Phospholipase C-linked receptors regulate the ATP-sensitive potassium channel by means of phosphatidylinositol 4,5-bisphosphate metabolism

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In the COS7 cells transfected with cDNAs of the Kir6.2, SUR2A, and M1 muscarinic receptors, we activated the ATP-sensitive potassium (K\textsubscript{ATP}) channel with a K\textsuperscript{+} channel opener and recorded the whole-cell K\textsubscript{ATP} current. The K\textsubscript{ATP} current was reversibly inhibited by the stimulation of the M\textsubscript{1} receptor, which is linked to phospholipase C (PLC) by the G\textsubscript{q} protein. The receptor-mediated inhibition was observed even when protein kinase C (PKC) was inhibited by H-7 or by chelating intracellular Ca\textsuperscript{2+} with 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) included in the pipette solution. However, the receptor-mediated inhibition was blocked by U-73122, a PLC inhibitor. M\textsubscript{1}-receptor stimulation failed to inhibit the K\textsubscript{ATP} current activated by the injection of exogenous modulator of K\textsubscript{ATP} channel (PiP\textsubscript{2}) through the whole-cell patch pipette. The receptor-mediated inhibition became irreversible when the replenishment of PiP\textsubscript{2} was blocked by wortmannin (an inhibitor of phosphatidylinositol kinases), or by including adenosine 5'--[\beta,\gamma-imido]triphosphate (AMPPNP, a nonhydrolyzable ATP analogue) in the pipette solution. In inside-out patch experiments, the ATP sensitivity of the K\textsubscript{ATP} channel was significantly higher when the M\textsubscript{1} receptor in the patch membrane was stimulated by acetylcholine. The stimulatory effect of pinacidil was also attenuated under this condition. We postulate that stimulation of PLC-linked receptors inhibited the K\textsubscript{ATP} channel by increasing the ATP sensitivity, not through PKC activation, but most probably through changing PiP\textsubscript{2} levels.

The ATP-sensitive potassium (K\textsubscript{ATP}) channels play a key role in the coupling between cellular metabolism and electrical activity in a wide range of tissues. Although the primary characteristic of the K\textsubscript{ATP} channel is the inhibition by intracellular ATP, various cytosolic substances, such as divalent cations, protons, anions, and nucleoside diphosphates, are known as modulators of K\textsubscript{ATP} channel (1--4).

In addition to the cytosolic factors listed above, a component of membrane lipid bilayer also modulates the K\textsubscript{ATP} channel; phosphatidylinositol (PI)-4,5-bisphosphate (PiP\textsubscript{2}) is known to increase the open probability and decrease the ATP sensitivity of the cardiac and reconstituted K\textsubscript{ATP} channel (5--8).

Methods

Molecular Biology. cDNAs of Kir6.2, SUR2A, and green fluorescent protein [GFP(S65A)] were subcloned into the pCI vector (Promega). The M\textsubscript{1} muscarinic receptor cDNA was subcloned into an expression vector carrying the simian virus 40 promoter. Mixtures of the following amounts of vectors (\mu g per dish) were cotransfected into COS7 cells with Lipofectamine reagent and Opti-MEM (GIBCO/BRL): 0.8 Kir6.2 + 0.8 SUR2A + 0.4 GFP + 1.6 receptor. COS7 cells were cultured in DMEM supplemented with 10% (vol/vol) FCS.

Electrophysiology. After the transfection (24--48 hr), the whole-cell and inside-out patch experiments were performed in GFPPositive cells with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The current and voltage signals were stored on a DAT tape recorder (TEAC, Tokyo, Japan) as an on-line computer (PC-98; NEC, Tokyo, Japan) through an analog--digital converter (ADX98E; Canopus, Osaka, Japan). The current signals were also played back and digitized with a Digipack 1200A interface (Axon Instruments). Data analysis was carried out by using in-house-made programs and commercial software (pClamp 6 & 7; Axon Instruments).

