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 (\(I_{\text{Cl,UTP}}\)) were studied in mouse ventricular myocytes with the whole-cell clamp technique. UTP\(_o\) (0.1 to 100 \(\mu\)M) 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 (CFTRinh-172, 10 \(\mu\)M) of cystic fibrosis transmembrane conductance regulator (CFTR), but not by a disulfonic stilbene derivative (DIDS, 100 \(\mu\)M). Suramin (100 \(\mu\)M), 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 \(\mu\)M), another P2Y receptor antagonist, induced little inhibition of \(I_{\text{Cl,UTP}}\). The activation of \(I_{\text{Cl,UTP}}\) was sensitive to protein kinase C (PKC) inhibitor, phospholipase C (PLC) inhibitor, intracellular GDP\(_S\) (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. \(I_{\text{Cl,UTP}}\) was persistently activated with a mixture of ATP\(_S\) + ATP in the pipette, suggesting the involvement of phosphorylation reaction in the current activation process. Our results strongly suggest that \(I_{\text{Cl,UTP}}\) is due to the activation of CFTR Cl\(^-\) channels through Gq/11-coupled P2Y receptor-PLC-PKC signaling and ATP hydrolysis in mouse heart.

Key words: Cl\(^-\) current, ventricular cell, P2Y receptor, CFTR, ATP hydrolysis.
CFTR-like Cl\textsuperscript{−} current in mouse cardiac myocytes [14, 17]. The subtype of P2Y receptor involved in the activation of CFTR Cl\textsuperscript{−} current is not clear. It has been reported that extracellular UTP (UTP\textsubscript{a}) 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\textsuperscript{−} current by UTP\textsubscript{a} is still unknown.

In the heart, studies with the RT-PCR method have revealed expressions of P2Y\textsubscript{1,2,4,6} receptors in neonatal rat [21] and of P2Y\textsubscript{1,2,4,6,11,12,13,14} receptors in human [22]. For mouse cardiomyocytes, recent studies [23, 24] suggest that at least P2Y\textsubscript{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\textsubscript{1}), UTP/ATP (P2Y\textsubscript{2}), UTP (P2Y\textsubscript{3}), UDP (P2Y\textsubscript{4}), ATP (P2Y\textsubscript{11}), 2-MeSADP (P2Y\textsubscript{12}), 2-MeSADP (P2Y\textsubscript{13}), and UDP-glucose (P2Y\textsubscript{14}) [2, 7]. The rat P2Y\textsubscript{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 P2Y\textsubscript{1,2,4,6,11,12,13} receptors, but counteracts P2Y\textsubscript{4,6} receptors only weakly [7]. Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) is a popular nonselective P2X receptor antagonist and inhibits P2Y\textsubscript{1} receptor potently, P2Y\textsubscript{4,6,13} receptors moderately, and P2Y\textsubscript{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 (P2Y\textsubscript{1,2,4,6,11}) that cause both an activation of protein kinase C (PKC) and Ca\textsuperscript{2+} mobilization through the activation of phospholipase C (PLC), and a family of Gi-coupled receptors (P2Y\textsubscript{12,13,14}) that inhibit adenyl cyclase protein kinase A (PKA) signaling [2, 4, 6]. It should be noted that P2Y\textsubscript{11} receptor can be coupled to Gs and adenyl cyclase activation [23, 25].

