Depletion of Phosphatidylinositol 4,5-Bisphosphate by Activation of Phospholipase C-coupled Receptors Causes Slow Inhibition but Not Desensitization of G Protein-gated Inward Rectifier K⁺ Current in Atrial Myocytes*

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G protein-gated inward rectifier K⁺ current in atrial myocytes (IK(ACh)) upon stimulation with acetylcholine (ACh) shows a fast desensitizing component (t½ ≈ 5 s). After washout of ACh, IK(ACh) recovers from fast desensitization within < 30 s. A recent hypothesis suggests that fast desensitization is caused by depletion of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂), resulting from costimulation of phospholipase C (PLC)-coupled M₂ receptors (M₂AChR). The effects of stimulating two established PLC-coupled receptors, α-adrenergic and endothelin (ET₁), on IK(ACh) were studied in rat atrial myocytes. Stimulation of these receptors caused activation of IK(ACh) and inhibition of the M₂AChR-activated current. In myocytes loaded with GTP S (guanosine 5'-3-O-(thio)triphosphate), causing stable activation of IK(ACh), inhibition via α-agonists and ET-1 was studied in isolation. Stimulation of either type of receptor under this condition, via G₁₁, caused a slow inhibition (t½ ≈ 50 s) by about 70%. No comparable effect on GTP S-activated IK(ACh) was induced by ACh, suggesting that PLC-coupled M₂AChRs are not functionally expressed in rat atrial myocytes, which was supported by the finding that M₂AChR transcripts were not detected by reverse transcriptase-polymerase chain reaction in identified atrial myocytes. Supplementing the pipette solution with PtdIns(4,5)P₂ significantly reduced inhibition of IK(ACh) but had no effect on fast desensitization. From these data it is concluded that stimulation of PLC-coupled receptors causes slow inhibition of IK(ACh) by depletion of PtdIns(4,5)P₂ whereas fast desensitization of IK(ACh) is not related to PtdIns(4,5)P₂ depletion. As muscarinic stimulation by ACh does not exert inhibition of IK(ACh), comparable to stimulation of α₁ and ETA receptors, expression of functional PLC-coupled muscarinic receptors in rat atrial myocytes is unlikely.

In the heart, predominantly in supraventricular tissue, in various neurons and endocrine cells, stimulation of receptors coupled to pertussis toxin-sensitive G proteins (G₁₁) activates G protein-gated inward rectifying K⁺ (GIRK) channels, resulting in reduction of excitability (1–3). Upon exposure of a myocyte to an appropriate receptor agonist, the current is activated within less than 1 s. Activation is followed by desensitization, i.e. a decay of current, made up of several components with different kinetics of onset and recovery (4). Slow components seem to represent receptor desensitization, presumably mediated by phosphorylation via receptor kinases(s) and subsequent events such as receptor internalization and down-regulation (5, 6), but a fast component, developing with a time constant in the range of seconds, is localized downstream of the activating receptor. Several mechanisms have been proposed for fast desensitization. Shui et al. (7) suggested channel dephosphorylation as the underlying mechanism, whereas Hong et al. (8) reported the contribution of a nonidentified cytosolic protein. The major properties of fast desensitization, such as the rate of current decay, its membrane-delimited nature, and its dependence on receptor density, can be accounted for by a model relating fast desensitization, analogous to activation, to the nucleotide exchange and hydrolysis cycle of the G protein (9). More recently, an attractive novel mechanism has been proposed (10). These authors suggested that activation and fast desensitization of atrial IK(ACh) result from costimulation of two muscarinic receptors: the M₂ subtype (M₂AChR) which causes activation via G₁₁, whereas stimulation of the M₃ subtype (M₃AChR) via activation of G₁₃ stimulates phospholipase C (PLC) and subsequent depletion of phosphatidylinositol bisphosphate (PtdIns(4,5)P₂) in the inner leaflet of the membrane. Such a mechanism would be in line with the finding that PtdIns(4,5)P₂ is an important cofactor for activation of GIRK channels (11–13) and other members of the Kir channel family (14–16) and supports the notion that PtdIns(4,5)P₂ meets the criteria of a receptor-controlled second messenger (17). However, this hypothesis is inconsistent with one major property of acute desensitization, namely its heterologous nature. In atrial cells IK(ACh) can be activated not only by M₂AChR but also, e.g. by A₁ adenosine receptors (18, 19) and a sphingolipid receptor of the EDG family (20, 21). These studies have shown that ACh rapidly desensitizes the response to Ado and vice versa; correspondingly, sphingosine 1-phosphate, presumably via a receptor of the EDG family (22), desensitizes the response to ACh upon stimulation with acetylcholine; M₂AChR and M₃AChR, muscarinic M₂ and M₃ receptor, respectively; PLC, phospholipase C; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; GTP S, guanosine 5'-3-O-(thio)triphosphate; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ET, endothelin; Phe, phenylephrine; InsP₃, inositol (3,4,5)bisphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid.