Solutions. The physiological saline contained (in mM): 140 NaCl, 5.4 KCl, 1 NaHPO\textsubscript{4}, 1.8 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 5 glucose, and 5 Heps (pH 7.4 with NaOH). The high K\textsuperscript{+}--whole cell-pipette solution contained (in mM): 110 aspartic acid, 10 NaCl, 1 MgCl\textsubscript{2}, 4 ATP, 0.1 GTP, 10 1,2-bis(2-aminophenoxy)ethane-N,N',N',N'-tetraacetate (BAPTA), 5 Heps (pH 7.4 with KOH). In some experiments, ATP was replaced with the same amount of adenosine 5'--[\beta,\gamma-imido]triphosphate (AMPPNP), or 0.1 mM GTP was replaced with 1 mM guanosine 5'--[\beta-thio]triphosphate (GDP\textsubscript{βS}). The inside-out patch pipette solution contained (in mM): 140 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 5 Heps (pH 7.4 with KOH). The control internal solution for the inside-out patch experiments contained: 140 KCl, 2 MgCl\textsubscript{2}, 2 EGTA, and 5 Heps (pH 7.2 with KOH). Li\textsubscript{4}AMPPNP, pinacidil, acetylcysteine (ACh), and H-7 were purchased from Sigma. PiP\textsubscript{2} (Calbiochem) was prepared with 10% (vol/vol) FCS.

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Abbreviations: K\textsubscript{ATP}, ATP-sensitive potassium; PLC, phospholipase C; PI, phosphatidylinositol; PiP\textsubscript{2}, PI-4,5-bisphosphate; PiP, PI-4-monophosphate; GFP, green fluorescent protein; CHO, Chinese hamster ovary; PKC, protein kinase C; ACh, acetylcholine; WNN, wortmannin; DAG, diacylglycerol; i-V, current–voltage; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N',N'-tetraacetate (BAPTA); AMPPNP, adenosine 5'-[\beta,\gamma-imido]triphosphate; GDP\textsubscript{βS}, guanosine 5'-[\beta-thio]triphosphate.

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was dissolved in the control internal solution by sonication for 30 min on ice. U-73122, (Wako Pure Chemicals, Osaka) wortmannin (WMN; Wako), LY 294002 (Tocris Neuramin, Bristol, U.K.), and diacylglycerol (DAG; Serdary Research Laboratories, Englewood Cliffs, NJ) were first dissolved in DMSO as a stock solution, and then used at the final concentration in the solution. The test solutions were applied either by bath perfusion or by a Y-tube apparatus (16). The whole-cell experiments were conducted at 37°C, and the inside-out patch experiments were conducted at 22–25°C.

Data Analysis. The whole-cell current–voltage (I–V) relationship was measured with ramp pulses (120 mV/s) applied every 6 s from the holding potential of −60 mV; initial positive slope from −60 mV to 0 mV was followed by a negative slope to −120 mV, and the I–V relationship was determined from the negative limb of the ramp pulse. The membrane potentials were corrected for the liquid-junction potential (∼10 mV) between the pipette solution and the physiological saline. In the inside-out patch experiments, the amplitude of mean patch current (MPC; the integral of open-channel current divided by the time for integration) was measured for 5–10 s, before (MPCCTR) and after (MPCATP) the application of ATP. The magnitude of inhibition was calculated as: (MPCCTR − MPCATP)/MPCCTR. The data points are expressed as mean ± SEM. The Hill equation fitted to the data points is described in the legend of Fig. 1. The statistical analyses were performed using the Student’s unpaired t test, with P < 0.05 being considered significant. The mean value was obtained from at least five observations in each experiment.

Results

Stimulation of the PLC-Linked Receptor Inhibited the Whole-Cell KATP Current. COS7 cells are known to possess the PI signal transduction pathway (17). We have confirmed that the application of ACh produced Ca2+ mobilization in the COS7 cells transfected with the M1 muscarinic receptor by measuring fura-2 fluorescence (data not shown). In the experiment shown in Fig. 1, cloned KATP channels (Kir6.2 + SUR2A) were coexpressed with (Fig. 1A and C) or without (Fig. 1B) M1 receptors. It is well known that intracellular Ca2+ promotes the rundown of the KATP channel (1). To exclude the effects of intracellular Ca2+, we carried out whole-cell patch experiments using the pipette solution containing 10 mM BAPTA. Thereby, intracellular Ca2+ concentration of COS7 cells was clamped to ∼10−10 M. Even under this condition, ACh reversibly inhibited the KATP current, which was activated by 100 μM pinacidil (18), in the COS7 cells expressing the M1 receptor (Fig. 1A), whereas ACh was without the inhibitory effect in the COS7 cell that was not cotransfected with the M1 receptor (Fig. 1B). When a low-ATP pipette solution containing 10 μM ATP was used, the KATP current occurred spontaneously after the formation of whole-cell configuration. Under this condition, ACh also inhibited the KATP current in a reversible manner (Fig. 1C).