In the present study, we examined extracellular UTP-activated Cl\textsuperscript{−} current (I\textsubscript{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\textsubscript{Cl,UTP} is due to an activation of CFTR Cl\textsuperscript{−} channels via the P2Y\textsubscript{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 anesthetized 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\textsuperscript{2+}-free PSS until the heart ceased to beat, and finally with the Ca\textsuperscript{2+}-free solution containing 0.1% collagenase (CLS III, 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\textsuperscript{+}, low-Cl\textsuperscript{−} storage (modified KB) solution [19], and muscarinic potassium channels [20]) in the tissue in high-K\textsuperscript{+}, low-Cl\textsuperscript{−} 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\textOmega 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\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 20 taurine, 5.0 creatine, 5.0 sodium pyruvate, 1.0 NaHPO\textsubscript{4}, 10 Hepes; pH 7.4 adjust with NaOH; 300 mosM with mannitol. Ca\textsuperscript{2+}-free PSS was prepared by simply omitting CaCl\textsubscript{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 Heps; pH 7.2 with KOH; 300 mM osm with mannitol. For \( I_{\text{ATP}} \) 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 solution contained (mM): 77 NaCl, 0.8 MgCl₂, 1.0 CaCl₂, 5.0 CsCl, 2.0 BaCl₂, 0.2 CdCl₂, 5.5 glucose, 10 Heps, 0.01 nicardipine; pH 7.4 adjusted with NaOH; total [Cl⁻] = 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 containing 10 mM MgATP, or 5 MgATP + 5 mM AMP-PNP, or 5 mM MgATP + 5 mM ATP₆S were also prepared. The osmolarity 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'-NaOH; total [Cl⁻]o = 90 mM; 310 mOsm with mannitol. In others, 0.1 mM Tris-GTP phosphate (GTP

**Data analysis.** Permeability ratios (Fig. 2C) were calculated from the shifts in \( E_{\text{rev}} \) according to the modified Goldman-Hodgkin-Katz equation: \( P_x / P_{\text{Cl}^-} = ([\text{Cl}^-]_o / [\text{Cl}^-]_i) \exp[-\Delta E_{\text{rev}} F \{(\text{RT})^{-1} - [\text{Cl}^-]_o / [\text{Cl}^-]_i\}^{-1}] \), where \( \Delta E_{\text{rev}} \) is the shift of reversal potential, [Cl⁻]o, and [Cl⁻]i, are the extracellular Cl⁻ concentrations in the control (standard) and anion-substituted solutions, respectively, and \([X^-]_i\) 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: 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 ± 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 (ATPo) or UTP (UTPo)-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 µM ATPo and reached an almost steady level within ~3 min. The inward current at −80 mV also increased after ATPo, but the increase appeared to occur with little delay. In this experiment, ATPo was replaced with UTPo (100 M) when the ATPo-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 UTPo in 6 cells was 99.8 ± 2.7% and 60.3 ± 9.4% of the magnitude of ATPo-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 \( f^{-1} \) relationship of the different currents between the current observed after ATP and that in control and the mean \( f^{-1} \) relationship of the current component, which was sensitive to the ago-
nis t replacement from ATP to UTP, are shown in Fig. 1C. The latter (b–c) exhibited inward rectification with a reversal potential ($E_{\text{rev}}$) of positive voltage, $E_{\text{rev}}$ being more positive than +100 mV in all tested cells ($n = 6$). In contrast, the $I–V$ relationship of the “UTP-induced current” (c–a), like that of the ATP-induced Cl$^-$ current ($I_{\text{Cl,ATP}}$) [15], was almost linear. The mean $E_{\text{rev}}$ of the UTP-induced current was –1.7 ± 4.4 mV, 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 activated both a nonselective cation current and a Cl$^-$ current, and they further suggest that UTP activates the latter almost exclusively in mouse cardiac cells. We attempted to further characterize the UTP-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 [Cl\(^-\)]\(_o\) on the UTP-induced current. When the bath solution was changed from high-[Cl\(^-\)]\(_o\) (90 mM) to low-[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 [Cl\(^-\)]\(_o\) obtained in several of the experiments are shown in Fig. 2B (solid circles and open triangles). There was a positive shift of \(E_{\text{rev}}\) with a reduction of [Cl\(^-\)]\(_o\), and \(E_{\text{rev}}\) at low [Cl\(^-\)]\(_o\) was +45 ± 5 mV (n = 4), the value being close to \(E_{\text{Cl}}\) (+49.6 mV) predicted for the low [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 (\(I_{\text{UTP}}\)) in high-[Cl\(^-\)]\(_o\), Cl\(^-\)-deficient bath solution (77 mM I\(^-\) + 13 mM Cl\(^-\)). Its current–voltage (I–V) relationship is shown in Fig. 2B (open squares). \(E_{\text{rev}}\) was +20 ± 6 mV in this case. The \(E_{\text{rev}}\) 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\(^-\)) \(P_x/P_{\text{Cl}}\) according to a modified Goldman-Hodgkin-Katz equation. In this calculation, \(P_x/P_{\text{Cl}}\) and \(P_{\text{Cl}}/P_{\text{Cl}}\) were 0.47 and 0.14, respectively. The anion selectivity of the current, Cl\(^-\) > iodide > aspartate, is similar to that of PKA-activated CFTR Cl\(^-\) current [28] in cardiac myocytes of many species and that of \(I_{\text{Cl}}\) [15] in mouse ventricular cells. This finding, together with the observation that both \(I_{\text{UTP}}\) and \(I_{\text{Cl}}\) exhibit similar current characteristics (Fig. 1), suggest that both ATP\(_o\) and UTP\(_o\) activate the same Cl\(^-\) current. Since ATP\(_o\) is thought to activate CFTR Cl\(^-\) current through a stimulator 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 blockers on persistently activated \(I_{\text{UTP}}\) 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 potently antagonize P2 purinergic receptors [1, 28, 30]. Therefore we attempted to obtain a receptor-independent Cl\(^-\) current, using GTP\(_7\)S (a nonhydrolyzable GTP analogue)-loaded cells. Since GTP hydrolysis is essential to terminate the signal transduction processes linking receptor occupation and activation of effector protein, once the effector protein is activated by an agonist, the activation persists even after the removal of ATP\(_o\), and GTP is still effective even after the removal of ATP\(_o\) [15]. In the present study, similar persistent \(I_{\text{UTP}}\) could be observed, and we examined the effects of Cl\(^-\) channel inhibitors on the \(I_{\text{UTP}}\) in the absence of ATP\(_o\).