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1 The abbreviations used are: GIRK, G protein-gated inward rectifier K⁺ channel; ACh, acetylcholine; IK(ACh) inwardly rectifier K⁺ current; M₂AChR and M₃AChR, muscarinic M₂ and M₃ receptor, respectively; PLC, phospholipase C; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; GTP S, guanosine 5'-3-O-(thio)triphosphate; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ET, endothelin; Phe, phenylephrine; InsP₃, inositol (3,4,5)bisphosphate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid.
and vice versa. In rat atrial myocytes overexpression of A1-receptors, a classic G<sub>o</sub>-coupled species, not only increased density of Ado-induced I<sub>K(ACh)</sub>, but also substantially enhanced fast desensitization of current activated via stimulation of this receptor (19).

In the present study we investigated the effect of stimulating two different intrinsic G<sub>q/11</sub>-coupled receptors on I<sub>K(ACh)</sub> in atrial myocytes. The results support the notion that stimulation of the PLC pathway via depletion of PtdIns(4,5)<sub>P2</sub> causes inhibition of I<sub>K(ACh)</sub>. However, this inhibition is slower than fast desensitization by a factor of 10. Moreover there is no evidence of significant expression of functional expression of a G<sub>q/11</sub>-coupled muscarinic receptor, such as M<sub>3</sub>AChR, in rat atrial myocytes.

**EXPERIMENTAL PROCEDURES**

**Isolation and Culture of Atrial Myocytes**—Experiments were performed with local ethics committee approval. Wistar Kyoto rats of either sex (around 200 g) were anesthetized by intravenous injection of urethane (1 g/kg). The chest was opened, and the heart was removed and mounted on the cannula of a sterile Langendorff apparatus for coronary perfusion at constant flow. The method of enzymatic isolation of atrial myocytes has been described elsewhere (see Ref. 4). The culture medium was fetal calf serum-free bicarbonate-buffered M199 (Life Technologies, Inc.) containing 25 μg/ml gentamycin (Sigma) and 25 μg/ml kanamycin (Sigma). Cells were plated at a low density (several thousand cells/dish) on 36-mm culture dishes. Medium was changed 24 h after plating and then every 2nd day. Myocytes were used experimentally from day 0 until day 4 after isolation. No effects of time in culture were found for the key experiments. No effects of time in culture were found for the key experiments. Myocytes were used experimentally from day 0 until day 4 after isolation. No effects of time in culture were found for the key experiments.

**Solutions and Chemicals**—For the patch clamp measurements an extracellular solution of the following composition was used (in mM): 120 NaCl, 20 KCl, 1.0 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 10 Hepes/NaOH, pH 7.4. The solution for filling the patch clamp pipettes for whole cell voltage clamp experiments contained (in mM) 110 potassium aspartate, 20 KCl, 1.0 NaCl, 1.0 MgCl<sub>2</sub>, 2.0 MgATP, 2.0 EGTA, 0.01 GTP or 0.5 GTP, 1.0 Hepes/KOH, pH 7.4. Standard chemicals for electrophysiological experiments were from Merck (Darmstadt, Germany), ECTE, Hepes, MgATP, adenosine, GTP, acetylcholine chloride, phenylephrine, methoxamine, and endothelium-1 were from Sigma; PtdIns(4,5)<sub>P2</sub>, Pasteurella multocida toxin and U23187 were from Calbiochem. 4-Diphenylacetoxy-N-methylpiperidine (4-DAMP) was from Tocris. Drugs were prepared as concentrated stock solutions either in distilled water or dimethyl sulfoxide. PtdIns(4,5)<sub>P2</sub> was dissolved in pipette solution at a nominal concentration of 500 μM. The solution was sonicated intermittently on ice for 30 min. Sonication was repeated each time before filling a new pipette. PtdIns(4,5)<sub>P2</sub> solutions were used for 1 day only.

