THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 31, Issue of August 2, pp. 27742–27747, 2002
Printed in U.S.A.
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

Acetylcholine-induced Phosphatidylinositol 4,5-Bisphosphate Depletion Does Not Cause Short-term Desensitization of G Protein-gated Inwardly Rectifying K+ Current in Mouse Atrial Myocytes*

Received for publication, April 16, 2002, and in revised form, May 15, 2002
Published, JBC Papers in Press, May 17, 2002, DOI 10.1074/jbc.M203660200

Hana Cho‡, Ji-Young Hwang§, Daesoo Kim§¶, Hee-Sup Shin§¶, Yangmi Kim, Yung E. Earm, and Won-Kyung Ho‖

From the National Research Laboratory for Cellular Signaling and Department of Physiology and Biophysics, Seoul National University College of Medicine, 28 Yonkeun-Dong, Chongno-Ku, Seoul 110-799, Korea, §Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea and the ¶National Creative Research Initiatives Center for Calcium and Learning, Korea Institute of Science and Technology, Seoul, 136-791, Korea

Depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2) induced by phenylephrine or endothelin causes the inhibition of acetylcholine-activated K^+ current (I_{KACh}) in atrial myocytes. In the present study, we have investigated the hypothesis that muscarinic receptor induced PIP_2 depletion also causes inhibition of I_{KACh}, resulting in desensitization. We confirmed the expression of G_q-coupled muscarinic receptors in mouse atrial myocytes using reverse transcriptase-polymerase chain reaction. The involvement of M_1 and M_3 receptors in desensitization is examined using specific antagonists, 4-DAMP and pirenzepine, but they significantly reduced peak I_{KACh}, implying nonspecific M_2 blockade. When ACh-induced phosphoinositide depletion was specifically inhibited using PLCβ1 knock-out mice, the extent of desensitization during 4 min was 47.5 ± 3.2%, which was not different from that in wild type (46.8 ± 2.1%). Phenylephrine-induced phosphoinositide hydrolysis and phenylephrine-induced inhibition of I_{KACh} were not affected by PLCβ1 knock-out. To facilitate PIP_2 depletion, replenishment of PIP_2 was blocked by wortmannin. Wortmannin did not affect the desensitization and the recovery from desensitization. These results suggest that PIP_2 depletion by acetylcholine does not contribute to short-term desensitization of I_{KACh}. The differential regulation of I_{KACh} by different phospholipase C-linked receptors may imply that receptor co-localization is required for PIP_2 to act as a signaling molecule.

Phosphatidylinositol 4,5-bisphosphate (PIP_2)[1] not only serves as a precursor of phospholipase C (PLC) to produce diacylglycerol and inositol trisphosphate, but it also exerts a direct role on the regulation of various cellular function. The underlying mechanism of PIP_2 action was investigated in detail for G protein-gated inwardly rectifying K^+ (GIRK) channels, and it was shown that the activation of GIRK channels by $G\beta\gamma$ depends on the presence of PIP_2 (1–5). It has recently been reported that atrial GIRK currents evoked by acetylcholine via M_2 muscarinic receptors (I_{KACh}) are inhibited by the α_1-adrenergic receptors or by endothelin receptors by means of PIP_2 depletion (6, 7). These result suggest that PIP_2 depletion caused by PLC activation can function as a signaling mechanism in I_{KACh} regulation. This mechanism is of particular interest because it provides a novel pathway by which cross-talk between different signaling pathways take place.

It is well known that the activation of I_{KACh} is not sustained in the continuous presence of acetylcholine but undergoes desensitization. The mechanism of desensitization has been widely investigated, and it is understood as a complex process involving receptor phosphorylation, the nucleotide exchange and hydrolysis cycle of the G protein, and channel dephosphorylation (8–12). Because the role of PIP_2 on I_{KACh} has been demonstrated, PIP_2 depletion via PLC-coupled muscarinic receptors has been proposed as a possible mechanism of I_{KACh} desensitization (13, 14). This hypothesis was supported by the finding that GIRK current expressed in COS-1 cells showed desensitization only when coexpressed with PLC-coupled muscarinic receptors. It was also shown that I_{KACh} desensitization in rat neonatal myocytes was inhibited by 4-diphenylacetoxy-*N*-methypiperidine (4-DAMP) and U73122, which were used for inhibiting M_3 muscarinic receptors and PLC, respectively (14). However, it is not yet clear whether intrinsic M_3 muscarinic receptors are involved in the desensitization of I_{KACh}. The validity of the conclusion of their study appears to depend primarily on the specificity of the drugs, but recent studies indicate that action of 4-DAMP and U73122 may not be specific as originally thought (7, 15, 16). Furthermore, the issue of the expression and function of PLC-coupled muscarinic receptors in cardiac myocytes are still controversial (7, 17).

