Regulation of Extracellular UTP-Activated Cl[−] Current by P2Y-PLC-PKC Signaling and ATP Hydrolysis in Mouse Ventricular Myocytes

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Abstract: The intracellular signaling pathways responsible for extracellular uridine-5'-triphosphate (UTP_o)-induced chloride (Cl[−]) currents ($I_{Cl,UTP}$) were studied in mouse ventricular myocytes with the whole-cell clamp technique. UTP_o (0.1 to 100 μM) activated a whole-cell current that showed a time-independent activation, a linear current-voltage relationship in symmetrical Cl[−] solutions, an anion selectivity of Cl[−] > iodide > aspartate, and an inhibition by a thiazolidinone-derived specific inhibitor (CFTR_{inh}-172, 10 μM) of cystic fibrosis transmembrane conductance regulator (CFTR), but not by a disulfonic stilbene derivative (DIDS, 100 μM), these properties matching those of CFTR Cl[−] channels. The potency order of nucleotides for an activation of the Cl[−] current was UTP = ATP > uridine-5'-diphosphate (UDP) = ADP. Suramin (100 μM), a P2Y receptor antagonist, strongly inhibited the UTP_o-activation of the Cl[−] current, where-

as pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 100 μM), another P2Y receptor antagonist, induced little inhibition of $I_{Cl,UTP}$. The activation of $I_{Cl,UTP}$ was sensitive to protein kinase C (PKC) inhibitor, phospholipase C (PLC) inhibitor, intracellular GDPβS (nonhydrolyzable GDP analogue) or anti-Gq/11 antibody. UTP_o failed to activate the Cl[−] current when the cells were dialyzed with nonhydrolyzable ATP analogues (ATPS or AMP-PNP) without ATP, suggesting that ATP hydrolysis is a prerequisite for the current activation. $I_{Cl,UTP}$ was persistently activated with a mixture of ATP_γS + ATP in the pipette, suggesting the involvement of phosphorylation reaction in the current activation process. Our results strongly suggest that $I_{Cl,UTP}$ is due to the activation of CFTR Cl[−] channels through Gq/11-coupled P2Y₂ receptor-PLC-PKC signaling and ATP hydrolysis in mouse heart.

Key words: Cl[−] current, ventricular cell, P2Y receptor, CFTR, ATP hydrolysis.

Extracellular nucleotides (purines and pyrimidines) are continually present in quite variable amounts in the extracellular space of the heart [1]. It is well known that the concentration of purines such as ATP is increased by hypoxia, ischemia, increased blood flow, and mechanical stretch [1–3]. The released purines, as paracrine or autocrine mediators, may regulate cardiac contraction, intracellular free-Ca²⁺ concentration, and cardiac ion channels [4] probably via cell-surface receptor families termed purine (or purinergic) receptors, which consist of a group of ligand-gated ion channels and G protein-coupled receptors, termed P2X and P2Y receptor families, respectively [2–7]. Pyrimidines such as uridine-5'-triphosphate (UTP) have been shown to be released from cardiac cells under certain pathophysiological conditions [8–10]. However, only a few studies have focused on the cardiac effect of UTP.

With respect to the regulation of cardiac chloride (Cl[−]) channels by purines, it has been reported that extracellular ATP (ATP_o) activates Cl[−] currents ($I_{Cl,ATP}$) in guinea pig atrial cells [11] and ventricular cells from rat [12] and mouse [13–15] hearts. Yamamoto-Mizuma *et al.* [15]

showed that $I_{Cl,ATP}$ is regulated by pertussis toxin (a selective Gi-protein inhibitor)-independent and GDPS (a nonhydrolyzable GDP analogue)-sensitive intracellular signal pathways and is inhibited by suramin (a purinergic receptor antagonist). Further, the several properties [current-voltage (I – V) relationships, anion permeability, effect of Cl[−] channel blockers, and single channel conductance] of $I_{Cl,ATP}$ were similar to those of cystic fibrosis transmembrane conductance regulator (CFTR) Cl[−] channels [14, 15]. These results suggest that ATP_o activates CFTR Cl[−] current via P2Y receptors in mouse ventricular cells. In line with this, an RT-PCR study clearly confirmed an expression of the homologue of CFTR in mouse heart [14], and ATP_o failed to activate any detectable Cl[−] currents in ventricular cells of *cfr* null mice [15]. It should be noted, however, that several protein kinase A (PKA) activators (isoprenaline, forskolin, and isobutyl methylxanthine), which are known to activate CFTR Cl[−] current in cardiac myocytes of many species, cannot activate any detectable Cl[−] current in mouse heart [14, 16]. It is interesting that the stimulation of endogenous protein kinase C (PKC) with phorbol 12,13-dibutyrate (PDBu) activated

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CFTR-like Cl^- current in mouse cardiac myocytes [14, 17].

The subtype of P2Y receptor involved in the activation of CFTR Cl^- current is not clear. It has been reported that extracellular UTP (UTP_o) modulates some cationic currents (ATP-sensitive potassium current [18], sodium current [19], and muscarinic potassium channels [20]) in heart, but the regulation of cardiac Cl^- current by UTP_o is still unknown.