**Current Measurement**—Membrane currents were measured using whole cell clamp. Pipettes were pulled from borosilicate glass and were filled with the solution listed above (DC resistance 4–6 megohms). Currents were measured by means of a patch clamp amplifier (LM/EPC 7, Darmstadt, Germany). Signals were analog filtered (corner frequency of 1–3 kHz), digitally sampled at 5 kHz, and stored on a computer, equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control and data acquisition. Experiments were performed at ambient temperature (22–24 °C). Cells were voltage clamped at −90 mV, i.e. negative to E<sub>K<sub>(−50 mV), resulting in inward K<sup>+</sup> currents. Current-voltage relations were determined by means of voltage ramps from −120 mV to +60 mV. Rapid superfusion of the cells for application and withdrawal of different solutions was performed by means of a custom made solenoid-operated flow system that permitted switching between up to six different solutions (t<sub>1/2</sub> ~100 ms). Performance of this system was dependent on the positionings at the outlet tube in relation to the cell studied. This was routinely optimized by measuring the time course of the blocking action of Ba<sup>2+</sup> on I<sub>K(ACh)</sub>. As shown previously, activation kinetics of I<sub>K(ACh)</sub> was not limited by the rate of agonist application (19).

**RT-PCR**—RNA from cell samples was isolated using Trizol reagent (Life Technologies, Inc.). RNA in 20 μl of RNase-free H<sub>2</sub>O (Qiagen, Hilden, Germany) was treated with 7.5 units of DNase I (Amersham Pharmacia Biotech) for 15 min at 37 °C. DNase was inactivated by adding 2.5 μl of 25 mM EDTA and heating at 65 °C for 10 min.

The culture medium was replaced by rinsing the cells for 30 min with a solution containing (in mM): 120 NaCl, 20 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Hepes/NaOH, pH 7.4. 0.1% diethyl pyrocarbonate was added. The solution was autoclaved before use. 10 myocytes were aspirated into glass capillaries with a ~30-μm tip diameter using a water hydraulic micromanipulator attached to an inverted microscope. Alternatively, the cells were scratched from the dish, and a corresponding volume of the cell suspension was aspirated, which should contain also nonmyocyte cells. Capillaries were rinsed with hexamethydisilazane (Sigma) and baked for 7 h at 240 °C before use. The content of the capillary (<0.5 μl) was expelled into a tube filled with 5 μl of lysis buffer containing: 0.8% (w/v) Nonidet P-40 substitute (Fluka, Buchs, Switzerland), 100 μg/ml yeast RNA in 20 μl of RNase-free H<sub>2</sub>O, pH 7.4. 0.1% diethyl pyrocarbonate was added. The solution was autoclaved before use. 10 myocytes were aspirated into glass capillaries with a ~30-μm tip diameter using a water hydraulic micromanipulator attached to an inverted microscope. Alternatively, the cells were scratched from the dish, and a corresponding volume of the cell suspension was aspirated, which should contain also nonmyocyte cells. Capillaries were rinsed with hexamethydisilazane (Sigma) and baked for 7 h at 240 °C before use. The content of the capillary (<0.5 μl) was expelled into a tube filled with 5 μl of lysis buffer containing: 0.8% (w/v) Nonidet P-40 substitute (Fluka, Buchs, Switzerland), 100 μg/ml yeast RNA in 20 μl of RNase-free H<sub>2</sub>O, pH 7.4. 0.1% diethyl pyrocarbonate was added. The solution was autoclaved before use. 10 myocytes were aspirated into glass capillaries with a ~30-μm tip diameter using a water hydraulic micromanipulator attached to an inverted microscope. Alternatively, the cells were scratched from the dish, and a corresponding volume of the cell suspension was aspirated, which should contain also nonmyocyte cells. Capillaries were rinsed with hexamethydisilazane (Sigma) and baked for 7 h at 240 °C before use. The content of the capillary (<0.5 μl) was expelled into a tube filled with 5 μl of lysis buffer containing: 0.8% (w/v) Nonidet P-40 substitute (Fluka, Buchs, Switzerland), 100 μg/ml yeast RNA in 20 μl of RNase-free H<sub>2</sub>O, pH 7.4. 0.1% diethyl pyrocarbonate was added. The solution was autoclaved before use. 10 myocytes were aspirated into glass capillaries with a ~30-μm tip diameter using a water hydraulic micromanipulator attached to an inverted microscope. Alternatively, the cells were scratched from the dish, and a corresponding volume of the cell suspension was aspirated, which should contain also nonmyocyte cells.
Inhibition of Atrial GIRK Current by PtIns(4,5)P_2 Depletion

**RESULTS**

**Definition of Fast Desensitization of Atrial I_{K(ACh)}**—As shown previously, atrial I_{K(ACh)} in the presence of an agonist shows different components of desensitization, depending on multiple factors such as agonist concentration, receptor density, speed, and duration of agonist exposure (4, 19, 23). The slower components, occurring upon agonist exposure on a time scale of minutes to hours, are supposed to reflect receptor desensitization, analogous to other receptor-G protein-effector pathways (4, 23–25). The fastest component, which occurs without a measurable delay upon exposure to ACh, represents a specific property of the pathway under study.