In the present study, we confirmed the presence and functional activity of PLC-coupled muscarinic receptor in mouse atrial myocytes by using reverse transcriptase-polymerase chain reaction (RT-PCR) and by measuring acetylcholine (ACh)-induced phosphoinositide (PI) hydrolysis. To search for the evidence of the role of PIP_2 depletion on I_{KACh} desensitization, we used PLCβ1 knock-out mice in which ACh-induced PI

* This work was supported in part by the Advanced Backbone Information Technology Development Project from the Korean Ministry of Information and Communication (IMT2000-C3-3) and the National Creative Research Initiative Program from the Korean Ministry of Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported as a postgraduate by the BK21 Program from the Korean Ministry of Education.

‖ To whom correspondence should be addressed: Dept. of Physiology and Biophysics, Seoul National University College of Medicine, 28 Yonkeun-Dong, Chongno-Ku, Seoul, 110-799, Korea. Tel.: 82-2-740-8227; Fax: 82-2-763-9667; E-mail: wonkyung@snu.ac.kr.

[1] The abbreviations used are: PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; GIRK, G-protein gated inwardly rectifying K^+; I_{KACh}, acetylcholine-activated K^+ current; 4-DAMP, 4-diphenylacetoxy-*N*-methypiperidine; RT-PCR, reverse transcriptase PCR; ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor; PI, phosphoinositide; mAChR, muscarinic acetylcholine receptors; PE, phenylephrine; WMN, wortmannin; I_{peak}, amplitude of I_{KACh} at peak; I_{ss}, amplitude of I_{KACh} at steady state.

This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org/ by guest on January 1, 2018

hydrolysis was significantly attenuated and the pharmacological method of potentiating PIP_2 depletion. The results showed that neither of these conditions significantly affected I_{KACh} desensitization, indicating that depletion of PIP_2 is not related to the ACh-induced desensitization of I_{KACh}. The differential regulation of I_{KACh} by different PLC-linked receptors suggests that receptor co-localization is required for PIP_2 to act as a signaling molecule.

EXPERIMENTAL PROCEDURES

Cell Isolation—Mouse atrial myocytes were isolated by perfusing Ca^{2+}-free normal Tyrode solution containing collagenase (0.14 mg ml^{-1}, Yakult, Tokyo) on a Langendorff column at 37 °C as described previously (16). Isolated atrial myocytes were used for RNA extraction or kept in high K^+, low Cl^- solution at 4 °C until use for electrophysiological study.

Solutions and Chemicals—Normal Tyrode solution contained (mM): 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 5 HEPES, titrated to pH 7.4 with NaOH. Ca^{2+}-free solution contained (mM): 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 10 glucose, and 5 HEPES, titrated to pH 7.4 with NaOH. The high K^+, low Cl^- solution contained (mM): 70 KOH, 40 KCl, 50 L-glutamic acid, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 10 glucose, 10 HEPES, and 0.5 EGTA. The pipette solution contained (mM): 140 KCl, 10 HEPES, 1 $MgCl_2$, and 5 EGTA, titrated to pH 7.2 with KOH. Neomycin and wortmannin were from Biomol, 4-DAMP was from Tocris, and other chemicals were from Sigma. Drugs were prepared as concentrated stock solutions either in distilled water or dimethyl sulfoxide. All experiments were conducted at 35 ± 1 °C. When required, 10 μM glibenclamide was applied in the presence of ACh to inhibit the ATP-sensitive K^+ channel. To ensure a rapid solution turn-over, the rate of superfusion was kept above 5 ml min^{-1}, which corresponded to 50 times bath volume (100 μl)/min.

Voltage Clamp Recording and Analysis—Membrane currents were recorded from single isolated myocytes in a perforated patch configuration using nystatin (ICN, 200 μg/ml). Voltage clamp was performed using an Axopatch-1C amplifier (Axon Instruments). The patch pipettes were pulled from borosilicate capillaries (Harvard Apparatus) using a Narishige puller (PP-83, Tokyo). The patch pipettes used had a resistance of 2–3 megaohms when filled with the above pipette solutions. Electrical signals were displayed during experiments using an oscilloscope (Tektronix, TDS 210) and a chart recorder (Gould). Voltage clamp and data acquisitions were performed using a digital interface (Digidata 1200, Axon Instruments) coupled to an IBM-compatible computer at a sampling rate of 1–2 kHz, filtered at 5 kHz. Cells were voltage-clamped at −40 mV, and the activation of I_{KACh} by the application of ACh resulted in a shift in holding current level in outward direction. We have shown in our previous study (6) that during I_{KACh} desensitization the current decrease occurs uniformly over the voltage range tested, and that the current change recorded at −40 mV well represents the change in I_{KACh}. In the present study, we did not show the current-voltage relationship, but it was checked in all experiments in the same way as described in the previous study (6) to confirm that the continuous current recording obtained at −40 mV represents I_{KACh}.