In both experiments shown in Fig. 1 A and C, the onset of receptor-mediated inhibition of the KATP current was very slow; it took ∼1 min to reach the steady state. Recovery from the receptor-mediated inhibition also proceeded in a slow time course (∼5 min). The recovery was sometimes incomplete, particularly when the KATP current was activated by a low ATP pipette solution. Time courses of the receptor-mediated inhibition and recovery of the KATP current closely resembled those of receptor-mediated depletion and replenishment of PIP2 reported in CHO cells and human neuroblastoma cells (11, 12).

The dose-inhibition relationship for ACh was examined in Fig. 1A. Because the recovery from the receptor-mediated inhibition was sometimes incomplete, various concentrations of ACh were applied in a cumulative manner; the amplitude of the current deflection became smaller as ACh concentration increased. The magnitude of the receptor-mediated inhibition (% inhibition) was determined as follows: % inhibition = (ICTR − ISACh)/ICTR, where ICTRL is the amplitude of the glibenclamide-sensitive current (i–vi), and IACh is the amplitude during ACh application (e.g., for 1 μM ACh, iv–vi). The concentration-inhibition relationships are shown in Fig. 1E. The concentration producing the half-maximal inhibition (IC50) was 0.54 ± 0.12 μM, and nH was 0.98 ± 0.22 (n = 6). The line was drawn with these values.

I–V relationships measured at the times i–vi are shown in Fig. 1D. All of the I–V curves showed weak inward rectification and intersected each other at the potential close to the equilibrium potential for K+. The amplitude of the KATP current was measured at 0 mV before and during the application of ACh. The magnitude of the receptor-mediated inhibition (% inhibition) was determined as follows: % inhibition = (ICTR − ISACh)/ICTR, where ICTRL is the amplitude of the glibenclamide-sensitive current (i–vi), and IACh is the amplitude during ACh application (e.g., for 1 μM ACh, iv–vi). The concentration-inhibition relationships are shown in Fig. 1E. The concentration producing the half-maximal inhibition (IC50) was 0.54 ± 0.12 μM, and the Hill coefficient (nH) was 0.98 ± 0.22.

Under the same experimental conditions, stimulation of PLC by endothelin or by the α1-adrenergic agonist inhibited the KATP current in rat ventricular myocytes, but not in guinea-pig and rabbit myocytes (Xie et al., unpublished observation). It has been reported that in rat ventricular myocytes, stimulation of endothelin receptor antagonizes the production of cAMP by means of the pertussis toxin (PTX)-sensitive mechanism, and simulta-
Under this condition, it seems unlikely that Ca$^{2+}$ completely. Therefore, it seems unlikely that Ca$^{2+}$ was activated during the receptor stimulation. To elucidate the mechanisms for the receptor-mediated inhibition, we blocked the effect of 1 mM ACh successfully inhibited the KATP current. After the KATP current was activated by the application of 100 μM pinacidil (started at 1). (A) The effect of H-7 on M₁ receptor-mediated inhibition. The applications of 1 μM ACh, 100 μM H-7, and 10 μM glibenclamide are indicated by the bars above the recording. (B) The effect of 1 μM U-73122. (C) A COS7 cell was dialyzed with the pipette solution containing 1 mM GDPβS. (D) Summary of the magnitudes of 1 μM ACh-induced maximal inhibition of the KATP current in control, H-7-, U-73122-, and GDPβS-treated cells. The numerals in parentheses indicate numbers of experiments. **, $P < 0.01$ vs. control.

neously promotes PI turnover by the PTX-insensitive pathway (19). The latter signal transduction pathway might not be significant in guinea pig and rabbit myocytes.

**PLC, but Not PKC, Was Involved in the Inhibition.** In all of the experiments in the present study, the pipette solutions contained 10 mM BAPTA, which should chelate intracellular Ca$^{2+}$ completely. Therefore, it seems unlikely that Ca$^{2+}$-dependent PKC was activated during the receptor stimulation. To elucidate the mechanisms for the receptor-mediated inhibition, we blocked each step of the signal transduction pathway. In Fig. 2A, the KATP current was activated by pinacidil. The following application of 100 μM H-7, a blocker of PKC, slightly inhibited the current. Under this condition, 1 μM ACh successfully inhibited the KATP current. The magnitude of the inhibition was not significantly different from the control experiments (65.5 ± 5.5%, $P > 0.05$ vs. control; 66.4 ± 10.1%; Fig. 2D), suggesting that the activation of PKC was not involved in the receptor-mediated inhibition of the KATP current.