The key properties of fast desensitization are defined in Fig. 1. A typical experiment, qualitatively representative of 20 myocytes studied using such a protocol, is illustrated in Fig. 1C. After rapid activation of inward I_{K(ACh)} the current decayed to a quasi steady-state level of 54% of the peak value. The decay could be approximated by a single exponential with a half-time of 5.28 ± 0.43 s (n = 30). A second slower phase of desensitization within the short period of time contributed to the total decay in current with less than 5% and thus could be ignored when exposure to ACh did not exceed 1 min or so.

We have shown previously that this second component is likely to reflect desensitization of the M_2_AChR (4). A characteristic feature of fast desensitization of atrial I_{K(ACh)} is its rapid reversibility. 30 s after starting washout of ACh-containing solution, a second challenge by the agonist resulted in a current of identical amplitude, i.e. rapid desensitization was reversed as soon as I_{K(ACh)} had decayed to its basal level. Longer periods of exposure to ACh and/or substantially higher concentrations could result in a significant reduction of the second response, most likely because of receptor desensitization.

A typical experiment, qualitatively representative of 20 myocytes studied using such a protocol, is illustrated in Fig. 1C. The cell was challenged by a high concentration of ACh (100 μM) for about 2 min. A second exposure after an ACh-free period of 140 s resulted in a current of 68% of the initial peak amplitude (C, a); the fast desensitizing component was almost completely abolished. The half time of activation, which, at

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**Fig. 2. Simultaneous activation and inhibition of I_{K(ACh)} by Phe.** 2 μM ACh and 100 μM Phe were superfused as indicated. The arrowhead denotes a gap of 30 s in duration in the recording. The identities of currents activated by ACh and Phe were verified by their identical current-voltage relations (not shown).
saturating agonist concentrations, is closely related to functional density of the receptor (4, 19, 23, 26), was increased from 120 to 210 ms (C, b). A slowing in activation rate, however, per se results in a diminution of the fast desensitizing component (19). Therefore, comparing peak currents of two responses elicited in succession provides little reliable information on fast desensitization because receptor desensitization, i.e. a reduction in density of functional receptors, might contribute to reduction of the amplitude of the second response (10).

Another property defining fast desensitization, in contrast to receptor desensitization, is represented by its heterologous nature. Fig. 1B shows that activation of \( I_{K_{ACh}} \) by 10 \( \mu M \) Ado, a concentration that is saturating for this agonist, via stimulation of \( \alpha_1 \) receptors, induced a current of about 35\% of the peak current that could be induced by ACh. As shown previously, sensitivity to Ado in this system is limited by low density of functional \( \alpha_1 \) receptors (19). Superfusion of 10 \( \mu M \) ACh in the presence of Ado resulted in a peak inward current of only 70\% of the current induced by 2 \( \mu M \) ACh alone. Thus, although the Ado-induced current did not show desensitization in terms of a visible kinetic component, the system was desensitized by exposure to Ado within few seconds. This subadditive behavior, first described by Kurachi et al. (27), has been found for various combinations of receptor agonists, such as \( M_2AChR/\)sphingolipid, or \( M_3AChR/\beta\)-adrenergic (21, 28). These properties of fast desensitization are contradictory to the hypothesis that it is mediated by stimulation of a muscarinic \( M_i \) receptor, unless one would assume that stimulation of each receptor causing activation of \( I_{K_{ACh}} \) is paralleled by costimulation of a G\(_{q/11}\)-coupled subtype causing desensitization.

**Inhibition of \( I_{K_{ACh}} \) by \( \alpha_1\)-Adrenergic Receptors and ET\(_A\) Receptors**—On principle, information on how activation of PLC interferes with \( I_{K_{ACh}} \) can be obtained by stimulation of any type of putative PLC-linked receptor expressed in the system under study. In line with a previous publication (29), stimulation of \( \alpha_1\)-adrenergic receptors resulted in a small activation of \( I_{K_{ACh}} \), and, concomitantly, an inhibition of \( I_{K_{ACh}} \) activated by brief pulses of ACh. A representative example is illustrated in Fig. 2. Whereas there was distinct inhibition in all experiments of this type, the activation could be very small or absent. Effects comparable to those observed upon \( \alpha_1\)-adrenergic stimulation were evoked by endothelin-1 (ET-1) (not shown, compare Fig. 3) in line with a previous publication (30).