RT-PCR—Total RNA was isolated from freshly isolated atrial myocytes using TRIzol reagent (Invitrogen). Total RNA yield and purity were analyzed spectrophotometerically (Beckman-Coulter, Fullerton, CA). RT-PCR was performed using a one-step RT-PCR kit (Invitrogen), which combines cDNA synthesis and PCR in a single reaction. 4 μl of total RNA was added to a final reaction volume of 50 μl consisting of 25 μl of 2× reaction mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM $MgSO_4$), 0.2 μM each of sense and antisense primer, 1 μl of RT/Platinum *Taq* mix. For the one-step RT-PCR, the reverse transcriptase step (50 °C/30 min) was followed by an initial denaturation at 94 °C/5 min followed by PCR amplification. The cycling profiles used were 30-s denaturing at 94 °C, 1 min-annealing at 55 °C, and 2-min extension at 72 °C, for 40 cycles followed by a final extension step of 8 min at 72 °C. The following primers were synthesized by Bionics: M_1 isoform, gctgtactggcgcatctacc (sense) and gcctgtgcctcaggatctac (antisense); M_2 isoform, ggatccctcagtctggtgac (sense) and ggtccgcttaactgggtagg (antisense); M_3 isoform, gacacctccagtgaccctct (sense) and ggtaggtgagtggcctggta (antisense); M_4 isoform, cagagctgtccaccacagag (sense) and gtagggtgtccaggtgagga (antisense); and M_5 isoform, ggtctccttcatcctctggg (sense) and gatctgagcagggtcctgtg (antisense). All primers were designed to avoid significant secondary and complementary structures and to have a GC content of 50–60%. The gene-specific primer pairs were designed on the basis of cDNA sequences of mouse muscarinic receptors (GenBank™ accession numbers NM007698, AF264049, NM033269, NM007699, and AF264051), and unique oligonucleotide sequences were chosen from cDNA regions with minimal homology among different muscarinic receptors. The specificity of primer pairs was verified by searching the GenBank™ data base using BLAST and by amplifying the PCR products of the predicted size, visualized as a single discrete band by ethidium bromide staining on an agarose gel.

Animals—The PLCβ1 mutation (18) has been maintained in two different heterozygous genetic backgrounds, C57BL/6J and 129/sv. Heterozygous animals from the two backgrounds were mated to obtain homozygous mutant mice in the F1 background between C57BL/6J and 129/sv. The genotype of the progeny was determined by PCR as described previously (18). Wild type littermates served as controls for the mutants. The mice had *ad libitum* access to food and water and were kept on a 14:10-h light-dark cycle with lights on at 6 a.m. Animals were housed and cared for according to the guidelines of the Korea Institute of Science and Technology for the care of experimental animals.

PI Hydrolysis in Atrial Tissues—The procedure described previously (18) was adapted for atrial tissues. Krebs buffer was composed of 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose (pH 7.4 at 37 °C) gassed with 95% O_2, 5% CO_2, and chilled on ice for 10 min. The atria were isolated from sacrificed mice, placed in a dish containing oxygenated Krebs buffer kept on ice, blotted to remove excess blood, and rinsed with Krebs buffer. The atrial preparation was pre-labeled with *myo*-[2-^{3}H]inositol (10 μCi/ml, Amersham Biosciences, TRK911) in 300 μl of Krebs buffer saturated with 95% O_2, 5% CO_2 for 1 h, treated with LiCl (10 mM) and *myo*-inositol (5 mM) for 20 min, and then incubated with an appropriate agonist for 40 min. The concentrations of agonists were chosen to induce maximum PI hydrolysis: 1 mM carbachol, 1 mM norepinephrine, and 300 nM endothelin-1. All procedures were performed in capped 5-ml flat-bottomed tubes (Amicon) with periodical supplies of gas at 37 °C. The reaction was stopped with 1 ml of $CHCl_3$, CH_3OH (1:2) and extracted with 0.6 ml of $CHCl_3$, H_2O (1:1). Total inositol polyphosphates in the aqueous phase were isolated on an AG1X-8 column and counted in a scintillation counter.

Statistics and Presentation of Data—Results in the text and the figures are presented as means ± S.E. (n = number of cells tested). Statistical analyses were performed using the Student's t test. The difference between two groups was considered to be significant when $p < 0.01$ and not significant when $p > 0.05$.