In the heart, studies with the RT-PCR method have revealed expressions of $\text{P2Y}_{1,2,4,6}$ receptors in neonatal rat [21] and of $\text{P2Y}_{1,2,4,6,11,12,13,14}$ receptors in human [22]. For mouse cardiomyocytes, recent studies [23, 24] suggest that at least $\text{P2Y}_{1,2,4,6,11}$ receptors are functionally expressed in these cells. In pharmacological studies, the major agonists of the cloned human P2Y receptors are ADP (P2Y_1), UTP/ATP (P2Y_2), UTP (P2Y_4), UDP (P2Y_6), ATP (P2Y_{11}), 2-MeSADP (P2Y_{12}), 2-MeSADP (P2Y_{13}), and UDP-glucose (P2Y_{14}) [2, 7]. The rat P2Y_4 receptor is activated by both ATP and UTP [7]. As with the antagonism, subtype-selective P2 receptor antagonists are not known. However, it has been reported that suramin, a popular P2 receptor antagonist, effectively counteracts $\text{P2Y}_{1,2,11,12,13}$ receptors, but counteracts $\text{P2Y}_{4,6}$ receptors only weakly [7]. Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) is a popular nonselective P2X receptor antagonist and inhibits P2Y_1 receptor potently, $\text{P2Y}_{4,6,13}$ receptors moderately, and $\text{P2Y}_{2,11,12}$ receptors only weakly [7]. Thus pharmacological studies with various receptor agonists and antagonists would serve to identify the subtype of P2Y receptors.

On the other hand, the intracellular signaling reaction triggered by receptor stimulation may be different among the P2Y receptor subtypes. Based on the functional coupling to particular G proteins and effector proteins, the cloned P2Y receptors can broadly be categorized into a family of Gq/11-coupled receptors ($\text{P2Y}_{1,2,4,6,11}$) that cause both an activation of protein kinase C (PKC) and Ca^{2+} mobilization through the activation of phospholipase C (PLC), and a family of Gi-coupled receptors ($\text{P2Y}_{12,13,14}$) that inhibit adenylyl cyclase-protein kinase A (PKA) signaling [2, 4, 6]. It should be noted that P2Y_{11} receptor can be coupled to Gs and adenylyl cyclase activation [23, 25].

In the present study, we examined extracellular UTP-activated Cl^- current ($I_{\text{Cl,UTP}}$) in mouse ventricular cells to identify the subtype of P2Y receptor and intracellular signaling pathway involved by using electrophysiological and pharmacological techniques. Our results strongly suggest that the cardiac $I_{\text{Cl,UTP}}$ is due to an activation of CFTR Cl^- channels via the P2Y_2 receptor-Gq/11-PLC-PKC signaling pathway and ATP hydrolysis. A preliminary account of this work has appeared in abstract form [26].

MATERIAL AND METHODS

Cell preparation. The Saga University Animal Care and Use Committee approved the use and treatment of all animals used in the experiments described here. The investigation confirms also with the Guiding Principles of the Physiological Society of Japan. Single ventricular myocytes from mouse hearts were isolated by using an enzymatic dispersion technique as previously described [15]. Briefly, mice (18–25 g, C-57BL/6J/black inbred, male) were anaesthetized with sodium pentobarbitone (50 mg/ml, 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 to wash out blood, then with a nominally Ca^{2+} -free PSS until the heart ceased to beat, and finally with the Ca^{2+} -free solution containing 0.1% collagenase (CLSII, Worthington, Lakewood, NJ, USA) and 1.0% bovine serum albumin (BSA) for 20–30 min. The ventricles were removed and cell dissociation was achieved by gentle mechanical agitation of the tissue in high- K^+ , low- Cl^- storage (modified KB) solution (see “Solutions and drugs”), and the dissociated cells were stored in a refrigerator (4°C) for later use (within 8 h). Only rod-shaped myocytes with clear cross-striations and no blebs were used in the experiments.

Electrophysiological techniques. The tight-seal whole-cell patch-clamp technique was used to record whole-cell currents. Patch pipettes (1.5 mm O.D. borosilicate glass electrodes) had a tip resistance of 1–3 M Ω when filled with pipette solution. Voltage-clamp recordings were performed using patch-clamp amplifiers (Model TM-1000; ACT ME, Tokyo, Japan), and membrane currents were filtered at a frequency of 2.5 kHz and sampled at 5 kHz with a Digidata 1322A and pCLAMP 9.0 software (Axon Instruments, Foster City, CA, USA). A 3 M KCl-agar bridge between the bath and the Ag-AgCl reference electrode was used to minimize changes in liquid junction potential. Unless otherwise stated, current recordings were made by applying voltage pulses of 400 ms duration to various potentials (from –100 to +100 mV in +20 mV steps) from a holding potential of 0 mV every 2 s. When necessary, the current density was calculated by membrane capacitance, which was obtained using pCLAMP 9.0 software. Usually, 5 min was allowed for adequate cell dialysis after membrane rupture before the beginning of the voltage clamp protocol. All experiments were performed at room temperature, as previously described [15].

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 NaHPO_4 , 10 HEPES; pH 7.4 adjust with NaOH; 300 mOsm with mannitol. Ca^{2+} -free PSS was prepared by simply omitting CaCl_2 from the PSS. The modified KB solution for cell storage contained (mM): 70 potassium