Figure 3A shows the effect of CFTRinh-172 (a thiazolidinone-derived selective CFTR Cl\(^-\) channel inhibitor [31]) on the persistent \(I_{\text{UTP}}\). \(I_{\text{UTP}}\) was almost completely inhibited by 10 mM CFTRinh-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 \(I_{\text{UTP}}\). Fifty micromolar
lar glibenclamide, 500 µM DPC, and 100 µM NPPB inhibited \( I_{\text{CTP}} \) in a voltage-independent manner, whereas \( I_{\text{CLTP}} \) was only slightly sensitive to 100 µM DIDS. These results support the view that UTP 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 \( n_H \) (Hill coefficient) were calculated. EC50 and \( n_H \) were 1.6 µM and 0.98 for ATP\(_o\), and 1.1 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 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 P2Y\(_{2}\) and P2Y\(_{4}\) receptors. To further identify the P2Y receptor subtype involved, we examined the effects of P2Y receptor-antagonist on the activation of \( I_{\text{CLTP}} \). An earlier report [15] showed that the activation of cardiac \( I_{\text{CTP}} \) 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 \( I_{\text{CLTP}} \). Figure 4C summarizes the results. Suramin (100 µM) strongly inhibited \( I_{\text{CLTP}} \), whereas PPADS (100 µM) exerted little effect on the \( I_{\text{CLTP}} \). The magnitude of \( I_{\text{CLTP}} \) at +100 mV observed after antagonists was 27.1 ± 8.8% (\( n = 4 \)) and 92.9 ± 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 \( I_{\text{CLTP}} \) (Fig. 4C) are taken into consideration together, our data appear to suggest the involvement of the P2Y\(_{2}\) receptor in the activation of \( I_{\text{CLTP}} \) 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 \( I_{\text{CLTP}} \) involves PTX (a selective Gi protein inhibitor)-insensitive G protein and that \( I_{\text{CLTP}} \) is regulated
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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 \( I_{Cl.UTP} \), 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 \( I_{Cl.UTP} \).

When the cell was pretreated with U-73122, 100 µM UTP activated \( I_{Cl.UTP} \) only a small amount or slightly (Fig. 5Ac). The application of 100 nM PDBu, an agent that activates PKC without involving PLC, could induce \( I_{Cl.UTP} \) of large amplitude in the presence of 5 µM U-73122 (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.

