Regulation of the slowly activating component of delayed rectifier K⁺ current (I_{Ks}) by membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) was examined in guinea pig atrial myocytes using the whole-cell patch clamp method. I_{Ks} was elicited by depolarizing voltage steps given from a holding potential of −50 mV, and the effect of various test reagents on I_{Ks} was assessed by measuring the amplitude of tail current elicited upon return to the holding potential following a 2-s depolarization to +30 mV. Intracellular application of 50 μM wortmannin through a recording pipette evoked a progressive increase in I_{Ks} over a 10–15-min period to 208.5 ± 14.6% (n = 9) of initial magnitude obtained shortly after rupture of the patch membrane. Intracellular application of anti-PtdIns(4,5)P₂ monoclonal antibody also increased the amplitude of I_{Ks} to 198.4 ± 19.9% (n = 5). In contrast, intracellular loading with exogenous PtdIns(4,5)P₂ at 10 and 100 μM produced a marked decrease in the amplitude of I_{Ks} to 54.3 ± 3.8% (n = 5) and 44.8 ± 8.2% (n = 5), respectively. Intracellular application of neomycin (50 μM) or aluminum (50 μM) evoked an increase in the amplitude of I_{Ks} to 161.0 ± 13.5% (n = 4) and 150.0 ± 8.2% (n = 4), respectively. These results strongly suggest that I_{Ks} channel is inhibited by endogenous membrane PtdIns(4,5)P₂ through the electrostatic interaction with the negatively charged head group on PtdIns(4,5)P₂. Potentiation of I_{Ks} by P2Y receptor stimulation with 50 μM ATP was almost totally abolished when PtdIns(4,5)P₂ was included in the pipette solution, suggesting that depletion of membrane PtdIns(4,5)P₂ is involved in the potentiation of I_{Ks} by P2Y receptor stimulation. Thus, membrane PtdIns(4,5)P₂ may act as an important physiological regulator of I_{Ks} in guinea pig atrial myocytes.

The delayed rectifier K⁺ current (I_{K})¹ is activated by membrane depolarization and thereby provides an outward current, which is essential for initiating phase 3 repolarization of the action potential in cardiac muscle. Two kinetically and pharmacologically distinct components of I_{K}, I_{Ka} (rapid) and I_{Ks} (slow), have been identified in cardiac myocytes from various mammalian species (1, 2) including humans (3). The KCNQ1 gene encodes the pore-forming a subunit, K_{a}LQT1, that assembles with an accessory β subunit minK (I_{Kr}) protein (encoded by KCNE1 gene) to produce the I_{Kr} channel (4, 5), whereas the HERG (human ether-a-go-go-related gene) product forms the pore-forming subunit of the I_{Kr} channel (6, 7). Mutations in any of these genes have been linked to long QT syndrome, an inherited cardiac arrhythmia characterized by abnormal ventricular repolarization and a high risk for sudden cardiac death (8).

Previous studies have demonstrated that both I_{K} and I_{Kr} represent relevant targets for the actions of autonomic neurotransmitters, hormones, intracellular messengers, and exogenous drugs. I_{K} is modulated by protein kinase A, protein kinase C (PKC), and intracellular free Ca^{2+} (9–11); whereas I_{Kr} is sensitive to inhibition not only by methanesulfonamide drugs but also by a wide range of other medications including some anti-arrhythmic, anti-histamic, antibiotic, and psychoactive agents (8). These modulations of I_{K} and I_{Kr} profoundly affect the repolarization process of the cardiac action potential and thereby mediate the regulation of cardiac function by these intracellular signaling molecules or exogenous pharmacological compounds.

Stimulation of many G_{q}-coupled receptors activates phosphoinositide-specific phospholipase C (PLC), leading to the hydrolysis of PtdIns(4,5)P₂ to form inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol. These two second messengers, respectively, activate InsP₃ receptors on intracellular Ca^{2+} stores and PKC and thereby play a pivotal role in transmitting the extracellular signals to the cellular responses. In addition to serving as a precursor for the production of second messengers, membrane PtdIns(4,5)P₂ has been shown to directly interact with many ion channels and transporters. Hilgemann and Ball (12) revealed for the first time that the Na⁺/Ca^{2+} exchanger and ATP-sensitive K⁺ channels are positively regulated by endogenous PtdIns(4,5)P₂ in guinea pig cardiac cell membranes. Recent studies have demonstrated that PtdIns(4,5)P₂ modulates the function of a number of ion channels, including many inwardly rectifying K⁺ channels (13–15), the HERG K⁺ channel (16) and voltage-gated P/Q-type Ca^{2+} channels (17).