glutamate, 20 KCl, 1.0 MgCl₂, 10 KH₂PO₄, 10 taurine, 10 EGTA, 10 glucose, 0.1% albumin, 10 β -hydroxybutyric acid, and 10 Hepes; pH 7.2 with KOH; 300 mOsm with mannitol. For $I_{Cl(P2Y)}$ recording, the extracellular and intracellular solutions were chosen to maximize the recording of Cl⁻ currents and to reduce possible contamination with cation currents and Ca²⁺-dependent currents. The standard extracellular solutions contained (mM): 77 NaCl, 0.8 MgCl₂, 1.0 CaCl₂, 5.0 CsCl, 2.0 BaCl₂, 0.2 CdCl₂, 5.5 glucose, 10 Hepes, 0.01 nifedipine; pH 7.4 adjusted with NaOH; total [Cl⁻]_o = 90 mM; 310 mOsm with mannitol. In some experiments, extracellular NaCl was replaced by an equimolar concentration (77 mM) of sodium iodide or sodium aspartate. The standard intracellular pipette solution contained (mM) 140 NMDG, 90 HCl, 5 MgATP, 0.1 Tris-GTP, 20 EGTA, 10 Hepes; pH 7.3 adjusted with NMDG; total [Cl⁻]_i = 90 mM; 290 mOsm using mannitol. In some other experiments, MgATP was replaced with an equimolar amount (5 mM) of adenosine 5'-(β,γ -imido)triphosphate tetralithium salt hydrate (AMP-PNP; Sigma, St. Louis, MO, USA) or adenosine 5'-[γ -thio]triphosphate tetralithium salt (ATPS; Sigma). In others, 0.1 mM Tris-GTP was replaced with 0.1 mM guanosine 5'-[γ -thio]triphosphate (GTP γ S; Sigma) or 1.0 mM guanosine 5'-[β -thio]diphosphate (GDP β S; Sigma). Pipette solutions containing 10 mM MgATP, or 5 MgATP + 5 mM AMP-PNP, or 5 mM MgATP + 5 mM ATP γ S were also prepared. The osmolality of all solutions was measured by using freezing point depression osmometers (Model OM-801; Vogel, Giessen, Germany).

The chemicals used were glibenclamide (Sigma), 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB; Sigma), diphenylamine-2-carboxylate (DPC; Sigma), 5-[4-Carboxyphenyl]methylene-2-thioxo-3-[(3-trifluoromethyl)phenyl]-4-thiazolidinone (CFTR_{inh}-172; Sigma), 4,4'-diisothiocyanostilben-2,2'-disulphonate (DIDS; Sigma), suramin (Sigma), pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS; Sigma), phorbol 12,13-dibutyrate (PDBu; Sigma), 4 α -phorbol 12,13-didecanoate (4 α PDD; Sigma), 1-(6-[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino) hexyl-1H-pyrrole-2,5-dione (U-73122; Sigma), 1-(6-[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino) hexyl-2,5-pyrrolidine-dione (U-73343; Calbiochem), bisindolylmaleimide-I (BIM-I; Sigma), and bisindolylmaleimide-V (BIM-V; Sigma). Extracellular nucleotides used were adenosine 5'-triphosphate disodium salt, (ATP; Sigma), uridine 5'-triphosphate disodium salt (UTP; Sigma), adenosine 5'-diphosphate sodium salt (ADP; Sigma), uridine 5'-diphosphate sodium salt (UDP; Sigma), and guanosine 5'-triphosphate tris salt (GTP; Sigma). Intracellular dialysis with anti-Gq/11 antibody has been described previously [27]. In brief, affinity-purified polyclonal antibody directed against 19 amino acids within the extreme carboxyl termini of Gq/11 (sc-392) was purchased from Santa Cruz Biotechnology (San-

ta Cruz, CA, USA). The cells were dialyzed for ~10 min with a pipette solution containing a 1:100 dilution (2 μ g ml⁻¹) of anti-Gq/11 antibody. Heat-inactivated anti-Gq/11 antibody (100°C for 20 min) was prepared as control. The osmolality of the pipette solution was not altered by the inclusion of the antibody.

Data analysis. Permeability ratios (Fig. 2C) were calculated from the shifts in E_{rev} according to the modified Goldman-Hodgkin-Katz equation: $P_x P_{Cl}^{-1} = ([Cl^-]_c \exp[-\Delta E_{rev} F / RT] - [Cl^-]_s) [X^-]_s^{-1}$, where ΔE_{rev} is the shift of reversal potential, $[Cl^-]_c$ and $[Cl^-]_s$ are the extracellular Cl⁻ concentrations in the control (standard) and anion-substituted solutions, respectively, and $[X^-]_s$ is the concentration of the substituting anion X⁻. F/RT is the usual thermodynamic parameter. Concentration-response curves (Fig. 3A) to extracellular ATP or UTP were analyzed by using a Hill equation: $\text{response} = 1/(1 + [EC50/[A]]^{nH})$, where $[A]$ is the agonist concentration, EC50 is the agonist concentration to achieve 50% of the maximum response, and nH is the Hill coefficient. The currents were normalized to the maximum difference current in each tested cell. The data are expressed as mean $\pm$ SEM; n indicates the number of cells. Statistical comparisons were performed either by one-way ANOVA with a post-hoc test (Scheffé's multiple comparison test) for group data, or by Student's t test when only two groups were compared. A two-tailed probability of <0.05 is taken to indicate statistical significance.

RESULTS

Figure 1A shows the time course of extracellular ATP (ATP_o) or UTP (UTP_o)-induced whole-cell currents observed at +80 mV (solid circles) and -80 mV (open circles) in a mouse ventricular cell. The outward current at +80 mV began to increase after the application of 100 μ M ATP_o and reached an almost steady level within ~3 min. The inward current at -80 mV also increased after ATP_o, but the increase appeared to occur with little delay. In this experiment, ATP_o was replaced with UTP_o (100 M) when the ATP_o-induced currents reached a steady level. This agonist replacement had little effect on the outward current at +80 mV, whereas it resulted in a decrease of the inward current at -80 mV. The average magnitude of outward and inward currents observed after UTP_o in 6 cells was $99.8 \pm 2.7\%$ and $60.3 \pm 9.4\%$ of the magnitude of ATP_o-induced outward and inward currents, respectively. Similar results were obtained when the order of agonist application was reversed (data not shown).