However, when PLC was blocked by 1 μM U-73122, the receptor-mediated inhibition was significantly attenuated. In the experiment shown in Fig. 2B, we first inhibited the KATP current by the application of 1 μM ACh. After the KATP current was recovered to the control level, 1 μM ACh was applied in the presence of U-73122. Clearly, 1 μM U-73122 abolished ACh-induced inhibition. As summarized in Fig. 2D, the magnitude of inhibition was 12.4 ± 4.4% under this condition ($P < 0.01$). Blocking GTP-binding protein also attenuated the receptor-mediated inhibition. In the experiment shown in Fig. 2C, the pipette solution contained 1 mM GDPβS (a nonspecific blocker of GTP-binding proteins) instead of 0.1 mM GTP. Under this condition, the inhibitory effect of 1 μM ACh was significantly attenuated (8.1 ± 3.1%, $P < 0.01$). Based on the above findings, we postulated that the receptor-mediated inhibition was the result of the activation of PLC, but not the activation of PKC. The most plausible mechanism might be the depletion of PI$^{3,4,5}$P$_3$.

**The Blockade of PI$^{3,4,5}$P$_3$ Synthesis Obstructed the Recovery from the Receptor-Mediated Inhibition.** In CHO cells and human neuroblastoma cells, the receptor-mediated activation of PLC produced a depletion of PI$^{3,4,5}$P$_3$ in the plasma membrane. WMN (an inhibitor of PI 3-kinase and PI 4-kinase) inhibited the replenishment of PI$^{3,4,5}$P$_3$ after the depletion (12). Therefore, we examined whether the inhibition of PI$^{3,4,5}$P$_3$ depletion by WMN was reflected in the recovery of the KATP current from the receptor-mediated inhibition. In Fig. 3A, we first applied 10 μM ACh to inhibit the KATP current and confirmed the recovery after washout of ACh. As summarized in Fig. 3C (●), the KATP current
was recovered to 90.8 ± 6.6% of the preceding level 4 min after the washout of ACh. In the presence of 100 μM WMN, the KATP current was no longer recovered after the washout of ACh (Fig. 3A). The amplitude of the KATP current measured at the end of the experiment was 5.0 ± 0.2% of the preceding level (□ in Fig. 3C, P < 0.01 vs. control). When we applied 10 μM WMN, the receptor-mediated inhibition was partially reversible (○ in Fig. 3C, 46.0 ± 7.3%, P < 0.01). These results are in agreement with those obtained in our previous inside-out experiments on the MgATP-dependent recovery of the channel (20).

We next tried to block the replenishment of PIP2 by replacing ATP with a nonhydrolyzable analogue, AMP-PNP. In the experiment shown in Fig. 3 B and C (○), the pipette solution contained 4 mM AMP-PNP instead of ATP. Because AMP-PNP and ATP equally inhibit the KATP channel, no potassium current was activated after the formation of the whole-cell patch. The KATP current was then activated by 100 μM pinacidil. The following application of 10 μM ACh produced an irreversible inhibition of the KATP current. AMP-PNP is commercially available only as the lithium salt. Jenkinson et al. (11) reported that Li+ disrupts the supply of PIP2 in CHO cells during the stimulation of PLC with a slow time course; the PIP2 level became significantly lower 10–30 min after the stimulation of PLC (Fig. 4). However, the recovery of the KATP current was 86.6 ± 9.1% (n = 6, P > 0.05 vs. control) 4 min after the washout of ACh, when the pipette solution contained 4 mM ATP and 16 mM LiCl (data not shown). Li+ may not inhibit the replenishment of PIP2 during a short period of agonist application. Therefore, we presumed that the inhibition of the recovery of the KATP current was not because of Li+, but because of AMP-PNP itself.