We have previously shown that, in guinea pig cardiac myocytes, the stimulation of P2Y receptor (G protein-coupled ATP receptor) by extracellular ATP markedly enhances I_{Ks} through a mechanism that appears to be independent of either the activation of PKC or the elevation of intracellular Ca^{2+} (18, 19). Moreover, it was recently demonstrated in guinea pig ventricular myocytes that the stimulation of P2Y receptor evokes a pronounced reduction in the activity of ATP-sensitive K⁺ channels through a PLC-induced depletion of membrane PtdIns(4,5)P₂ (20). Therefore, this study was designed to extend...
explore the possible role for membrane PtdIns(4,5)P$_2$ in the regulation of Ik$_{Ca}$ in native mammalian cardiac myocytes. Our results provide the first detailed evidence to suggest that endogenous membrane PtdIns(4,5)P$_2$ has a potent inhibitory action on Ik$_{Ca}$ channels in guinea pig cardiac myocytes.

**EXPERIMENTAL PROCEDURES**

**Isolation of Atrial Myocytes—**All of the experiments conformed with *The Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication number 85–23, revised 1996) and were approved by the institution's Animal Care and Use Committee. Single atrial myocytes were obtained from the heart of adult Hartley guinea pigs using an enzymatic dissociation procedure as described previously (21, 22). The hearts were retrogradely perfused with nominally Ca$^{2+}$-free Tyrode solution containing 0.4 mg ml$^{-1}$ collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 7–10 min through the coronary artery. After the enzyme treatment, single myocytes were dissociated and stored in a high K$^+$, low Cl$^-$ Krebs-buffre solution (22).

**Solutions and Chemicals—**Normal Tyrode solution contained (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 0.33 NaH$_2$PO$_4$, 5.5 glucose, and 5.0 Hepes (pH adjusted to 7.4 with NaOH). The extracellular solution used for measuring whole-cell $I_{Ca}$ was normal Tyrode solution supplemented with 0.4 μM nisoldipine (a generous gift from Bayer) and 5 μM E-4031 (N-(4-((1-(2-(6-methyl-2-pyridinyl)ethyl)-4-piperidinyl)carbonyl)phenyl)methanesulfonamide, dihydrochlordihydurate) (Wako). In some experiments, chromanol 293B (trans-6-cyano-4-(N-ethylsulphonyl-N-methylamino)-3-hydroxy-2,2-dimethyl-chromane) (a generous gift from Aventis Pharma Deutschland GmbH, Frankfurt, Germany) was added to the extracellular solution to selectively block Ik$_{Ca}$ (23, 24). The control pipette solution contained (in mM): 70 potassium aspartate, 50 KCl, 10 KH$_2$PO$_4$, 3 Na$_2$ATP (Sigma), 0.1 Li$_2$GTP (Roche Diagnostics), 5 EGTA, and 5 Hepes (pH adjusted to 7.2 with KOH). The concentration of free Ca$^{2+}$ and Mg$^{2+}$ in the pipette solution was calculated to be $1.5 \times 10^{-5}$ M ($\mu$Ca $= 9.8$) and $3.7 \times 10^{-5}$ M, respectively (25, 26). When used, wortmannin (Wako), LY-294002 (a specific inhibitor of phosphatidylinositol 3-kinase (PtdIns 3-kinase), resulting in a substantial reduction in the cellular levels of both PtdIns(4,5)P$_2$ and phosphatidylinositol 4-phosphate (28–30). To elucidate the action of endogenous membrane PtdIns(4,5)P$_2$ on Ik$_{Ca}$, we first examined the effect of 50 μM wortmannin on Ik$_{Ca}$ in guinea pig atrial myocytes. Fig. 1A demonstrates a representative result showing the time course of changes in the amplitude of Ik$_{Ca}$.recorded from an atrial myocyte dialyzed with a pipette solution containing 50 μM wortmannin. Immediately after gaining access to the cell interior (rupture of the patch membrane), the membrane potential was held at −50 mV and was then repetitively (every 20 s) depolarized to a test potential of +50 mV for 2 s to evoke Ik$_{Ca}$. The amplitude of the Ik$_{Ca}$ tail current elicited upon repolarization to a holding potential of −50 mV, which reflects the degree of Ik$_{Ca}$ activation during the preceding depolarizing step to +30 mV, was progressively increased as wortmannin was dialyzing into the cell and typically reached a maximum response −10–15 min after rupture of the patch membrane (Fig. 1A). The effect of internal application of wortmannin on Ik$_{Ca}$ was quantitatively assessed by normalizing the amplitude of Ik$_{Ca}$ tail current measured −10–15 min after establishing whole-cell mode with reference to that obtained shortly (<30 s) after whole-cell mode. In a total of nine myocytes, the addition of 50 μM wortmannin to the pipette solution increased the amplitude of Ik$_{Ca}$ tail current to 208.5 ± 14.6% within a period of 10–15 min (see also Fig. 3).