Figure 1B shows the whole cell currents elicited by various voltage pulses obtained during the course of the experiment shown in Fig. 1A. The mean $I-V$ relationship of the different currents between the current observed after UTP and that in control and the mean $I-V$ relationship of the current component, which was sensitive to the ago-

Fig. 1. Extracellular ATP- or UTP-induced membrane currents in mouse ventricular myocytes. **A**, the time course of extracellular ATP- or UTP-induced whole-cell currents at +80 mV (solid circles) and -80 mV (open circles) in a mouse ventricular cell exposed to ATP and UTP in standard extracellular solution. Either 100 μ M ATP or 100 μ M UTP was applied to the cell during the period indicated by a bar. Here and in subsequent similar figures, the pulse protocol is the same as shown in the inset of **A**. **B**, whole-cell currents induced by applying 400 ms voltage-clamp steps to membrane potentials from -100 mV to +100 mV in +20 mV steps from a holding potential of 0 mV every 2 s. Currents were recorded at the points of a (control), b (ATP), and c (UTP) indicated in **A**. In subsequent similar figures, the step-pulse protocol is the same as that shown in the inset of **B**. **C** shows the mean I - V relationship (c-a) of the difference current between the current observed after UTP and that in control ($n = 6$). The mean I - V relationship (b-c) of the current fraction representing the current decrease observed on the agonist replacement from ATP to UTP is also shown ($n = 6$).

Fig. 2. Anion selectivity of extracellular UTP-induced currents in mouse ventricular myocytes. **A**, the time course of changes in whole-cell currents during the application of 100 μ M UTP (bar), as affected by switching the bathing solution from NaCl-rich (NaCl) to Na-aspartate-rich (Na-Asp) solution. The solution was changed as indicated by a bar. The Na-aspartate-rich solution was prepared by replacing 77 mM of 90 mM NaCl in standard (NaCl-rich) solution with an equimolar amount of Na-aspartate. **B**, the mean I - V relationships of UTP-induced currents (difference currents, e.g., b-a and c-a in **A**) obtained in NaCl-, NaI-, and Na-aspartate-rich solutions in 8, 4, and 4 cells, respectively. NaI-rich and Na-aspartate-rich solutions were prepared in a way similar to the above (**A**).

nist replacement from ATP to UTP, are shown in Fig. 1C. The latter (b-c) exhibited inward rectification with a reversal potential (E_{rev}) of positive voltage, E_{rev} being more positive than +100 mV in all tested cells ($n = 6$). In contrast, the I - V relationship of the "UTP_o-induced current" (c-a), like that of the ATP_o-induced Cl⁻ current ($I_{\text{Cl,ATP}}$) [15], was almost linear. The mean E_{rev} of the UTP_o-induced current was -1.7 ± 4.4 mV, of which value being close to the predicted Cl⁻ equilibrium potential ($E_{\text{Cl}} = 0$ mV under symmetrical Cl⁻ conditions). These findings confirm the previous results [15], which showed that ATP_o activated both a nonselective cation current and a Cl⁻ current, and they further suggest that UTP_o activates the latter almost exclusively in mouse cardiac cells. We attempted to further characterize the UTP_o-induced current in the following way.

We confirmed the Cl^- -selectivity of the UTP_o -induced current. Figure 2A shows the effect of aspartate-rich solution with reduced $[\text{Cl}^-]_o$ on the UTP-induced current. When the bath solution was changed from high- $[\text{Cl}^-]_o$ (90 mM) to low- $[\text{Cl}^-]_o$ (13 mM Cl^- + 77 mM aspartate) solution, the outward component of the UTP_o -induced current decreased markedly (Fig. 2A). Mean I - V relationships for the UTP_o -induced current at high and low $[\text{Cl}^-]_o$ obtained in several of the experiments are shown in Fig. 2B (solid circles and open triangles). There was a positive shift of E_{rev} with a reduction of $[\text{Cl}^-]_o$, and E_{rev} at low $[\text{Cl}^-]_o$ was $+45 \pm 5$ mV ($n = 4$), the value being close to E_{Cl} ($+49.6$ mV) predicted for the low $[\text{Cl}^-]_o$ condition. These findings support the view that UTP_o , like ATP_o [13, 15], activates the Cl^- current in mouse ventricular cells. The inward rectification of the UTP_o -induced current in aspartate-rich bath solution (Fig. 2B, open triangles) can be explained by assuming that extracellular aspartate ions little or only slightly flow inwardly, but that intracellular Cl^- ions substantially flow outwardly, generating a sizable inward current in this solution.

We examined also the UTP_o -induced Cl^- current ($I_{\text{Cl,UTP}}$) in I^- -rich, Cl^- -deficient bath solution (77 mM I^- + 13 mM Cl^-). Its current-voltage (I - V) relationship is shown in Fig. 2B (open squares). E_{rev} was $+20 \pm 6$ mV in this case. The E_{rev} values obtained in Cl^- -, aspartate-, and I^- -rich solutions were used to calculate the permeability ratio of these anions (permeability ratio of ion X^- with respect to Cl^- , P_X/P_{Cl}) according to a modified Goldman-Hodgkin-Katz equation. In this calculation, $P_{\text{I}}/P_{\text{Cl}}$ and $P_{\text{Asp}}/P_{\text{Cl}}$ were 0.47 and 0.14, respectively. The anion selectivity of the current, $\text{Cl}^- > \text{iodide} > \text{aspartate}$, is similar to that of PKA-activated CFTR Cl^- current [28] in cardiac myocytes of many species and that of $I_{\text{Cl,ATP}}$ [15] in mouse ventricular cells. This finding, together with the observation that both $I_{\text{Cl,ATP}}$ and $I_{\text{Cl,UTP}}$ exhibit similar current characteristics (Fig. 1), suggest that both ATP_o and UTP_o quite likely activate the same Cl^- current. Since ATP_o is thought to activate CFTR Cl^- current through a stimulation of P2Y receptors in mouse ventricular cells [14, 15], UTP_o was considered to activate CFTR Cl^- current in these cells.