Fig. 5. Role of PLC and PKC in the activation of \( I_{Cl.UTP} \). 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.

Fig. 6. Involvement of Gq/11 protein in the activation of \( I_{Cl.UTP} \). 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.
pretreated with BIM-I (100 nM) for 10 min (Fig. 5C), 100 µM UTP activated \(I_{\text{Cl,UTP}}\) only slightly (\(n = 6\)), whereas 100 µM UTP\(_o\) induced a current of 3.1 ± 0.4 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 \(I_{\text{Cl,UTP}}\). 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 \(I_{\text{Cl,UTP}}\) 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 ± 0.6 pA pF\(^{-1}\), whereas UTP\(_o\) + 100 nM PDBu induced a current of 3.7 ± 0.7 pA pF\(^{-1}\). In the cells with the inactivated antibody (\(n = 4\)), UTP\(_o\) or UTP\(_o\) + PDBu induced a current whose amplitude (4.1 ± 0.8 or 4.2 ± 1.0 pA pF\(^{-1}\), respectively) was comparable to that of control \(I_{\text{Cl,UTP}}\) (Fig. 4). We confirmed that the activation of \(I_{\text{Cl,UTP}}\) 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 \(I_{\text{Cl,UTP}}\) 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 \(I_{\text{Cl,UTP}}\) also involves such processes by using hydrolysis-resistant ATP analogues (ATP\(_y\)S and AMP-PNP). We prepared pipette solutions of five types, i.e., those containing 5 mM ATP\(_y\)S or 5 mM AMP-PNP without ATP, and those containing 10 mM ATP or 5 mM ATP + 5 mM ATP\(_y\)S or 5 mM ATP + 5 mM AMP-PNP.

When the cells were dialyzed with pipette solutions containing only ATP\(_y\)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 \(I_{\text{Cl,UTP}}\). When the pipette solution containing ATP\(_y\)S + 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 \(I_{\text{Cl,UTP}}\) after the withdrawal of UTP\(_o\) was very slow in this case (Fig. 7A). The magnitude of \(I_{\text{Cl,UTP}}\) at +100 mV was 6.6 ± 0.7 pA pF\(^{-1}\) (\(n = 4\)) in the presence of UTP\(_o\) and it was 5.6 ± 0.8 pA pF\(^{-1}\) at 15 min after the agonist removal (Fig. 7C). This contrasted with the observation that \(I_{\text{Cl,UTP}}\) activated in the cells dialyzed only with ATP disappeared almost completely within 5
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MIN after agonist removal (Figs. 1A, 2A, and 5C). The slow deactivation may be explained as follows. Since ATP/PiS 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/PiS 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 $I_{\text{Cl,UTP}}$.

On the other hand, when the pipette solution contained AMP-PNP + ATP, the deactivation of $I_{\text{Cl,UTP}}$ was also slow, though its rate of deactivation was faster than that observed with ATP/PiS (Fig. 7B). The magnitude of $I_{\text{Cl,UTP}}$ still remaining 5 min after agonist removal was $3.5 \pm 0.3$ pA pF$^{-1}$ ($n = 4$), of which the value was about 50% of that seen before agonist removal ($6.7 \pm 0.7$ 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 ($I_{\text{Cl,UTP}}$) in mouse ventricular myocytes (Fig. 1A); (ii) an inhibition of $I_{\text{Cl,UTP}}$ by CFTRinh-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 $I_{\text{Cl,UTP}}$ by suramin, but not by PPADS (Fig. 4C); (v) an inhibition of $I_{\text{Cl,UTP}}$ activation by PKC or PLC inhibitors (Fig. 5) and anti-Gq/11 antibody (Fig. 6); and (vi) a modulation of $I_{\text{Cl,UTP}}$ activation by the intracellular nonhydrolyzable ATP analogues (Fig. 7). These results were believed to suggest that $I_{\text{Cl,UTP}}$ 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 $I_{\text{Cl,UTP}}$ after agonist removal was slow when the cells were dialyzed with a solution containing a mixture of ATP and ATP/PiS (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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