RESULTS

Expression of Muscarinic Receptor Isoforms in Mouse Atrial Myocytes—We first examined the presence of PLC-coupled muscarinic acetylcholine receptors (mAChR). It is known that odd-numbered muscarinic receptors (M_1, M_3, and M_5) stimulate PLC via $G_{q/11}$, whereas even-numbered muscarinic receptors (M_2 and M_4) stimulate effectors such as GIRK channels via $G_{i/o}$ protein (19). Pharmacological identification of the subtypes suffers from the limitation in selectivity of ligands presently available (7). Molecular techniques allow more sensitive identifications, and in human atrial cells, transcripts of all five subtypes have been detected by RT-PCR, a finding that was confirmed by Western blot and immunocytochemistry (17). On the other hand, Meyer *et al.* (7) reported that M_3AChR transcripts were not detected by RT-PCR in rat atrial myocytes. Corresponding data are not available for mouse atrial myocytes. In Fig. 1, we identified, using RT-PCR, that all five mAChR isoforms (M_1–M_5) are expressed in mouse atrial myocytes. Note the bands present at the expected molecular weights for M_1 (320 base pairs (bp), *lane 1*), M_2 (317 bp, *lane 3*), M_3 (369 bp, *lane 5*), M_4 (319 bp, *lane 7*), and M_5 (287 bp, *lane 9*). Similar results were obtained from the other two mRNA samples from atrial myocytes. Note also the absence of bands in the RT-negative controls (*lanes 2*, *4*, *6*, *8*, and *10*). The identities of all PCR fragments were confirmed by sequencing.

Effects of Specific Antagonists for PLC-linked Muscarinic Receptors—To investigate whether ACh-induced PIP_2 hydrolysis leads to I_{KACh} desensitization, we examined the effect on the I_{KACh} desensitization of the substance known as specific antagonists for PLC-linked muscarinic receptors. I_{KACh} was activated by applying 10 μM ACh and recorded using a perforated

Downloaded from http://www.jbc.org/ by guest on January 1, 2018

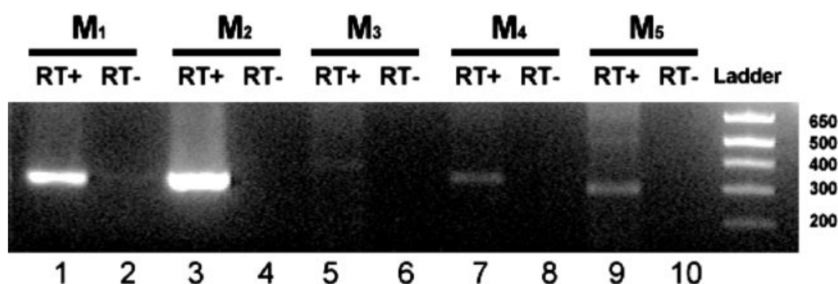

FIG. 1. **Detection of mRNA coding for various types of mAChRs in mRNA samples purified from mouse atrial myocytes.** A representative gel with ethidium bromide staining of PCR-amplified products is shown. *Lanes 1–10*, expected PCR products for M_1 (320-bp band), M_2 (317-bp band), M_3 (369-bp band), M_4 (319-bp band), and M_5 (287-bp band). *RT+* indicates reaction with reverse transcriptase, and *RT−* indicates the omission of reverse transcriptase from the reaction to exclude possible contamination by genomic DNAs.