The above view was further supported by the following experiments in which the effects of various Cl^- channel inhibitors on $I_{\text{Cl,UTP}}$ were examined. To see the direct effect of the inhibitors on the Cl^- channel in these experiments, it was important to exclude the possible interaction of the inhibitors on the receptors. Especially, it is noteworthy that DIDS and other stilbene derivatives, which are popular Cl^- transport inhibitors, do not affect CFTR Cl^- channels [29], but potentially antagonize P2 purinergic receptors [1, 28, 30]. Therefore we attempted to obtain a receptor-independent Cl^- current, using $\text{GTP}\gamma\text{S}$ (a nonhydrolyzable GTP analogue)-loaded cells. Since GTP hydrolysis is essential to terminate the signal transduction

Fig. 3. Effects of Cl^- channel inhibitors on $I_{\text{Cl,UTP}}$. The pipette solution contained 0.1 mM $\text{GTP}\gamma\text{S}$ to give a Cl^- current persisting after the removal of UTP. **A**, a representative I - V relationship of the whole-cell current obtained in control (open circles), after an application of UTP [solid circles; UTP (+)], 10 min after a washout of the UTP [open squares; UTP (-)] and after a further application of 10 μM CFTR_{inh}-172 (solid squares). **B**, a summary of the inhibitory effect of Cl^- channel blockers on persistently activated $\Delta I_{\text{Cl,UTP}}$ in $\text{GTP}\gamma\text{S}$ -loaded cells. We examined 50 μM glibenclamide (Glib, $n = 5$), 500 μM DPC ($n = 4$), 100 μM NPPB ($n = 4$), 10 μM CFTR_{inh}-172 ($n = 5$), and 100 μM DIDS ($n = 4$). $\Delta I_{\text{drug}}/\Delta I_{\text{UTP}}$, which represents the ratio of inhibitor-sensitive current components (ΔI_{drug}) to control ΔI_{UTP} was determined at +100 and -100 mV. The data are expressed as mean $\pm$ SEM.

processes linking receptor occupation and activation of effector protein, once the effector protein is activated by an agonist, the activation persists after the removal of the agonist in $\text{GTP}\gamma\text{S}$ -loaded cells. Indeed, it has been shown that in such cells $I_{\text{Cl,ATP}}$ once activated by ATP_o , persists even after the removal of ATP_o [15]. In the present study, similar persistent $I_{\text{Cl,UTP}}$ could be observed, and we examined the effects of Cl^- channel inhibitors on the $I_{\text{Cl,UTP}}$ in the absence of UTP_o .

Figure 3A shows the effect of CFTR_{inh}-172 (a thiazolidinone-derived selective CFTR Cl^- channel inhibitor [31]) on the persistent $I_{\text{Cl,UTP}}$. $I_{\text{Cl,UTP}}$ was almost completely inhibited by 10 μM CFTR_{inh}-172 in a voltage-independent manner. The inhibitory effect developed quickly (within ~ 3 min) and was reversible ($n = 4$). Figure 3B summarizes the effects of several inhibitors on $I_{\text{Cl,UTP}}$. Fifty micromo-

Fig. 4. Dependence of cardiac Cl^- current on agonists and antagonists of purinergic receptors. **A**, the dose-response relationships of extracellular ATP- or UTP-induced (difference) currents. Each point represents the mean of the data obtained in 4 different cells. For each curve, responses were normalized to the maximum response obtained at a maximal agonist concentration ($100 \mu M$). The EC₅₀ (concentration for half-maximal activation) and nH (Hill coefficient) values were obtained by fitting the curve to the Hill equation. **B**, the mean current density of the Cl^- current at +100 mV obtained with a maximal concentration ($100 \mu M$) of various agonists (ATP, $n = 43$; UTP, $n = 18$; ADP, $n = 4$; UDP, $n = 4$; GTP, $n = 4$). The symbols * and **, significantly smaller than the value for ATP with $P < 0.05$ and 0.01 , respectively. **C**, the mean current density of UTP-induced (difference) current obtained at +100 mV in the presence and absence of $100 \mu M$ suramin ($n = 4$) or $100 \mu M$ PPADS ($n = 4$). The symbol *, significant ($P < 0.05$) reduction of the current after suramin. n.s., not significantly different.

lar glibenclamide, $500 \mu M$ DPC, and $100 \mu M$ NPPB inhibited $I_{Cl,UTP}$ in a voltage-independent manner, whereas $I_{Cl,UTP}$ was only slightly sensitive to $100 \mu M$ DIDS. These results support the view that UTP_o activates CFTR Cl^- current in mouse ventricular cells.

We compared the potency of ATP_o and UTP_o for an activation of the Cl^- current. Figure 4A shows the dose-response relationship for each agonist to activate the Cl^- current, where the magnitude of the Cl^- current activated at +100 mV in standard bath solution was evaluated at various agonist concentrations. By fitting the curve to a Hill equation, the values of EC₅₀ (concentration for half-maximal activation) and nH (Hill coefficient) were calculated. EC₅₀ and nH were $1.6 \mu M$ and 0.98 for ATP_o, and $1.1 \mu M$ and 1.14 for UTP_o, indicating that ATP_o and UTP_o had an almost equal potency for activation of the Cl^- current. The similar dose-response relationship of ATP_o and UTP_o for the activation of Cl^- current is in line with the view that these agonists stimulate the receptor of the same type to activate the same Cl^- current. The agonist potency was examined also for other nucleotides (ADP, UDP, and GTP). In the graph shown in Fig. 4B, the magnitude of the Cl^- current at +100 mV activated by $100 \mu M$ of various agonists is compared. The data suggest that the potency order for activation of the Cl^- current is UTP = ATP > UDP = ADP.