The Stimulation of the PLC-Linked Receptor Shifted the ATP Sensitivity. In the previous section, we demonstrated that the stimulation of the M1 receptor inhibited the pinacidil-induced KATP current without PKC activation. However, the mechanism beyond this point remains unclear. This mechanism might be explained in two ways. First, stimulation of the M1 receptor may change the intracellular ATP concentration. Second, M1 receptor stimulation may modulate the properties of the KATP channel. It has been reported that exogenous application of PIP2 promoted the opening of the KATP channel and decreased the ATP sensitivity (7, 8). Therefore, when the stimulation of the M1 receptor depletes the PIP2 pool of the plasma membrane, it may produce the opposite effect on the KATP channel. We first confirmed that 10 μM PIP2 markedly decreased the ATP sensitivity in the inside-out patch experiments. PIP2 restored KATP-channel activity in a slow time course (Fig. 4A). In a different patch, ATP sensitivity was markedly decreased 3 min after PIP2 application; 10 mM ATP was required to inhibit the KATP channel completely (Fig. 4B). Fig. 4D summarizes the dose-inhibition relationship; ATP concentration for the half-maximal inhibition (IC50) was 23.4 ± 5.9 μM in control (○; also see Fig. 5A and B). Forty seconds after the application of PIP2, IC50 was 246.8 ± 18.1 μM (□, P < 0.01 vs. control). Prolonged application (3 min) produced a larger change in IC50 (△: 1276.5 ± 224.2 μM, P < 0.01).

Baukrowitz et al. (8) reported that injection of PIP2 into Xenopus oocytes expressing Kir6.2 + SUR2A promoted a persistent activation of the KATP current. However, in the whole-cell patch experiments, we failed to activate the KATP current with the pipette solutions containing 100, 200, and 500 μM PIP2. This was presumably because micellar PIP2 solution could not passively diffuse into the cells. Therefore, we tried to inject the pipette solution containing 100 μM PIP2 by applying positive pressure (~10 cmH2O) to the patch pipette. In most cases, we lost the whole-cell patch by this manipulation. In 3 of 24 experiments, we could activate the KATP current (Fig. 4C). Under this condition, M1-receptor stimulation failed to inhibit the KATP current.

We next stimulated M1 receptors by including 30 μM ACh in the pipette solution and measured the ATP sensitivity. As shown in Fig. 5A Upper, 1 μM ATP did not inhibit the KATP channel under control conditions. In contrast, 1 μM ATP produced a sizable inhibition when the pipette solution contained 30 μM ACh (Fig. 5A, Lower). Fig. 5B summarizes the dose-inhibition relationship. IC50 was 23.4 ± 5.9 μM under control condition (○). In the presence of 30 μM ACh, ATP sensitivity was significantly higher (□; IC50 = 2.6 ± 0.4 μM, P < 0.01).

In the previous whole-cell experiments, the stimulation of the PLC-linked receptor inhibited the pinacidil-induced KATP current. Therefore, PLC activation may also increase the ATP sensitivity in the presence of pinacidil. In the experiments shown in Fig. 5 C and D, we applied 100 μM pinacidil to the cytoplasmic surface of the patch membrane and measured the ATP sensitivity in the comparable condition with the previous whole-cell experiments. Pinacidil altered IC50 from 23.4 ± 5.9 μM to 297.3 ± 56.5 μM (△; P < 0.01). When M1 receptors were stimulated, the ATP sensitivity increased significantly in the presence of pinacidil (○; IC50 = 7.9 ± 2.2 μM). Thus, it seemed likely that the receptor-mediated inhibition of the whole-cell KATP current was the result of the increase in ATP-sensitivity.
Discussion

The present study has provided convincing evidence that the activation of the PLC-linked receptor inhibits the \( \text{K}_{\text{ATP}} \) channel (\( \text{Kir}6.2 \) + \( \text{SUR}2A \)), as previously suggested by Baukrowitz et al. (8). It has been reported that receptor-mediated activation of PLC reduces PIP2 concentration by 84.9 ± 7.3% in human neuroblastoma cells (12). Although we have not carried out the biochemical measurements of PIP2 concentration, it is likely that similar magnitude of PIP2 depletion also occurs in COS7 cells. The stimulation of the PLC-linked receptor produces inositol trisphosphate (IP3) and DAG. Exogenous application of IP3 had no effect on \( \text{K}_{\text{ATP}} \) channel activity (6, 7). In the present study, 10 \( \mu \text{M} \) DAG did not inhibit the \( \text{K}_{\text{ATP}} \) channel in inside-out patch experiments (data not shown), although 1,2-diacyl-sn-glycerol (DOG), a membrane-permeant analogue of DAG, inhibited the \( \text{K}_{\text{ATP}} \) channel (6). It should be noted that other types of PI metabolites, such as IP5 and IP6, regulate the \( \text{Ca}^{2+} \) channel (20). Therefore, the involvement of such metabolites cannot be excluded in the present study. However, stimulation of the PLC-linked receptor did not inhibit the \( \text{K}_{\text{ATP}} \) current when exogenous PIP2 was applied in the whole-cell patch experiment (Fig. 4C). This may partly support the idea that the mechanism of the receptor-mediated inhibition was the depletion of PIP2, rather than the production of PI metabolites that may inhibit the \( \text{K}_{\text{ATP}} \) channel. This idea may also be supported by the finding that the receptor-mediated inhibition became irreversible when the replenishment of PIP2 was blocked by WMN or AMPPNP.