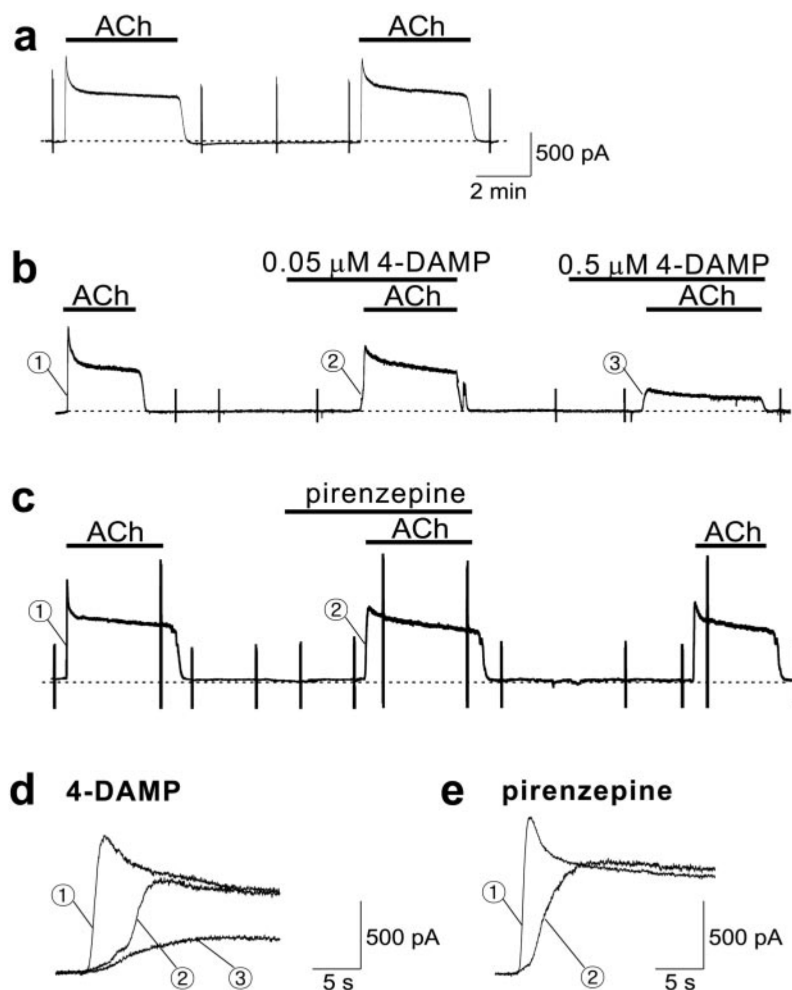

FIG. 2. **4-DAMP and pirenzepine inhibit peak I_{KACh} and blunt the desensitization.** *a*, chart recordings of the whole cell current at a holding potential of −40 mV. The *dotted line* indicates zero current level. The application of 10 μM ACh is indicated by the *horizontal bar above* the current trace. ACh was applied for 4 min at 6-min intervals. The vertical deflections of the current trace are responses to voltage ramps, which were applied to check the current-voltage relationship. In most figures, these have been cut off for graphical reasons. *b*, after the control I_{KACh} was recorded, 4-DAMP (0.05 and 0.5 μM) was applied 3 min before the second and third activation of I_{KACh} as indicated. *c*, 10 μM ACh and 1 μM pirenzepine were applied as indicated. *d* and *e*, The traces in *b* and *c* are expanded and superimposed in *d* and *e*, respectively, to show the effect of the drugs on the rising phase of I_{KACh} activation. The *numbers* correspond to the labeling in *b* and *c*.

patch clamp configuration (Fig. 2). When the membrane potential was held at −40 mV, the application of 10 μM ACh induced a rapid activation of outward currents followed by a decrease in currents due to desensitization. After a 6-min wash-out, the second application of ACh induced a current with the same amplitude, indicating full recovery from desensitization (Fig. 2*a*). These characteristics are typical for I_{KACh} as reported previously (6, 20, 21). We also confirmed, by applying voltage ramps between +60 and −120 mV, that the current-voltage relationship and the reversal potential were typical for I_{KACh} (data not shown).

The hypothesis that desensitization in atrial cells is mediated by M_3 receptors is based in part on the observation that the desensitization was largely abolished by 0.5 μM 4-DAMP, an M_3 receptor antagonist (14). However, the results of subsequent studies have suggested that the abolishment of desensitization by 4-DAMP is not caused by the selective inhibition of desensitization but by the inhibition of fast activation caused by nonspecific antagonistic action of 4-DAMP on M_2 receptor (7, 15). To test whether 4-DAMP can be used in mouse atrial myocytes for the purpose of selective inhibition of the M_3 receptor without affecting I_{KACh} activation mediated by the M_2 receptor, we compared I_{KACh} profiles in control and in the presence of the drug from the same cell. When 0.05 μM 4-DAMP was pretreated before the second application of ACh, the amplitude of peak I_{KACh} was significantly reduced, and the fast phase of desensitization was almost abolished (Fig. 2*b*). When the current trace was seen in the expanded time scale, a significant slowing of the activation rate was recognized (Fig. 2*d*). When the concentration of 4-DAMP was increased to 0.5 μM,

Downloaded from http://www.jbc.org/ by guest on January 1, 2018

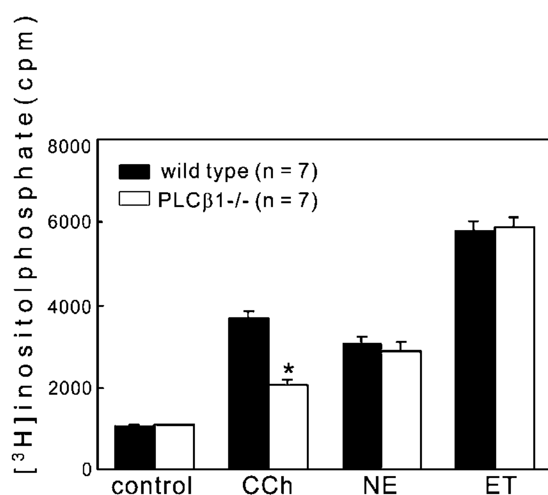

FIG. 3. **LiCl-amplified agonist-induced PI hydrolysis in atrial myocytes from wild type and PLCβ1−/− mice.** Agonist-induced PI hydrolysis was assayed as described under "Experimental Procedures." Treatments with carbachol (*CCh*, 1 mM), norepinephrine (*NE*, 1 mM), and endothelin-1 (*ET*, 300 nM) were performed three times in triplicate. Carbachol-induced PI hydrolysis was dramatically reduced in the PLCβ1−/− mice compared with the wild type control. *, indicates $p < 0.01$.