To study the inhibitory effect of the \( \alpha_1\)-adrenergic agonist or ET-1 in isolation and to obtain information on its kinetic properties, \( I_{K_{ACh}} \) was routinely activated by loading the cells with the hydrolysis-resistant GTP analogue GTPyS. To accelerate activation, the cells were repetitively exposed to ACh until a steady current level was reached. Fig. 3A shows a control trace. Once activated, the current remained fairly constant for several minutes of recording. The sample traces in Figs. 3, B and C, illustrate that 100 \( \mu M \) phenylephrine (Phe) and 10 nm ET-1 both caused an inhibition of \( I_{K_{ACh}} \). A comparable effect was also found if 100 \( \mu M \) methoxamine was used as \( \alpha \)-agonist (not shown). The summarized data in Fig. 4A demonstrate that both \( \alpha_1\)-adrenergic stimulation as well as stimulation of ET\(_A\) receptors caused inhibition of \( I_{K_{ACh}} \) in GTPyS-loaded myocytes to about the same degree and with identical time course. The mean half-times of inhibition were 53.4 ± 7.9 s (Phe, \( n = 10 \)) and 57.3 ± 8.8 s (ET-1, \( n = 10 \)), respectively. On average, the GTPyS-activated current in the absence of Phe or ET-1, respectively, decayed spontaneously by ~20\% within 3 min (Fig. 4A). The identical kinetics of inhibition by the two receptor agonists suggest that it is not determined by the receptor species but by downstream reactions of a common signaling pathway. Compared with fast desensitization (compare Fig. 1), inhibition of \( I_{K_{ACh}} \) by activation of \( \alpha_1\)-adrenergic or ET\(_A\) receptors was slower by about 1 order of magnitude. The slow time course of inhibition is unlikely to reflect slower activation of the G protein coupling to these receptors when GTP is exchanged by GTPyS. At least for the \( M_2AChR-G_{i/o} \) pathway, using activation of \( I_{K_{ACh}} \) as an assay, activation rates were identical few seconds after breaking the patch under the pipette, i.e. before equilibration of the cytosol with GTPyS, and about 30 s later, when the response to ACh was completely irreversible, as shown in Fig. 4B, which is qualitatively representative of 10 experiments using such a protocol.

The slow decay of GTPyS-activated \( I_{K_{ACh}} \) by about 20\% within 3 min in the control group could reflect an inhibitory effect caused by costimulation of putative intrinsic G\(_{q/11}\)-coupled muscarinic receptors. However, exposure of a GTPyS-loaded cell to a high concentration of ACh (100 \( \mu M \)), following activation of \( I_{K_{ACh}} \) either by ACh at a low concentration (2 \( \mu M \)) or by adenosine via \( \Lambda_1 \) receptors, i.e. without prior muscarinic stimulation, did not cause any inhibition, as shown in Fig. 5.
Inhibition of Atrial GIRK Current by PtIns(4,5)P₂ Depletion

After activation by adenosine and subsequent exposure to 100 μM ACh, the decrease in current after 3 min was 16.2 ± 4.8% (n = 6), which was not significantly different from the decrease without exposure to ACh (18.1 ± 4.2%, n = 12). We propose that the slight "spontaneous" decay of I_{K(ACh)} resulted from slow receptor-independent activation by GTP·S of the G protein involved in the inhibitory pathway. In contrast to a recent report (10), from the data presented so far there is no evidence that intrinsic M₃AChRs are involved in regulating I_{K(ACh)} in adult atrial myocytes.

Whereas the M₂AChR represents the classic cardiac ACh receptor, the issue of expression of other subtypes in cardiac myocytes is controversial. Identification of subtypes on pharmacological grounds only is problematic, as selectivity of the ligands presently available is limited (see Fig. 7). Moreover, positive data on global M₃AChR expression in the heart, without identification of the cell type, are functionally meaningless. In rat ventricular myocytes no M₃AChR transcripts were detected using single cell RT-PCR, which was confirmed by Southern blot analysis and immunocytochemistry, whereas these cells were positive for both M₄AChR and M₃AChR (31). Corresponding data are not available for atrial myocytes.

In atrial myocytes collected under optical control no M₃AChR transcripts could be detected in the RT-PCR products, as shown in Fig. 6A. This gel, which is representative of five experiments, is negative for M₃AChR transcripts in identified atrial myocytes, whereas transcripts for GAPDH and M₄AChR could be detected. On the other hand, using the same primers, M₄AChR transcripts could be detected in samples obtained from suspended cultures (Fig. 6B), which might reflect expression of this subtype in vascular smooth muscle and/or endothelial cells, which contaminate the cultures, though at a low level.

