**, 284–286.

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P & Williams M (1994). Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46**, 143–156.

Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U & Williams M (1997). Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol Sci* **18**, 79–82.

Gadsby DC & Nairn AC (1999). Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol Rev* **79**, S77–S107.

Hool LC, Oleksa LM & Harvey RD (1997). Role of G proteins in α1-adrenergic inhibition of the β-adrenergically activated chloride current in cardiac myocytes. *Mol Pharmacol* **51**, 853–860.

Hume JR, Duan D, Collier ML, Yamazaki J & Horowitz B (2000). Anion transport in heart. *Physiol Rev* **80**, 31–81.

Hwang TC, Horie M, Nairn AC & Gadsby DC (1992). Role of GTP-binding proteins in the regulation of mammalian cardiac chloride conductance. *J General Physiol* **99**, 465–489.

Inscho EW (2001). P2 receptors in regulation of renal microvascular function. *Am J Physiol Renal Physiol* **280**, F927–F944.

Iyadomi I, Hirahara K & Ehara T (1995). α-Adrenergic inhibition of the β-adrenoceptor-dependent chloride current in guinea-pig ventricular myocytes. *J Physiol* **489**, 95–104.

Jentsch TJ, Stein V, Weinreich F & Zdebik AA (2002). Molecular structure and physiological function of chloride channels. *Physiol Rev* **82**, 503–568.

Jia Y, Mathews CJ & Hanrahan JW (1997). Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. *J Biol Chem* **272**, 4978–4984.

Kaneda M, Fukui K & Doi K (1994). Activation of chloride current by P2-purinoceptors in rat ventricular myocytes. *Br J Pharmacol* **111**, 1355–1360.

Kottgen M, Loffler T, Jacobi C, Nitschke R, Pavenstadt H, Schreiber R, Frische S, Nielsen S & Leipziger J (2003). P2Y6 receptor mediates colonic NaCl secretion via differential activation of cAMP-mediated transport. *J Clin Invest* **111**, 371–379.

Lee SY & O'Grady SM (2003). Modulation of ion channel function by P2Y receptors. *Cell Biochem Biophys* **39**, 75–88.

Levesque PC & Hume JR (1995). ATP_o but not $cAMP_i$ activates a chloride conductance in mouse ventricular myocytes. *Cardiovasc Res* **29**, 336–343.

Matsubayashi T, Matsuura H & Ehara T (1999). On the mechanism of the enhancement of delayed rectifier K^+ current by extracellular ATP in guinea-pig ventricular myocytes. *Pflugers Arch* **437**, 635–642.

Matsuura H & Ehara T (1992). Activation of chloride current by purinergic stimulation in guinea pig heart cells. *Circ Res* **70**, 851–855.

Matsuura H, Tsuruhara Y, Sakaguchi M & Ehara T (1996). Enhancement of delayed rectifier K^+ current by P2-purinoceptor stimulation in guinea-pig atrial cells. *J Physiol* **490**, 647–658.

Nilius B & Droogmans G (2001). Ion channels and their functional role in vascular endothelium. *Physiol Rev* **81**, 1415–1459.

Nilius B, Oike M, Zahradnik I & Droogmans G (1994). Activation of a Cl^- current by hypotonic volume increase in human endothelial cells. *J General Physiol* **103**, 787–805.

North RA (2002). Molecular physiology of P2X receptors. *Physiol Rev* **82**, 1013–1067.

Perez-Samartin AL, Miledi R & Arellano RO (2000). Activation of volume-regulated Cl^- channels by ACh and ATP in *Xenopus* follicles. *J Physiol* **525**, 721–734.

Puceat M, Bony C, Jaconi M & Vassort G (1998). Specific activation of adenylyl cyclase V by a purinergic agonist. *FEBS Lett* **431**, 189–194.

Puceat M, Hilal-Dandan R, Strulovici B, Brunton LL & Brown JH (1994). Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J Biol Chem* **269**, 16938–16944.

Schultz BD, Singh AK, Devor DC & Bridges RJ (1999). Pharmacology of CFTR chloride channel activity. *Physiol Rev* **79**, S109–S144.

Shoda M, Hagiwara N, Kasanuki H & Hosoda S (1997). ATP-activated cationic current in rabbit sino-atrial node cells. *J Mol Cell Cardiol* **29**, 689–695.

Tominaga M, Horie M, Sasayama S & Okada Y (1995). Glibenclamide, an ATP-sensitive K^+ channel blocker, inhibits cardiac cAMP-activated Cl^- conductance. *Circ Res* **77**, 417–423.

Ugur M & Vassort G (2001). A novel nonspecific current activated by extracellular ATP in rat cardiomyocytes. *Biophys J* **80**, 586a (abstract).

Vandenberg JI, Yoshida A, Kirk K & Powell T (1994). Swelling-activated and isoprenaline-activated chloride currents in guinea pig cardiac myocytes have distinct electrophysiology and pharmacology. *J General Physiol* **104**, 997–1017.

Vassort G (2001). Adenosine 5′-triphosphate: a P2-purinergic agonist in the myocardium. *Physiol Rev* **81**, 767–806.

Yamazaki J & Hume JR (1997). Inhibitory effects of glibenclamide on cystic fibrosis transmembrane regulator, swelling-activated, and Ca^{2+}-activated Cl^- channels in mammalian cardiac myocytes. *Circ Res* **81**, 101–109.

Acknowledgements

The authors would like to thank Susan Tamowski, Paul Scowen, Linda Ye, and Phillip Keller for excellent technical assistance. This study was supported by NIH grants HL49254 and NCRR P20RR15581.

Author's present address

S. Yamamoto-Mizuma: Department of Anatomy and Physiology, Saga Medical School, 5-1-1 Nabeshima, Saga, 849–8501, Japan.