the inhibition of I_{KACh} became more significant, and both the peak and steady state currents were reduced. M_1 antagonist pirenzepine was also tested. As shown in Fig. 2*c*, this compound at 1 μM caused a reversible reduction of peak current as well as a loss of desensitization. Again, this was accompanied by slowing of the activation rate (Fig. 2*e*). These results indicate that both 4-DAMP and pirenzepine affect the activation phase in such a way that the initial fast phase of the desensitization is masked, and thus they are not suitable as pharmacological probes to investigate the role of M_1 and M_3 receptors in the desensitization phase.

Acetylcholine-induced PI Hydrolysis and I_{KACh} Desensitization in PLCβ1 Knock-out Mice—Because selective inhibition of PLC-coupled receptor using pharmacological probes failed, we next tried the knock-out mouse model. It was reported that muscarinic receptor-mediated PI hydrolysis in brain is selectively inhibited in PLCβ1−/− mice (18). In Fig. 3, we measured agonist-stimulated PI hydrolysis in mouse atrial myocytes isolated from wild type and PLCβ1−/− mice. PI hydrolysis was increased by carbachol as well as by norepinephrine and endothelin-1. Carbachol-induced increase in PI hydrolysis was markedly decreased (by 44.19%) in PLCβ1−/− mice. In contrast, norepinephrine or endothelin-1-induced PI hydrolysis increase did not change in PLCβ1−/− mice. These results indicate that in mouse atrial cells acetylcholine induces PI hydrolysis, and that about half of this activity is linked to PLCβ1. If I_{KACh} desensitization in atrial myocytes is mediated by the activation of PLC through G_q-coupled muscarinic receptors, it would be expected that in PLCβ1−/− mice desensitization is largely attenuated. To test this hypothesis, we compared the desensitization of I_{KACh} in atrial myocytes of PLCβ1−/− mice and that of wild type mice.

In atrial myocytes isolated from PLCβ1−/− mice, ACh-induced desensitization was not significantly different from that of the wild type (Fig. 4, *a* and *b*). I_{KACh} was recorded using the same protocol as described in the legend for Fig. 2. The results are summarized in Fig. 4*c*. When the extent of desensitization was determined to be the proportion of current decrease observed over a 4-min time span, it was found to be 46.8 ± 2.1% ($n = 9$) in wild type and 47.5 ± 3.2% ($n = 7$) in PLCβ1−/− mice. The amplitude of I_{KACh} at peak activation measured at −40 mV