Inhibition of ACh-induced GIRK current was also evaluated in atrial myocytes, which were obtained from isolated atrial preparations. As shown in Fig. 7, however, selectivity of 4-DAMP for muscarinic receptor subtypes is poor. This compound, at 10 nM, caused a reversible reduction of the current induced by 2 μM ACh by about 40%. As with any other muscarinic antagonist, such as atropine, inhibition of the current by 4-DAMP was paralleled by slowing of activation and blunting of the rapidly desensitizing component. The current in GTP·S-loaded myocytes was not sensitive to 4-DAMP (not shown) the compound most likely also acts as an M₃AChR antagonist. This is in line with the reported inhibition constants ranging from 4 × 10⁻⁹ M to 1.6 × 10⁻⁸ M for the M₄AChR and from 1.2 × 10⁻⁹ M to 5 × 10⁻¹⁰ for the M₃AChR (32). Thus, despite its frequent use as pharmacological tool, this compound is not sufficiently selective to be suitable for discriminating between M₄AChR and M₃AChR.

Inhibition of I_{K(ACh)} Is Mediated by the PLC-linked Signaling
**Pathway—α₁-Adrenergic receptors and ET₄ receptors are supposed to couple preferentially but not exclusively to G proteins of the Gq/11 family. The subsequent step in the common pathway controlled by this class of G proteins is activation of PLC, yielding the second messengers inositol (3,4,5)trisphosphate (InsP₃) and diacylglycerol from hydrolysis of PtIns(4,5)P₂**.

To verify the contribution of this class of G proteins to inhibition of IK(ACh), myocytes were pretreated with *P. multocida* toxin, a protein toxin that has been described to uncouple Gq proteins from their receptors, presumably by first causing activation followed by a modification that prevents reactivation after deactivation (33, 34). In cells pretreated with the toxin (1 mg/ml for 20 h), activation of IK(ACh) by M₃AChR and/or intracellular GTPgS was not affected (Fig. 8). However, inhibition of IK(ACh) by 100 μM Phe was completely abolished. This was also confirmed in four experiments using 10 nM ET-1 (not shown). Contribution of Gq/11 to mediating the inhibition is consistent with the notion that activation of PLC represents the subsequent signaling step, although contributions by alternative mechanisms cannot be ruled out a priori, such as direct inhibitory actions of Gbg dimers containing the β₅ subunit (35). A frequently used PLC inhibitor is the aminosteroid U 73122. Because many side effects not related to inhibition of PLC have been described for this compound, its use as pharmacological tool is limited and requires careful control experiments. This compound has been used previously to support the hypothesis that activation of PLC by M₃AChR and subsequent depletion of PtIns(4,5)P₂ represent the mechanism of fast desensitization. As shown in Fig. 9, however, U 73122 at 1 μM caused a reduction in GTPgS-activated IK(ACh) by about 50%. Although this was not investigated further in detail, this compound is likely to act as a blocker of Kir3.0 channels and thus is not suited as a tool to study PLC-related effects in this system.

**Receptor-induced Inhibition of IK(ACh) Is Reduced by PtIns(4,5)P₂ Depletion**

Inhibition of Atrial GIRK Current by PtIns(4,5)P₂ Depletion

*Fig. 5.* ACh does not cause inhibition of IK(ACh). Panel A, in a GTPγS-loaded myocyte IK(ACh), was activated by a brief (4-s) pulse of 2 μM ACh. Activation was maximal because before application of ACh the cell had been dialedyzed with GTPγS for 15 s, which caused some “spontaneous” inward current. The dashed line indicates the current level immediately after rupturing the membrane patch. 100 μM ACh applied for 2 min did not cause inhibition, but a subsequent exposure to 100 μM Phe resulted in a decrease in current by about 2 nA. Panel B, IK(ACh) in a GTPγS-dialyzed myocyte was activated maximally by three exposures to 100 μM Ado. 100 μM ACh failed to cause inhibition. 2 mM BaCl₂ was applied briefly in this experiment to measure the baseline current level.

*Fig. 6.* Panel A, gel electrophoresis of RT-PCR products amplified from identified atrial myocytes. As described under “Experimental Procedures,” 10 myocytes had been picked under optical control for the RT reaction. The solution containing the RT products was split and used in two different PCRs with either primers for the M₃AChR and GAPDH (second and third lanes from the left) or M₃AChR and GAPDH (fourth and fifth lanes). Panel B, the message for the M₃AChR is apparent in RT-PCR products from samples of suspended atrial cultures (rightmost lane) but not in atrial myocytes (second from left), which are positive for GAPDH (third lane).
nase C is not causally involved. As in the present study a moderate pipette Ca\(^{2+}\)-buffering capacity (2 mM EGTA) was used, Ca\(^{2+}\) released from InsP\(_3\)-sensitive stores as mediator cannot be excluded. Supplementing the pipette solution with a 10 mM concentration of the fast Ca\(^{2+}\) chelator BAPTA did not affect inhibition of IK(ACh) by Phe (10 cells, not shown), suggesting that Ca\(^{2+}\)-dependent processes are not involved.