In a separate set of experiments, the amplitude of Ik$_{Ca}$ tail current, measured using the same voltage-clamp protocol (2-s depolarization to +50 mV from a holding potential of −50 mV applied at a 20-s interval), was found to gradually decrease to 88.5 ± 2.1% (n = 11) of initial magnitude over the same time period (−10–15 min) when the cell was dialyzed with control pipette solution (see also Figs. 3 and 5A). Furthermore, the time course of changes in Ik$_{Ca}$ tail current was not appreciably affected by internal dialysis with the PtdIns 3-kinase inhibitor LY-294002 (31) at 10 μM (decrease to 89.4 ± 2.5% of initial values, n = 5, Fig. 3) or wortmannin at 50 nM (decrease to 87.1 ± 2.6% of initial values; n = 7, Fig. 3), a concentration that was demonstrated to fully inhibit PtdIns 3-kinase with a relatively small effect on PtdIns 4-kinase (28). Similarly, the myosin light chain kinase inhibitor ML-7 (5 μM) also did not mimic the stimulatory action of 50 μM wortmannin on Ik$_{Ca}$ over the same time period (decrease to 88.2 ± 2.1% of initial values, n = 5, Fig. 3). Taken together, it seems most reasonable to assume that a progressive increase in Ik$_{Ca}$ observed during internal dialysis with 50 μM wortmannin arises primarily from the inhibition of PtdIns 4-kinase, but not from the inhibition of the PtdIns 3-kinase or myosin light chain kinase, by this compound.

To evaluate whether the voltage dependence of Ik$_{Ca}$ activation is affected by internally applied wortmannin, depolarizing voltage steps to various test potentials between −40 and +50 mV were applied from a holding potential of −50 mV shortly (within −1 min) and approximately 15 min after a rupture of the patch membrane with pipette solution containing 50 μM wortmannin (Fig. 1B). Ik$_{Ca}$ recorded within −1 min of patch rupture (Fig. 1B, left panel) was expected to be little, if any, affected by internally applied wortmannin and was therefore...
Fig. 1. Enhancement of $I_{Ks}$ by internal dialysis with wortmannin. $A$, time course of changes in $I_{Ks}$ during dialysis of the cell interior with a pipette solution containing 50 μM wortmannin. An atrial myocyte was repetitively depolarized every 20 s from a holding potential of −50 mV to +50 mV for 2 s, and the amplitude of $I_{Ks}$ tail current elicited upon repolarization to the holding potential was plotted as a function of time after the rupture of the patch membrane. $B$, superimposed current traces during 2-s voltage-clamp steps to membrane potentials of −40 to +50 mV in 10-mV steps applied from a holding potential of −50 mV recorded shortly (within −1 min, left panel) and −15 min (right panel) after rupture of the patch membrane with a pipette solution containing 50 μM wortmannin. Current records shown in panels $A$ and $B$ were obtained from distinct myocytes. $C$, $I$–$V$ relationships for $I_{Ks}$ tail currents recorded shortly (open circles) and −15 min (filled circles) after a patch rupture from the data shown in panel $B$.

regarded as representing control traces. In this example, $I_{Ks}$, which was recorded −15 min after patch rupture (Fig. 1B, right panel), exhibited a steady-state response to wortmannin, which was confirmed by monitoring the changes in the amplitude of $I_{Ks}$ in response to repetitive depolarizing voltage steps to +30 mV (data not shown).

Fig. 1C shows $I$–$V$ relationships for $I_{Ks}$ tail current recorded under these two conditions (open circles, within −1 min after patch rupture; filled circles, −15 min after patch rupture). The amplitude of $I_{Ks}$ tail current was increased with internal dialysis of 50 μM wortmannin by a factor of more than two at each test potential. The smooth curves through the data points (Fig. 1C) represent the least-squares fit to a Boltzmann equation shown in Equation 1,

$$I_{Ks,tail} = I_{Ks,tail,max}(1 + \exp((V_{m} - V_{1/2})/k))$$

(Eq. 1)

where $I_{Ks,tail,max}$ is the fitted maximal tail current amplitude, $V_{1/2}$ is the half-maximal voltage, $V_{m}$ is the test potential, and $k$ is the slope factor. The $V_{1/2}$ and $k$ values were 5.5 ± 2.4 and 11.5 ± 1.4 mV, respectively, for the data obtained within −1 min after patch rupture and 3.3 ± 1.0 and 11.6 ± 1.8 mV for the data recorded −15 min after patch rupture ($n = 4$), thus showing that the voltage dependence of $I_{Ks}$ activation was not significantly affected during internal dialysis with 50 μM wortmannin.

Effects of Anti-PtdIns(4,5)P$_2$ Monoclonal Antibody, PtdIns(4,5)P$_2$, and PtdIns(4,5)P$_2$ plus Wortmannin on $I_{Ks}$—To assess whether a possible reduction in the level of membrane PtdIns(4,5)P$_2$ associated with an addition of wortmannin (50 μM) resulted in a marked increase in $I_{Ks}$ (Fig. 1), we examined the effects of the addition of anti-PtdIns(4,5)P$_2$ monoclonal antibody (32) and exogenous PtdIns(4,5)P$_2$ on $I_{Ks}$ in guinea pig atrial myocytes (Fig. 2). Previous patch clamp experiments have clearly demonstrated that anti-PtdIns(4,5)P$_2$ monoclonal antibody added to cytoplasmic solutions in either inside-out or whole-cell mode can prevent the PtdIns(4,5)P$_2$ interaction with several types of ion channel proteins by specifically binding to endogenous PtdIns(4,5)P$_2$ (14–17). Fig. 2A demonstrates a representative example of the $I_{Ks}$ response to internal application of the 40-fold diluted anti-PtdIns(4,5)P$_2$ monoclonal antibody. The amplitude of $I_{Ks}$ tail current elicited upon repolarization to −50 mV following 2-s depolarization to +30 mV was progressively increased to 198.4 ± 19.9% ($n = 5$, Fig. 3) of its initial magnitude over a period of 10–15 min after patch rupture. In contrast, the addition of control mouse IgG to the pipette solution did not appreciably affect the time course of changes in the amplitude of $I_{Ks}$ tail current (decrease to 87.9 ± 2.6% of initial values after 15 min of whole-cell mode, $n = 5$, Fig. 3). These results indicate that blocking the action of endogenous PtdIns(4,5)P$_2$ leads to a markedly potentiation of $I_{Ks}$.