According to the previous studies on P2Y receptor subtypes [7], the potency order of agonists for the activation of Cl^- current (Fig. 4B) appears to coincide with the property of P2Y₂ and P2Y₄ receptors. To further identify the P2Y receptor subtype involved, we examined the effects of P2Y receptor-antagonist on the activation of $I_{Cl,UTP}$. An earlier report [15] showed that the activation of cardiac $I_{Cl,ATP}$ was inhibited by suramin (a popular P2Y receptor antagonist). We attempted to see the effects of suramin as well as another P2Y antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), on $I_{Cl,UTP}$. Figure 4C summarizes the results. Suramin ($100 \mu M$) strongly inhibited $I_{Cl,UTP}$, whereas PPADS ($100 \mu M$) exerted little effect on the $I_{Cl,UTP}$. The magnitude of $I_{Cl,UTP}$ at +100 mV observed after antagonists was $27.1 \pm 8.8\%$ ($n = 4$) and $92.9 \pm 4.1\%$ ($n = 4$) of control for suramin and PPADS, respectively. It has been shown that PPADS can only weakly antagonize P2Y_{2,11,12} receptors, though it substantially inhibits other P2Y receptor subtypes [7]. Thus if the data for the agonist potency (Fig. 4B) and the ineffectiveness of PPADS on $I_{Cl,UTP}$ (Fig. 4C) are taken into consideration together, our data appear to suggest the involvement of the P2Y₂ receptor in the activation of $I_{Cl,UTP}$ in mouse heart.

Most P2Y receptor subtypes expressed in the heart may be coupled to Gq/11 protein and may regulate the production of PKC via an activation of phospholipase C (PLC) [1]. Although previous reports [14, 15] showed that an activation of $I_{Cl,ATP}$ involves PTX (a selective Gi protein inhibitor)-insensitive G protein and that $I_{Cl,ATP}$ is regulated

Fig. 5. Role of PLC and PKC in the activation of I_{CLUTP} . **A**, the original current tracings showing the effects of UTP (100 μM) and PDBu (100 nM) on the whole-cell currents in the presence of U-73122 (5 μM). Drugs were applied to the cell consecutively as indicated by a bar. **B**, a summary of the data showing the effect of UTP (100 μM) and PDBu (100 nM) on the membrane currents in the presence of U-73122 (5 μM, left) or U-73343 (5 μM, right). The effects of 4αPDD (100 μM) with U-73122 is also shown. **C**, The density of UTP (100 μM)-induced currents obtained in the cells that were pretreated for 10 min with BIM-I (100 nM, left) or BIM-V (100 nM, right). In B and C, the data represent the density of the currents activated by agonists (difference currents) at +100 mV. ** at $P < 0.01$. n.s., not significantly different.

by PKC, detailed signaling pathways linking purinergic stimulation to PKC activation are unknown. To clarify whether the process of Gq/11 protein-PLC-PKC signaling is responsible for an activation of I_{CLUTP} , we examined the effects of BIM-I (a specific PKC inhibitor), U-73122 (a specific PLC inhibitor), GDPS (nonhydrolyzable GDP analogue) and anti-Gq/11 antibody on the activation of I_{CLUTP} .

When the cell was pretreated with U-73122, 100 μM UTP_o activated I_{CLUTP} only a small amount or slightly

Fig. 6. Involvement of Gq/11 protein in the activation of I_{CLUTP} . **A**, the original current tracings showing the effects of UTP (100 μM) and PDBu (100 nM) on the whole-cell currents in a cell dialyzed with pipette solution containing anti-Gq/11 antibody (2 mg ml⁻¹). The drugs were applied to the cell consecutively as indicated by a bar. The records (a) and (b), which were obtained, respectively, 1 and 10 min after membrane rupture, show that the basal currents were not changed by intracellular dialysis of anti-Gq/11 antibody. **B**, the summary of the data showing the effects of UTP (100 μM) and PDBu (100 nM) on the membrane currents in cells dialyzed with anti-Gq/11 antibody (2 mg ml⁻¹, left) or heat-inactivated anti-Gq/11 antibody (2 mg ml⁻¹, right). The data represent the density of the currents activated by agonists (difference currents) at +100 mV. The symbol **, significant ($P < 0.01$) increase in the current after PDBu. n.s., not significantly different.

(Fig. 5Ac). The application of 100 nM PDBu, an agent that activates PKC without involving PLC, could induce I_{CLUTP} of large amplitude in the presence of 5 μM U-73122, as expected (Fig. 5Ad). Figure 5B summarizes the results of the experiments in which the effects of UTP_o, PDBu, and 4αPDD (inactive analogue of PDBu) on the whole cell current were examined in the presence of U-73122 or U-73343 (inactive analogue of U-73122). In the presence of 5 μM U-73122 ($n = 4$), 100 μM UTP_o or UTP_o + 100 nM 4PDD induced a current of 0.5 ± 0.2 or 0.4 ± 0.3 pA pF⁻¹, respectively, whereas UTP_o + 100 nM PDBu induced a current of 3.2 ± 0.8 pA pF⁻¹. In the presence of 5 μM U-73343 ($n = 4$), UTP_o or UTP_o + PDBu induced a current whose amplitude (3.8 ± 0.5 or 4.0 ± 0.5 pA pF⁻¹, respectively) was comparable to that of I_{CLUTP} in the absence of the PLC inhibitor (Fig. 4). When the cell was

pretreated with BIM-I (100 nM) for 10 min (Fig. 5C), 100 μM UTP_o activated I_{CLUTP} only slightly ($n = 6$), whereas 100 μM UTP_o induced a current of $3.1 \pm 0.4 \text{ pA pF}^{-1}$ in the presence of 100 nM BIM-V (inactive analogue of BIM-I, $n = 4$).