In a number of recent publications evidence has been provided that PtIns(4,5)P\(_2\) represents a cofactor for activation of Kir3.0 channels by Gbg (11, 36) and is also required for activity of other inward rectifying K\(^+\) channels (13, 16, 17, 37, 38). We therefore studied whether loading of atrial myocytes with PtIns(4,5)P\(_2\) via the pipette filling solution affects properties of macroscopic IK(ACh) and/or \(\alpha_1\)-adrenergic inhibition. Fig. 10A demonstrates that inclusion of PtIns(4,5)P\(_2\) (nominally 500 \(\mu\)M) in the pipette without GTP\(_\gamma\)S had no effect on amplitude and desensitization properties of IK(ACh). This was found in six out of six myocytes loaded with PtIns(4,5)P\(_2\). On the other hand, supplementation of the pipette solution with PtIns(4,5)P\(_2\) caused a significant reduction of the Phe-induced inhibition of GTP\(_\gamma\)S-activated IK(ACh). Representative traces from this series of experiments are shown in Fig. 10B. As shown in Figs. 2 and 4, 100 \(\mu\)M Phe caused an inhibition of steady-state IK(ACh) by about 70\% (C). These data suggest first that under normal conditions the concentration of PtIns(4,5)P\(_2\) in the membrane is not a limiting factor for activation of IK(ACh). Second, inhibition of IK(ACh) by heptahelical receptors activating PLC is caused by depletion of PtIns(4,5)P\(_2\). Moreover, depletion of PtIns(4,5)P\(_2\) is not causally related to fast desensitization.

**DISCUSSION**

The present study has provided evidence that activation of \(\alpha\)-adrenergic and ET\(_A\) receptors causes inhibition of atrial IK(ACh) by depletion of PtIns(4,5)P\(_2\). The rate of inhibition with a half-time of around 50 s was identical for the two receptors, suggesting a common signaling mechanism. Interestingly, the rate of inhibition was similar to the rate of inhibition of heterologously expressed K\(_{ATP}\) channels by stimulation of coexpressed M\(_2\)AChR receptors, described recently as an example of a receptor-controlled signal that is mediated via depletion of PtIns(4,5)P\(_2\) (17).

The GIRK (Kir3.0) channel complex represents the paradigmatic target of G protein \(\beta\gamma\) subunits. It is generally assumed that physiological activation of Kir3.0 channels is specific to \(\beta\gamma\) subunits of pertussis toxin-sensitive G proteins (Gtg\(_\alpha\)), although examples of activation under certain conditions of atrial IK(ACh) via the G\(_\gamma\) pathway have been presented recently (28, 39).

Apart from Gbg regulation, channel activity has been shown to be modulated by PtIns(4,5)P\(_2\) and intracellular [Na\(^+\)]. Sensitivity to PtIns(4,5)P\(_2\) has also been demonstrated for other members of the Kir channel family (12, 15, 16, 37). It remained unclear, however, whether PtIns(4,5)P\(_2\) is a necessary cofactor for Kir channels or if PtIns(4,5)P\(_2\) acts as a signaling molecule in terms of a second messenger. More recently, Xie et al. (17) demonstrated that PLC-coupled receptors can regulate K\(_{ATP}\) channels composed of Kir6.2 and SUR2A subunits via depletion of PtIns(4,5)P\(_2\) in an heterologous expression system, providing direct evidence that phosphoinositides act as messengers in a receptor-controlled signaling pathway. The physiological relevance of modulation of Kir channels by PtIns(4,5)P\(_2\) was also demonstrated in an elegant study on expressed K\(_{ATP}\) channels (37). These authors found a decrease in ATP sensitivity of K\(_{ATP}\) in cells coexpressing a phosphoinositide-4-phosphate 5-kinase, which contributes to up-regulating the levels of PtIns(4,5)P\(_2\) and other phosphoinositides.

The notion of a second messenger role of PtIns(4,5)P\(_2\) was supported by Kobrinsky et al. (10), who reported that activation of PLC-coupled receptors caused fast desensitization of native IK(ACh) in rat neonatal myocytes and Kir3.1/Kir3.4 currents expressed in a cell line. According to that study, fast desensitization of M\(_2\)AChR-activated IK(ACh) in atrial myocytes results from PLC activation via costimulation of a PLC-linked muscarinic receptor subtype (M\(_3\)AChR). The present findings confirm
that activation of PLC-coupled receptors causes inhibition of atrial $I_{K(ACh)}$ in a PtIns(4,5)P$_2$-sensitive manner. However, they disprove the hypotheses that intrinsic M$_3$AChRs are involved in regulation of atrial $I_{K(ACh)}$ and that PtIns(4,5)P$_2$-depletion causes fast desensitization.