We then tested the effects of exogenous PtdIns(4,5)P$_2$ at concentrations of 10 and 100 μM on $I_{Ks}$. The amplitude of $I_{Ks}$ tail current in myocytes loaded intracellularly with 10 and 100
Effects of anti-PtdIns(4,5)P$_2$ monoclonal antibody, PtdIns(4,5)P$_2$ or PtdIns(4,5)P$_2$ plus wortmannin on $I_{Ks}$. Atrial myocytes loaded with the 40-fold diluted anti-PtdIns(4,5)P$_2$ monoclonal antibody (A), 100 μM PtdIns(4,5)P$_2$ (B), or 50 μM wortmannin plus 100 μM PtdIns(4,5)P$_2$ (C) through a recording pipette were depolarized every 20 s from a holding potential of -50 to +30 mV for 2 s. The amplitude of the tail current elicited upon return to the holding potential was plotted as a function of time after gaining access to the cell interior.

Fig. 2. Effects of anti-PtdIns(4,5)P$_2$ monoclonal antibody, PtdIns(4,5)P$_2$ or PtdIns(4,5)P$_2$ plus wortmannin on $I_{Ks}$. Atrial myocytes loaded with the 40-fold diluted anti-PtdIns(4,5)P$_2$ monoclonal antibody (A), 100 μM PtdIns(4,5)P$_2$ (B), or 50 μM wortmannin plus 100 μM PtdIns(4,5)P$_2$ (C) through a recording pipette were depolarized every 20 s from a holding potential of -50 to +30 mV for 2 s. The amplitude of the tail current elicited upon return to the holding potential was plotted as a function of time after gaining access to the cell interior.
potent inhibitory action on \( I_{\text{Ks}} \) and that disruption of PtdIns(4,5)P2 interaction with \( I_{\text{Ks}} \) channels results in a marked increase in \( I_{\text{Ks}} \) in guinea pig atrial myocytes. It has previously been demonstrated in guinea pig cardiac myocytes that the activation of P2Y receptor stimulates PLC and thereby depletes plasma membrane PtdIns(4,5)P2 that is required for maintenance of the activity of the ATP-sensitive K+ channels (20).

We next checked whether the possible reduction of PtdIns(4,5)P2 levels mediates potentiation of \( I_{\text{Ks}} \) associated with P2Y receptor stimulation (18, 19). As demonstrated in Fig. 5, the amplitude of \( I_{\text{Ks}} \) current usually underwent some rundown (10–20% of initial magnitude, see also Fig. 3) over the first 5–10 min following establishment of whole-cell mode with control pipette solution. After this current rundown was allowed to reach a steady-state level, the cell was then exposed to 50 \( \mu \text{M} \) ATP, which typically evoked an increase in amplitude of \( I_{\text{Ks}} \) by 103.5 ± 10.6% (\( n = 5 \); Fig. 5D) when evaluated by monitoring the changes in tail current amplitude elicited upon repolarization to −50 mV following 2-s depolarization to +30 mV. In guinea pig atrial myocytes, increasing the concentration of ATP above 50 \( \mu \text{M} \) caused no further increase in \( I_{\text{Ks}} \) (18), showing that 50 \( \mu \text{M} \) ATP evokes a maximal enhancement of \( I_{\text{Ks}} \).

Previous whole-cell patch clamp experiments have demonstrated that suppression of the muscarinic K+ channels by \( \alpha_1 \)-adrenergic agonist phenylephrine is greatly attenuated when PtdIns(4,5)P2 is included in the pipette solution (34), thus indicating that substantial reductions of plasma membrane PtdIns(4,5)P2 associated with receptor-mediated PLC activation can be effectively compensated by exogenous PtdIns(4,5)P2 applied through a recording pipette. Therefore, we checked whether extracellular ATP at a maximally effective concentration (50 \( \mu \text{M} \)) can increase the amplitude of \( I_{\text{Ks}} \) in atrial myocytes loaded with 100 \( \mu \text{M} \) PtdIns(4,5)P2 (Fig. 5B) where membrane PtdIns(4,5)P2 level is expected to be kept relatively stable during PLC activation. In these myocytes, as expected, a progressive and marked decrease in the amplitude of \( I_{\text{Ks}} \) was consistently observed following rupture of the patch membrane. The effect of bath application of ATP was then tested after the declining response reached a steady-state level (10–15 min after whole-cell mode, see also Fig. 2B). In a total of five myocytes loaded with 100 \( \mu \text{M} \) PtdIns(4,5)P2, ATP at 50 \( \mu \text{M} \) increased the amplitude of \( I_{\text{Ks}} \) tail current only by 16.1 ± 9.9% (Fig. 5D), which is significantly smaller than the control response. The stimulatory effect of extracellular ATP was thus largely abolished by the exogenously applied PtdIns(4,5)P2. This result strongly suggests that a reduction of PtdIns(4,5)P2 levels in the plasma membrane is primarily involved in the potentiation of \( I_{\text{Ks}} \) associated with the stimulation of P2Y receptor.