In Fig. 6, we examined the effect of anti-Gq/11 antibody on the activation of I_{CLUTP} . Our antibody was an affinity-purified polyclonal antibody directed against 19 amino acids within the extreme carboxyl termini of Gq/11, and the inhibitory effect of its intracellular application on the Gq/11-dependent system has been described previously [27]. As shown in Fig. 6A, the intracellular dialysis with anti-Gq/11 antibody itself appeared to have little effect on the whole cell current (b). The application of 100 μM UTP_o activated I_{CLUTP} only slightly in this condition (c). Again, 100 nM PDBu could activate I_{CLUTP} of large amplitude (d). Figure 6B summarizes the results of the experiments in which the effects of UTP_o and PDBu on the whole cell current were examined in the presence of the intracellular antibody or heat-inactivated antibody. With the raw antibody ($n = 4$), 100 μM UTP_o induced a current of $1.8 \pm 0.6 \text{ pA pF}^{-1}$, whereas $\text{UTP}_o + 100 \text{ nM PDBu}$ induced a current of $3.7 \pm 0.7 \text{ pA pF}^{-1}$. In the cells with the inactivated antibody ($n = 4$), UTP_o or $\text{UTP}_o + \text{PDBu}$ induced a current whose amplitude (4.1 ± 0.8 or $4.2 \pm 1.0 \text{ pA pF}^{-1}$, respectively) was comparable to that of control I_{CLUTP} (Fig. 4). We confirmed that the activation of I_{CLUTP} was almost completely abolished in ventricular cells ($n = 5$) dialyzed with 1 mM GDPS (data not shown). These results suggest that UTP_o activates the Cl^- current via Gq/11-coupled P2Y_2 receptors linked to the PLC-PKC signaling pathway in mouse ventricular cells.

PKC may mediate protein phosphorylation as a final step in the process of I_{CLUTP} activation. It has been shown that the activation of CFTR Cl^- channels requires both ATP hydrolysis and phosphorylation reactions [32]. We tested whether the activation of I_{CLUTP} also involves such processes by using hydrolysis-resistant ATP analogues (ATP γ S and AMP-PNP). We prepared pipette solutions of five types, i.e., those containing 5 mM ATP γ S or 5 mM AMP-PNP without ATP, and those containing 10 mM ATP or 5 mM ATP + 5 mM ATP γ S or 5 mM ATP + 5 mM AMP-PNP.

When the cells were dialyzed with pipette solutions containing only ATP γ S or AMP-PNP, an application of UTP_o (100 μM) could induce little or no Cl^- current in these cells (data not shown), suggesting that ATP hydrolysis is a necessary event for the activation of I_{CLUTP} . When the pipette solution containing ATPS + ATP was used, the application of UTP_o (100 μM) induced the Cl^- current (Fig. 7A), as in the cells dialyzed with ATP alone. However, the deactivation of I_{CLUTP} after the withdrawal of UTP_o was very slow in this case (Fig. 7A). The magnitude of I_{CLUTP} at +100 mV was $6.6 \pm 0.7 \text{ pA pF}^{-1}$ ($n = 4$) in the presence of UTP_o , and it was $5.6 \pm 0.8 \text{ pA pF}^{-1}$ at 15 min

Fig. 7. The modulation of I_{CLUTP} activation by intracellular poorly hydrolyzable ATP analogues. **A**, original current tracings showing the effects of UTP (100 μM) on the whole-cell currents in a cell dialyzed with pipette solution containing 5 mM ATP and 5 mM ATP γ S. (a), control; (b), after application of 100 μM UTP . Records obtained 5 and 15 min after the withdrawal of UTP are also shown (c and d). **B**, similar to A, but the cell was dialyzed with a pipette solution containing 5 mM ATP and 5 mM AMP-PNP. **C**, a summary of the data showing the magnitude of UTP -induced currents (white columns) and the rate of current deactivation after agonist withdrawal (hatched columns) observed in cells dialyzed with a pipette solution containing 5 mM ATP + 5 mM ATP (ATP, $n = 4$), 5 mM ATP + 5 mM ATP γ S (ATPS, $n = 4$), or 5 mM ATP + 5 mM AMP-PNP (AMP-PNP, $n = 4$). The data represent the density of the currents activated by UTP (difference currents) at +100 mV. The symbol *, significantly smaller than the control UTP -induced current (ATP) at $P < 0.05$. The symbols ¶ and ¶¶, significantly smaller than the control UTP -induced current (AMP-PNP) at $P < 0.05$ and 0.01, respectively. n.s., not significantly different from the control UTP -induced current (+ATP γ S).

after the agonist removal (Fig. 7C). This contrasted with the observation that I_{CLUTP} activated in the cells dialyzed only with ATP disappeared almost completely within 5

min after agonist removal (Figs. 1A, 2A, and 5C). The slow deactivation may be explained as follows. Since ATP γ S can be a phosphate donor in phosphorylation reaction, and since thiophosphoprotein, a product of phosphorylation with -thiophosphate, is poorly hydrolyzable, phosphorylation, once induced with ATP γ S would persist after a withdrawal of stimulation, though phosphorylation induced with ATP would subside rapidly. Our findings suggest that a phosphorylation step is present in the activation of $I_{Cl,UTP}$.