First, fast desensitization of atrial $I_{K(ACh)}$ at room temperature has a half-time of in the order of 5 s, whereas inhibition of $I_{K(ACh)}$ by stimulation of PLC-linked receptors was slower by a factor of 10. The slow rate of inhibition is an intrinsic property of the signaling pathway and does not result from the experimental condition (loading with GTP$\gamma$S) because the rate of signaling via Gi/o was not affected by this condition.

Second, ACh did not induce a measurable inhibition of $I_{K(ACh)}$.

Third, we have shown previously that atrial $I_{K(ACh)}$, activated by A1 receptors, displays fast desensitization, which was increased in myocytes overexpressing the A1 receptor (19).

**Fig. 8.** $\alpha$-Receptor-induced inhibition of $I_{K(ACh)}$ is abolished by treatment with *P. multocida* toxin. Panel A, representative current recording from a myocyte treated with the 1 $\mu$g/ml toxin ($\geq$ 20 h). 10 $\mu$M ACh and 100 $\mu$M Phe were applied as indicated. Panel B, summarized data. Untreated cells were from the same batches as *P. multocida* toxin-treated cells. The difference between the control group and the *P. multocida* toxin-treated group was not significant.

**Fig. 9.** Inhibition of GTP$\gamma$S-activated $I_{K(ACh)}$ by U 73122. The current was activated by brief exposure to ACh as in Fig. 2 (not shown). U 73122 was applied as indicated.

Inhibition of Atrial GIRK Current by PtIns(4,5)P$_2$ Depletion

**Fig. 10.** Loading of myocytes with PtIns(4,5)P$_2$. Panel A, receptor-activated $I_{K(ACh)}$ in the absence of intracellular GTP$\gamma$S is not affected by supplementing the pipette solution with PtIns(4,5)P$_2$. The first response to ACh was induced within 6 s after rupturing the patch under the recording pipette. Panel B, inhibition of GTP$\gamma$S-activated $I_{K(ACh)}$ is reduced by adding PtIns(4,5)P$_2$ to the pipette solution (representative recording). Panel C, control measurement from a cell of the same batch as B without PtIns(4,5)P$_2$. Panel D, summarized data ($n = 6$ for either group).
With a background of these results any contribution of intrinsic PLC-coupled muscarinic receptors to fast desensitization can be safely excluded. Moreover, the lack of any inhibition of atrial I_K(ACh) by ACh, in contrast to α-agonists or ET-1, and the negative RT-PCR for M_2AChR transcripts argue against expression of this receptor in rat atrial myocytes.

GIRK currents generate inhibitory postsynaptic potentials, which are shaped by different phases of desensitization in terms of an adaptation to synaptic inputs. The term desensitization is a phenomenological one. It comprises ultrarapid conformational changes on the microsecond time scale of ionotropic receptor/channel proteins, such as the nicotinic ACh receptor, to phosphorylation-initiated reductions in sensitivity of a cell to a stimulus on the time scale of hours as, e.g. in case of the paradigmatic β-adrenergic receptors, but also in the pathway that is subject to the present investigation.

Both the instantaneous onset of acute desensitization and its fast recovery argue against phosphorylation/dephosphorylation of the channel complex, as suggested previously (7, 40). The most convincing concept to date suggests that fast desensitization is caused by the kinetics of the nucleotide exchange and hydrolysis cycle of the G protein (9). The model proposed by these authors takes into account that fast desensitization is closely linked to activation of the current. Although attractive, this model does not describe all of the properties of I_K(ACh) in a native myocyte. According to that model, GTPγS-induced I_K(ACh) should be larger, and in the presence of the GTP analog, fast desensitization should be abolished. Our data (e.g. Figs. 5 and 10) suggest that steady-state currents in GTPγS-loaded myocytes are smaller than peak currents activated by ACh. Moreover, desensitization is not abolished by GTPγS. Therefore further experimental work is required to clarify the mechanism of fast desensitization.

The present study confirms a second messenger role of PtIns(4,5)P_2. Dual regulation of Kir3.0 currents by stimulatory G_alpha_{11} coupled receptors via PtIns(4,5)P_2 and by inhibitory G_alpha_{11} coupled receptors via PtIns(4,5)P_2 depletion represents a novel concept of synaptic integration at the level of an ion channel.

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Addendum—While the present publication was under review, a paper was published which provided strong evidence for α-adrenergic inhibition of I_K(ACh) via depletion of PtIns(4,5)P_2 in mouse atrial myocytes (41).

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Depletion of Phosphatidylinositol 4,5-Bisphosphate by Activation of Phospholipase C-coupled Receptors Causes Slow Inhibition but Not Desensitization of G Protein-gated Inward Rectifier K$^+$ Current in Atrial Myocytes

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