We also checked whether extracellular ATP can further enhance \( I_{\text{Ks}} \) in wortmannin-treated myocytes in which membrane PtdIns(4,5)P2 level is expected to be substantially reduced (30). As demonstrated in Fig. 5C, extracellular ATP potentiated the amplitude of \( I_{\text{Ks}} \) tail current by 27.7 ± 12.4% (\( n = 6 \); Fig. 5D) in myocytes loaded with 50 \( \mu \text{M} \) wortmannin, thus showing that stimulatory effect of extracellular ATP was partly but not totally abolished in the presence of wortmannin. Assuming that endogenous membrane PtdIns(4,5)P2 was reduced to some extent by treatment with wortmannin (30), this result was also consistent with the view that extracellular ATP potentiates \( I_{\text{Ks}} \) through a reduction of membrane PtdIns(4,5)P2.

**Effect of \( I_{\text{Ks}} \) Potentiation by Extracellular ATP on Action Potentials in Guinea Pig Atrial Myocytes**—It has previously been demonstrated that bath application of ATP at micromolar concentrations significantly shortens the action potential duration (APD) in guinea pig atrial myocytes (35, 36). It is assumed that the APD shortening by extracellular ATP is primarily mediated through its stimulatory action on two distinct K+ channels, namely, \( I_{\text{Kss}} \) (18, 19) and the muscarinic K+ channels (35–38). Whereas potentiation of \( I_{\text{Ks}} \) by extracellular ATP remains rather stable over a period of at least 5 min during exposure to the agonist, activation of \( I_{\text{KACH}} \) by micro-}

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**Fig. 3. Summary of \( I_{\text{Ks}} \) response to internal dialysis with various test reagents.** \( I_{\text{Ks}} \) was repetitively activated by 2-s depolarization to +30 mV from a holding potential of −50 mV immediately after patch rupture with a control pipette solution or the pipette solution containing various test reagents as indicated. The amplitude of tail current elicited upon return to the holding potential at a steady-state response was normalized to that obtained briefly after patch rupture. The columns and bars denote the means ± S.E., respectively. **, \( p < 0.01 \) compared with control group.
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Fig. 4. Potentiation of IKs by internal dialysis with neomycin or AlCl3. Atrial myocytes were loaded with either 50 μM neomycin (A and B) or 50 μM AlCl3 (C and D) through a recording pipette. A and C, superimposed current traces during 2-s voltage-clamp steps to membrane potentials of −40 to +50 mV in 10-mV steps applied from a holding potential of −50 mV recorded shortly (within ~1 min, left panel) and ~10 min (right panel) after rupture of the patch membrane. B and D, I-V relationships for IKs tail currents obtained from the records in A and C, respectively, shortly after (within ~1 min, open circles) and ~10 min (filled circles) after rupture of patch membrane.

DISCUSSION

The present result that IKs in guinea pig atrial myocytes is markedly potentiated by the application of anti-PtdIns(4,5)P2 monoclonal antibody but is reduced by the addition of exogenous PtdIns(4,5)P2 (Fig. 2, A and B) strongly suggests that endogenous membrane PtdIns(4,5)P2 exerts an inhibitory action on IKs. Polyvalent cations (such as neomycin) that preferentially neutralize the anionic head of PtdIns(4,5)P2 were previously demonstrated to antagonize the actions of PtdIns(4,5)P2 on ion channels or transporters (12–14), suggesting a role for electrostatic interactions of PtdIns(4,5)P2 with ion channel or transporter proteins. In fact, site-directed mutagenesis studies on inwardly rectifying K+ channel subunits have identified several sites (basic residues) that are important for interaction with membrane PtdIns(4,5)P2, particularly within the proximal C terminus (13, 14). The present finding that the addition of neomycin or aluminum results in a marked potentiation of IKs (Fig. 4) also supports the view that an inhibitory action of PtdIns(4,5)P2 is mediated through electrostatic interactions with basic residues in IKs channel proteins. Whereas amino acid sequences of IKs channels expressed in guinea pig heart are presently unknown, similarities in electrophysiological and pharmacological properties of IKs between humans and guinea pigs (24) may indicate a high sequence homology of the channel protein between these two species. It should be noted that there are some basic residues cluster in the cytoplasmic faces of the C terminus in human KvLQT1 (e.g., KKKSVVVKKKKFKLDK298) (4, 5), which might be candidates for sites of interaction with PtdIns(4,5)P2. Previous studies have provided strong evidence to suggest that membrane PtdIns(4,5)P2 is fundamental for the activation of inwardly rectifying K+ channels (12–15) and some of voltage-dependent K+ channels (16, 39, 40). The present study identifies the presence of the K+ channel that is inhibited by membrane PtdIns(4,5)P2, and thus, it appears that PtdIns(4,5)P2 acts as both stimulatory and inhibitory factors in the regulation of K+ channel function.