On the other hand, when the pipette solution contained AMP-PNP + ATP, the deactivation of $I_{Cl,UTP}$ was also slow, though its rate of deactivation was faster than that observed with ATP γ S (Fig. 7B). The magnitude of $I_{Cl,UTP}$ still remaining 5 min after agonist removal was 3.5 ± 0.3 pA pF $^{-1}$ ($n = 4$), of which the value was about 50% of that seen before agonist removal (6.7 ± 0.7 pA pF $^{-1}$; Fig. 6C). Since AMP-PNP may not act as a phosphate donor, the slow current deactivation after this agent cannot be explained by a slow dephosphorylation. We consider the phenomenon observed here to be similar to the persistent opening of CFTR channels produced specifically with AMP-PNP [33]. To explain this sustained channel opening, a specific behavior of nucleotide-binding domains (NBDs) included in the channel protein has been proposed (see DISCUSSION).

DISCUSSION

The major findings of the present investigation include (i) a demonstration that UTP $_o$ activates a Cl $^-$ current ($I_{Cl,UTP}$) in mouse ventricular myocytes (Fig. 1A); (ii) an inhibition of $I_{Cl,UTP}$ by CFTR $_{inh}$ -172, but no inhibition by DIDS (Fig. 3); (iii) a potency order of agonists for activation of the Cl $^-$ current to be UTP = ATP > UDP = ADP (Fig. 4B); (iv) an inhibition of the activation of $I_{Cl,UTP}$ by suramin, but not by PPADS (Fig. 4C); (v) an inhibition of $I_{Cl,UTP}$ activation by PKC or PLC inhibitors (Fig. 5) and anti-Gq/11 antibody (Fig. 6); and (vi) a modulation of $I_{Cl,UTP}$ activation by the intracellular nonhydrolyzable ATP analogues (Fig. 7). These results were believed to suggest that $I_{Cl,UTP}$ is due to an activation of CFTR Cl $^-$ channels via P2Y $_2$ receptor linked to the Gq/11-PLC-PKC signaling pathway and ATP hydrolysis in mouse ventricular myocytes.

The coupling of several P2Y receptor subtypes, including the P2Y $_2$ receptor to PLC and PKC, is well established in many previous studies [1–4, 6, 7], and the cloned P2Y $_2$ receptor has been reported to be UTP/ATP-sensitive and coupled to Gq/11 protein to activate PKC through PLC stimulation [2, 4, 6]. The present results obtained from native mouse ventricular cells are in agreement with these concepts. The expression of P2Y $_2$ receptor in mouse heart has been confirmed with a real-time PCR method [24].

The deactivation of $I_{Cl,UTP}$ after agonist removal was

slow when the cells were dialyzed with a solution containing a mixture of ATP and ATP γ S (Fig. 7), and this finding was taken to support the view that the phosphorylation-dephosphorylation cycle is related to the activation and deactivation of the current. It is of interest that the current deactivation after agonist removal was also slow in the cells dialyzed with a mixture of ATP and AMP-PNP (Fig. 7). Since AMP-PNP cannot cause phosphorylation reaction, a mechanism other than the phosphorylation-dephosphorylation cycle must be considered to explain this slow current deactivation. Hwang *et al.* [33] observed in guinea pig ventricular cells that phosphorylated CFTR Cl $^-$ channels remained open for many minutes when AMP-PNP was present intracellularly. As an explanation of this phenomenon, it has been proposed that the opening and closing of CFTR channel gates are linked to ATP binding and hydrolysis at two nucleotide binding domains on the channel protein (NBD1 and NBD2) [32–35], and that the binding of AMP-PNP to one NBD specifically keeps the opening of the channels for a very long time (as if they were “locked open”). It is possible that a similar mechanism operated in the slow current deactivation observed in the present study. On the other hand, UTP $_o$ could hardly induce the Cl $^-$ current when the cells were dialyzed with only AMP-PNP or ATPS without ATP. This finding was taken to indicate that ATP hydrolysis is a prerequisite for the current activation. In line with this, it has been proposed that ATP hydrolysis at one NBD controls the opening of the CFTR Cl $^-$ channels [32–35].

Nucleotides are known to exert various electrophysiological and mechanical effects on heart, and some of these effects have been attributed to the stimulation of P2Y receptors [1, 2], but the receptor subtype responsible for each cardiac response induced by P2Y receptor stimulation has not necessarily been clear. Our results suggest that P2Y $_2$ receptor stimulation induces a definite electrophysiological response in mouse ventricular cells. Very recently, UTP $_o$ has been shown to exert a positive inotropic effect on mouse heart via P2Y $_2$ receptors [24]. It is interesting that P2Y $_2$ receptor mRNA levels in the left ventricle of rats were increased 4.7 fold under congestive heart failure, compared with control [36], suggesting a pathophysiological role of P2Y $_2$ receptor in the development of heart failure.

UTP could be a physiologically important modulator of cardiac ionic currents. UTP $_o$ has been reported to affect ATP-sensitive potassium current [18], sodium current [19], and muscarinic potassium channels [20] in heart. Our study demonstrates that UTP $_o$ activates a cardiac Cl $^-$ current. The involvement of Cl $^-$ currents in the regulation of cardiac cell volume has been given attention, and an activation of the Cl $^-$ currents is expected to shrink the cell [37, 38]. Under some pathological conditions, ischemia, for example, an osmotic stress induced by the intracellular accumulation of metabolites such as lactate may cause

swelling of cardiac cells [39]. On the other hand, UTP has been reported to be released from cardiac cells under certain pathological conditions. The release of UTP was increased in rat cardiac hypertrophy [8], in rat hyperthyroid heart [9], and in pig cardiac ischemia [10]. Therefore the released UTP might exert a beneficial effect on cardiac muscle under such pathological conditions, playing a cell volume-regulatory role. Although UTP as an agonist may largely mimic the action of ATP on cardiac function, further studies are necessary to elucidate the physiological as well as the pathophysiological roles of UTP in the regulation of cardiac function.

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