So far, specific blockers for PI 4-kinase or PI 5-kinase have not been reported. WMN, an inhibitor of PI 3-kinase, blocks PI 4-kinase at a higher concentration. The concentration of WMN used in the present study was higher than those in the biochemical experiments used to block PI 4-kinase (12, 21, 22). At a low concentration (200 nM), WMN did not inhibit the recovery from the receptor-mediated inhibition (data not shown). Because WMN has a nonspecific effect on the enzymes other than PI kinases at higher concentration (23), other unknown types of enzymes might be involved in the recovery from the receptor-mediated inhibition. Exogenous PIP3 also activated the \( \text{K}_{\text{ATP}} \) channel (7), raising the question of whether WMN effect might be the result of the block of PI 4-, rather than PI 3-, kinase. However, the receptor-mediated inhibition was readily reversible in the presence of 10 \( \mu \text{M} \) LY294002, a specific PI 3-kinase blocker (ref. 24, and data not shown). Therefore, this finding supports the hypothesis that the WMN effect was the result of the block of PI 4-kinase. We previously reported that WMN inhibited the MgATP-dependent recovery of the \( \text{K}_{\text{ATP}} \) channel, most likely by blocking PIP2 replenishment (25). LY294002 (10 \( \mu \text{M} \)) did not inhibit MgATP-dependent recovery (data not shown). WMN had similar dose-dependent effects on MgATP-dependent recovery and the receptor-mediated inhibition. These findings suggested that relevant enzymes may be involved in these phenomena.

Exogenous application of PIP2 decreased the ATP sensitivity of C-terminal-truncated Kir6.2 (Kir6.2C26), which was functionally expressed on the plasma membrane by itself (8, 26). The neutralization of positively charged amino acid residue R177 or R176 in Kir6.2 reduced the level of functional expression and delayed the time course of the activation by PIP2 (6, 7),
suggesting that the target of PIP$_2$ was Kir6.2. In the present study, M$_1$-receptor stimulation attenuated the effect of pinacidil (Fig. 5). Because the target of pinacidil is SUR (2), this finding supports the finding that PIP$_2$ modulated the function of SUR1 as well as Kir6.2 (27).

The receptor-mediated inhibition of the K$_{ATP}$ current appears contradictory to the previous report in cat atrial myocytes; using the nystatin-perforated patch method, M$_1$-receptor stimulation contradicted to the previous report in cat atrial myocytes; using the nystatin-perforated patch method, M$_1$-receptor stimulation activated the whole-cell K$_{ATP}$ current (28). This discrepancy might arise from different experimental conditions; in the nystatin-perforated patch experiment, intracellular metabolism and Ca$^{2+}$ transient are kept intact. Intracellular ATP consumption may increase by means of the extrusion of intracellular Ca$^{2+}$ and the phosphorylation by PKC during the activation of PI signal transduction pathway. In contrast, the pipette solution used in the present study contained 10 mM BAPTA and 5 mM MgATP. The change of ATP concentration and the activation of Ca$^{2+}$-dependent PKC may be minimized under this condition. Therefore, we hypothesized that the effects of PIP$_2$ depletion were observed separately from those of the change of intracellular ATP level and PKC activation. When PKC was directly applied to the patch membrane isolated from the cardiac myocytes, the open probability of the KATP channel was decreased, and the stoichiometry of the dose-inhibition curve for ATP was modified without changing IC$_{50}$ (29). In the whole-cell patch experiment, PKC activation by phorbol 12,13-didecanoate induced the K$_{ATP}$ current (30). However, in the present study, we have demonstrated that independent of phosphorylation by PKC, the change in PIP$_2$ level could drastically regulate the K$_{ATP}$ channel. The balance between these factors may determine the properties of the K$_{ATP}$ channel in the native tissues during the stimulation of PLC-linked receptors. PIP$_2$ has been reported to regulate the activity of the inward-rectifier K$^+$ channel, GIRK channel, and Na$^+$/Ca$^{2+}$ exchanger (31). Therefore, the receptor-mediated regulation of the PIP$_2$ pool may play an important role in the control of cellular function through the modulation of ion channels and transporters.

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