It has been demonstrated in bovine adrenal glomerulosa cells that PtdIns 4-kinase, which regulates the synthesis of hormone-sensitive pools of polyphosphoinositides, is susceptible to inhibition by micromolar concentrations of wortmannin (28). This wortmannin-sensitive isoform of PtdIns 4-kinase (PtdIns 4-kinase β) has been demonstrated to be widely expressed in a variety of tissues including the heart (29). The present result that the stimulatory action of 50 μM wortmannin on IKs is completely abolished by the concomitant presence of exogenous PtdIns(4,5)P2 (Fig. 2 C and D) strongly suggests that potentiation of IKs evoked by wortmannin arises primarily from a reduction in the membrane PtdIns(4,5)P2 levels, probably because of the inhibition of PtdIns 4-kinase. It has been shown in SH-SY5Y human neuroblastoma cells that the blockade of PtdIns 4-kinase by 10 μM wortmannin alone results in a substantial reduction in the level of PtdIns(4,5)P2 as well as phosphatidylinositol 4-phosphate in the plasma membrane under...
basal conditions (30). Furthermore, incubation of these cells with 10 μM wortmannin for 10 min markedly attenuates transient peaks of both InsP₃ accumulation and intracellular Ca²⁺ elevation evoked by subsequent stimulation of M₃-muscarinic receptor. These data support the view that micromolar concentrations of wortmannin not only inhibits the replenishment of PtdIns(4,5)P₂ following PLC-coupled receptor-mediated depletion (30, 41, 42) but also substantially reduces membrane PtdIns(4,5)P₂ pool even under basal conditions through the inhibition of wortmannin-sensitive isoform of PtdIns 4-kinase (30). The present finding that extracellular ATP is still effective at potentiating $I_{Ks}$ in wortmannin-treated atrial myocytes (Fig. 5C) may reflect partial (but not total) depletion of membrane PtdIns(4,5)P₂ associated with the inhibition of $I_{Ks}$.

Whereas most metabotropic P2Y receptors are coupled to PLC through the pertussis toxin-insensitive G protein Gq, leading to the hydrolysis of PtdIns(4,5)P₂ to yield diacylglycerol and InsP₃ (43), an enhancement of $I_{Ks}$ through P2Y receptor stimulation in guinea pig atrial myocytes was not appreciably affected by the presence of either 10 μM H-7 or 20 mM BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid), thus suggesting that activation of PKC and elevation of intracellular Ca²⁺ are not primarily involved (18, 19). It has recently been shown in guinea pig ventricular myocytes that extracellular ATP depletes membrane PtdIns(4,5)P₂ through the stimulation of the Gq-PLC pathway, leading to reductions of the activity of ATP-sensitive K⁺ channels (20). The present result that the addition of exogenous PtdIns(4,5)P₂ greatly prevents the stimulatory effect of extracellular ATP on $I_{Ks}$ (Fig. 5B) is consistent with the view that this stimulatory action of ATP is mediated through reductions in membrane PtdIns(4,5)P₂. Thus, alterations of membrane PtdIns(4,5)P₂ levels via P2Y receptor stimulation appear to represent an effective signaling mechanism for regulation of $I_{Ks}$ in guinea pig cardiac myocytes.