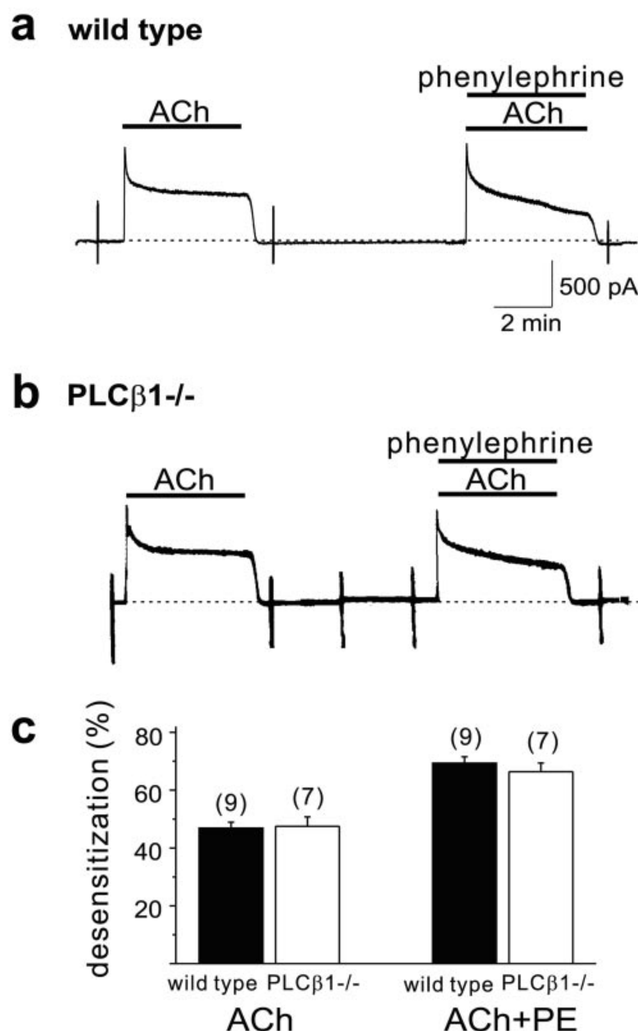

FIG. 4. **ACh-induced desensitization and PE effect on desensitization in PLCβ1−/− mice.** *a*, a recording of I_{KACh} at −40 mV from an atrial myocyte of wild type mice. The applications of 10 μM ACh and 100 μM PE are indicated by the *horizontal bar above* the current trace. *b*, a recording of I_{KACh} from an atrial myocyte of PLCβ1−/− mice. *c*, summary of the extent of ACh-induced desensitization and PE effect in the wild type and PLCβ1−/− mice cells. The *numbers* in *parentheses* indicate the numbers of cells tested. The extent of desensitization was indicated as % decrease of the current amplitude during 4 min of ACh application: $100 \times (I_{peak} - I_{4\ min})/I_{peak}$. The difference between wild type and PLCβ1 −/− was not significant.

was 954.4 ± 103.8 pA ($n = 9$) in the wild type and 824.8 ± 128.3 pA ($n = 7$) in PLCβ1−/− mice. After the second exposure to ACh, the peak amplitude of I_{KACh} recovered almost completely both in wild type and in PLCβ1−/− mice. The ratio of the amplitudes of the second to the first peak current (I_{peak2}/I_{peak1}) was 95.4 ± 4.7% in wild type and 97.2 ± 5.6% in PLCβ1−/− mice.

For comparison, the effect of α_1-adrenergic agonist phenylephrine (PE) on I_{KACh} was also tested in PLCβ1−/− mice. When PE was applied together with the second exposure of ACh, desensitization of I_{KACh} was significantly accelerated compared with that without PE (Fig. 4*b*). The magnitude of the PE effect in PLCβ1−/− mice was not significantly different from that in wild type mice (Fig. 4, *a* and *c*).

Considering that the inhibition of I_{KACh} by PE is caused by PIP_2 depletion (6), this result is comparable with PI hydrolysis data shown in Fig. 3, in which PI hydrolysis induced by PE was unchanged in PLCβ1−/− mice. Taken together, it is suggested that agonist-induced PIP_2 depletion is not always parallel to

Downloaded from http://www.jbc.org/ by guest on January 1, 2018

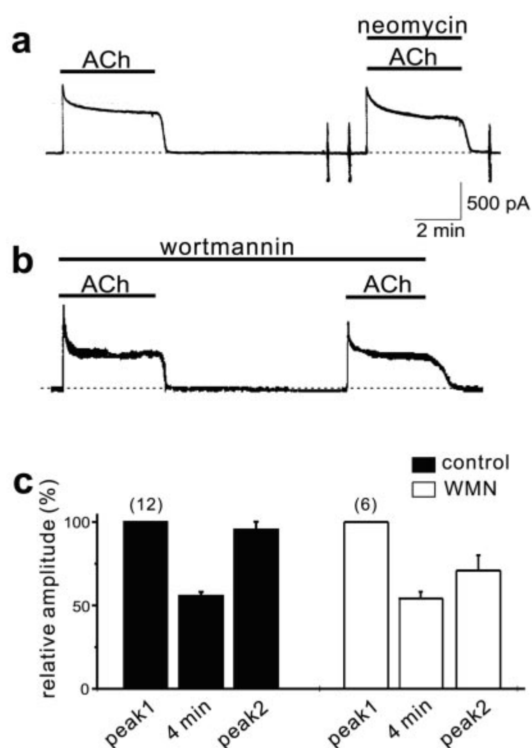

FIG. 5. **ACh-induced desensitization was not affected by neomycin or wortmannin.** *a*, 10 μM ACh and 500 μM neomycin were applied as indicated. *b*, 10 μM ACh and 100 μM WMN were applied as indicated. *c*, summary data of the relative amplitudes (%) of I_{4min} and I_{peak2} after a 6-min wash-out *versus* I_{peak1} · I_{KACh} was measured at −40 mV. The *numbers* in *parentheses* indicate the number of cells tested.

reduction of I_{KACh}, in that PIP_2 depletion caused by muscarinic receptor activation does not cause inhibition of I_{KACh}, whereas that by PE does inhibit I_{KACh}.

Effects of PLC Inhibitor and PI Kinase Inhibitor on I_{KACh} Desensitization—As shown in Fig. 3, ACh-induced PI hydrolysis was not completely inhibited by PLCβ1 knock-out. In Fig. 5*a*, we tested the effect of neomycin, an inhibitor of phosphatidylinositol-specific phospholipase C, on the ACh-induced desensitization of I_{KACh}. When 500 μM neomycin was applied with ACh at the second exposure, the activation and desensitization of I_{KACh} were not significantly affected. The extent of desensitization in the presence of neomycin was 48.6 ± 3.7% (n = 7). This value is not significantly different from that in control (44.2 ± 2.3%, n = 12). This result is consistent with the lack of a PLCβ1 knock-out effect, which suggests that in mouse atrial cells ACh-induced activation of the PLC-linked signaling pathway does not regulate GIRK channels.