However, it was previously demonstrated in guinea pig ventricular myocytes that endothelin enhances $I_{Ks}$ via PLC-mediated PKC activation and intracellular Ca²⁺ mobilization (44). It was also shown that α₁-adrenergic receptors, α₁-adrenergic receptors, and endothelin receptors seemed to be all coupled to the Gq-PLC pathway to potentiate $I_{Ks}$, but signaling molecule associated with P2Y receptors appeared to be distinct from that associated with α₁-adrenergic receptors or endothelin receptors. Although the precise mechanism for this difference remains unknown, it was recently demonstrated in mouse atrial myocytes that the muscarinic K⁺ current is greatly reduced by the membrane PtdIns(4,5)P₂ depletion induced by α₁-adrenergic receptors (through PLC activation) but is not significantly affected by the PtdIns(4,5)P₂ reduction evoked by PLC-coupled M₁ (or M₃/M₅) muscarinic receptors (46). This observation strongly suggests that the muscarinic K⁺ channel is preferentially co-localized with α₁-adrenergic receptor but not with M₁ (or M₃/M₅) muscarinic receptor. Because the concentration of PtdIns(4,5)P₂ has been suggested to change locally within the plasma membrane (47), PtdIns(4,5)P₂ metabolism coupled to P2Y receptor
might be more directly linked to the $I_{Ks}$ channel proteins. It has been demonstrated in guinea pig cardiac myocytes that raising the intracellular Ca$^{2+}$ concentrations over 0.1 mM evokes significant increase in the amplitude of $I_{Ks}$ (9, 11). This intracellular Ca$^{2+}$-dependent enhancement of $I_{Ks}$ has been assumed to play an important role in limiting an amount of Ca$^{2+}$ entry in Ca$^{2+}$-overloaded myocardium. However, the cellular mechanism underlying this Ca$^{2+}$-dependent enhancement of $I_{Ks}$ has yet to be fully characterized. It was previously shown that PLC in cardiac membranes is activated by intracellular free Ca$^{2+}$ at concentrations of $\gtrsim 0.5$ mM (12). Thus, the potentiation of $I_{Ks}$ and activation of PLC are evoked by intracellular free Ca$^{2+}$ within a relatively similar concentration range. Therefore, it is reasonable to assume that an elevation of intracellular Ca$^{2+}$ levels within physiological range ($\lesssim 1$–$2$ mM) is accompanied by the activation of PLC, which should result in substantial depletion of membrane PtdIns(4,5)$P_2$ and subsequent potentiation of $I_{Ks}$. Xie et al. (48) have suggested that intracellular Ca$^{2+}$-induced rundown of the ATP-sensitive K$^+$ channels is evoked by the Ca$^{2+}$-dependent activation of PLC and resultant depletion of membrane PtdIns(4,5)$P_2$ (48). Alternatively, it is probable that intracellular Ca$^{2+}$ interrupts the electrostatic interaction between membrane PtdIns(4,5)$P_2$ and $I_{Ks}$ channels (48), which is again expected to result in potentiation of $I_{Ks}$. Thus, it is of interest to elucidate whether such a PtdIns(4,5)$P_2$-dependent mechanism is indeed involved in the intracellular Ca$^{2+}$-evoked $I_{Ks}$ potentiation in cardiac myocytes.

In recent years, it has been shown that the KCNQ1/KCNE1 heteromeric channel, the molecular constituents of cardiac $I_{Ks}$ (4, 5), is activated by exogenously applied PtdIns(4,5)$P_2$ (40) and $I_{Ks}$ channels (48), which is again expected to result in potentiation of $I_{Ks}$. Thus, it is of interest to elucidate whether such a PtdIns(4,5)$P_2$-dependent mechanism is indeed involved in the intracellular Ca$^{2+}$-evoked $I_{Ks}$ potentiation in cardiac myocytes.
PtdIns(4,5)P_2 Regulation of Cardiac I_{Ks} Potassium Current

...ventral stimuli. It has recently been shown that the application of 20 \mu M PtdIns(4,5)P_2 increases the recombinant KCNQ2/3 heteromeric current but that even a higher concentration (100 \mu M) produces at best a weak effect on the native M current (51). Alternatively, under the present experimental conditions, membrane PtdIns(4,5)P_2 level might change within a relatively modest range when various compounds are applied through a recording pipette in the whole-cell configuration and the present results do not necessarily rule out the possibility that I_{Ks} can be inhibited by a drastic decrease in membrane PtdIns(4,5)P_2 levels, as expected for the stimulation of heterologously expressed G_\alpha_PLC-linked membrane receptors.

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REFERENCES

1. Sanguinetti, M. C., and Jurkiewicz, N. K. (1990) J. Gen. Physiol. 96, 195–215
2. Liu, D.-W., and Antzelevitch, C. (1995) Circ. Res. 76, 361–365
3. Li, G.-R., Feng, J., Yue, L., Carrier, M., and Nattel, S. (1996) Circ. Res. 78, 689–696
4. Barhanin, J., Lesage, F., Guillemare, E., Fink, M., Lazdunski, M., and Romey, G. (1996) Nature 384, 78–80
5. Sanguinetti, M. C., Curran, M. E., Zeu, A., Shen, J., Spector, P. S., Atkinson, D. L., and Keating, M. T. (1996) Nature 384, 80–83
6. Sanguinetti, M. C., Jiang, C., Curran, M. E., and Keating, M. T. (1995) Cell 81, 299–307
7. Trudeau, M. C., Warmke, J. W., Ganetzky, B., and Robertson, G. A. (1995) Science 269, 92–95
8. Keating, M. T., and Sanguinetti, M. C. (2001) Cell 104, 569–580
9. Tohse, N., Kameyama, M., and Irisawa, H. (1987) Am. J. Physiol. 253, H1321–H1324
10. Walsh, K. B., and Kass, R. S. (1988) Science 242, 67–69
11. Tohse, N. (1990) Am. J. Physiol. 258, H1200–H1207
12. Hilgemann, D. W., and Ball, R. (1996) Science 273, 956–959
13. Pan, Z., and Makielki, J. C. (1997) J. Biol. Chem. 272, 5388–5395
14. Huang, C. L., Feng, S., and Hilgemann, D. W. (1998) Nature 391, 803–806
15. Sui, J. L., Petit-Jacques, J., and Logothetis, D. E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1307–1312
16. Bian, J., Cui, J., and McDonald, T. V. (2001) Circ. Res. 89, 1168–1176
17. Wu, L., Bauer, C. S., Zhen, X.-G., Xie, C., and Yang, J. (2002) Nature 419, 947–952
18. Matsuura, H., Tsuruhara, Y., Sakaguchi, M., and Ehara, T. (1996) J. Physiol. (Lond.) 490, 647–658
19. Matsuura, H., and Ehara, T. (1997) J. Physiol. (Lond.) 503, 45–54
20. Oketani, N., Kakei, M., Ichinari, K., Okamura, M., Miyamura, A., Nakazaki, M., Ito, S., and Tei, C. (2002) Am. J. Physiol. 282, H757–H765
21. Powell, T., Terrar, D. A., and Twist, V. W. (1980) J. Physiol. (Lond.) 302, 131–153
22. Isenberg, G., and Klocckner, U. (1982) Pflügers Arch. Eur. J. Physiol. 395, 6–18
23. Busch, A. E., Suessbrich, H., Waldegg, S., Sailer, E., Gregor, R., Lang, H.-G., Lang, F., Gibson, K. J., and Maylie, J. G. (1996) Pflügers Arch. Eur. J. Physiol. 432, 1094–1096
24. Zhao, R. F., Gaspo, R., Bers, N. A., Shryock, J., and Belardinelli, L. (1991) Circ. Res. 69, 1177–1184
25. Tsien, R. Y., and Bink, T. J. (1980) Biochim. Biophys. Acta 599, 623–638
26. Hanill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Pflügers Arch. Eur. J. Physiol. 391, 85–100
27. Nakanishi, S., Catt, K. J., and Brown, D. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5317–5321
28. Meyers, R., and Cantley, L. C. (1997) J. Biol. Chem. 272, 4384–4390
29. Williams, C. (1998) J. Biol. Chem. 273, 5037–5046
30. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
31. Fukushima, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S., and Takenawa, T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9057–9061
32. Nakanishi, C., Feng, S., Mao, Y., Shrammat, I., Yamamato, M., Earnest, S., Lemmon, M., and Hilgemann, D. W. (2002) Am. J. Physiol. 283, C223–C234
33. Meyer, T., Wellner-Kienitz, M.-C., Biewald, A., Bender, K., Eickel, A., and Pott, L. (2001) J. Biol. Chem. 276, 5659–5668
34. Bagazzi, E., Wu, S.-N., Shroyock, J., and Belardinelli, L. (1991) Circ. Res. 68, 1035–1044
35. Har, Y., and Nakaya, H. (1997) Eur. J. Pharmacol. 324, 295–303
36. Matsuura, H., Sakaguchi, M., Tsuruhara, Y., and Ehara, T. (1996) J. Physiol. (Lond.) 490, 659–671
37. Matsuura, H., and Ehara, T. (1996) J. Physiol. (Lond.) 497, 379–393
38. Suh, B.-C., and Hille, B. (2002) Neuron 35, 507–520
39. Zhang, H., Craciun, L. C., Mirshahi, T., Rohnes, T., Lopes, C. M. B., Jin, T., and Logothetis, D. E. (2003) Neuron 37, 963–975
40. Xie, L. H., Horie, M., and Takano, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15292–15297
41. Cho, H., Nam, G. B., Lee, S. H., Earm, Y. E., and Ho, W. K. (2001) J. Biol. Chem. 276, 159–164
42. Ralevic, V., and Burnstock, G. (1998) Pharmacol. Rev. 50, 413–490
43. Habuchi, Y., Tanaka, H., Fukuraku, T., Suzuki, Y., Takahashi, H., and Yoshimura, M. (1992) Am. J. Physiol. 262, H345–H354
44. Tohse, N., Nakaya, H., and Kanno, M. (1992) Circ. Res. 71, 1141–1146
45. Cho, H., Hwang, J. Y., Kim, D., Shin, H. S., Kim, Y., Earm, Y. E., and Ho, W. K. (2002) J. Biol. Chem. 277, 27742–27747
46. Botelho, R. J., Feruela, M., Dierckman, R., Anderson, R., Wells, A., York, J. D., Meyer, T., and Grinstein, S. (2000) J. Cell Biol. 151, 1353–1367
47. Xie, L. H., Takano, M., Kakei, M., Okamura, M., and Noma, A. (1999) J. Physiol. (Lond.) 514, 655–665
48. Louisoumar, G., Park, K.-H., Bellocq, C., Baro, I., Charpentier, F., and Esca, D. (2003) EMBO J. 20, 5412–5421
49. Selyanko, A. A., Hatley, J. K., Woods, I. C., Abogadie, P. C., Jentsch, T. J., and Brown, D. A. (2000) J. Physiol. (Lond.) 522, 349–355
50. Ford, C. P., Stemkowski, P. L., Light, P. E., and Smith, P. A. (2003) J. Neurosci. 23, 4931–4941