The depletion of PIP_2 by the receptor-mediated activation of PLC can be potentiated by PI-4 kinase inhibitor, because the replenishment of PIP_2 after depletion is dependent on PI-4 kinase (22). Wortmannin (WMN) is a known inhibitor of PI-3 kinase and PI-4 kinase. We previously showed that in the presence of WMN, the inhibition of I_{KACh} by α_1-adrenergic activation is markedly potentiated, and its recovery was completely blocked (6). Fig. 5*b* was used to determine whether the inhibition of PIP_2 replenishment by WMN affects the desensitization and recovery of I_{KACh}. When the same series of experiments shown in Fig. 2*a* was performed in the presence of 100 μM WMN, no significant differences were observed in the activation and desensitization of I_{KACh} compared with the control. The extent of desensitization after 4 min was 44.2 ± 2.3% in the control group and 45.8 ± 4.0% in the WMN group (Fig. 5*c*). Furthermore, WMN did not block the recovery of I_{KACh}. In the presence of WMN, the peak amplitude of I_{KACh} during the second exposure to ACh (I_{peak2}) was larger than the current amplitude after desensitization, suggesting that recovery from desensitization still occurred in the presence of WMN. This result contrasted sharply with our previous result, which showed that recovery was completely blocked when WMN was treated with α_1-adrenergic agonist (6). However, the recovery in Fig. 5*c* was not complete, and the I_{peak2}/I_{peak1} ratio was 70.9 ± 9.1% (Fig. 5*c*). Considering that the magnitude of the reduction of basal PIP_2 levels in unstimulated cells by WMN in this period of time (10 min) was about 75% (22), it would appear that incomplete recovery of ACh-induced desensitization may be explained by the basal reduction of the PIP_2 level by WMN. These results suggest that in mouse atrial cells, PIP_2 depletion induced by ACh does not result in a reduction of I_{KACh}.

DISCUSSION

The main question addressed in the present study is whether ACh induced PIP_2 hydrolysis causes inhibition of I_{KACh}, resulting in current desensitization. The results obtained can be summarized as follows. 1) The expression of G_q-coupled muscarinic receptors in mouse atrial myocytes was confirmed using RT-PCR. 2) Despite the fact that acetylcholine-induced phosphoinositide hydrolysis was specifically inhibited by 44.19% using PLCβ1 knock-out mice, the extent of desensitization in PLCβ1−/− was not different from that in wild type. 3) The activation of a_1-adrenergic receptor caused inhibition of I_{KACh} in PLCβ1 knock-out mice to the same extent as that in wild type mice. 4) When wortmannin was applied together with ACh to facilitate PIP_2 depletion, desensitization and the recovery from desensitization were not affected. From these results it was suggested that mouse atrial GIRK channels are not regulated by muscarinic receptor-mediated PIP_2 depletion.

The question raised by the present study in relation to the previous study (6) is why PI hydrolysis (PIP_2 depletion) by PLC-coupled muscarinic receptor activation does not regulate GIRK channels, whereas PIP_2 depletion by α_1-adrenergic receptor does. Considering that both receptors induce similar amount of PI hydrolysis (Fig. 3) and thus are expected to cause similar amount of PIP_2 depletion, this question is most intriguing. Possible explanations can be given by assuming two conditions: 1) the effect of PIP_2 depletion triggered by a specific receptor is limited to a local microdomain, and 2) GIRK channels are preferentially co-localized with α_1 adrenergic receptor but not with M_1 (or M_3/M_5) muscarinic receptors in mouse atrial myocytes. Given these conditions, PIP_2 can act as a localized signal regulated by a specific receptor in the regulation of GIRK channels. Direct experiments to prove the first assumption have not yet been performed in cardiac myocytes, but a line of evidence suggests that degradation and accumulation of PIP_2 are confined to a microdomain, enabling PIP_2 to act as a localized signal (23–25). The second assumption is also supported by studies that investigated the mechanism of subtype-specific interaction of β-adrenergic receptors with the M_2 muscarinic receptor (26). It is well known that the β_1 adrenergic receptor-cAMP pathway shows a high degree of sensitivity to inhibitory modulation by M_2 muscarinic receptors, whereas β_2 adrenergic receptor stimulation of cAMP accumulation is not susceptible to inhibitory modulation by M_2 muscarinic receptors. The molecular mechanism contributing specificity is suggested to be that M_2 muscarinic receptors are preferentially co-localized with β_1 adrenergic receptor, but not with β_2 adrenergic receptor (27). The results of the present study, together with those of a previous study (6), also imply that the differential topographical arrangement of GIRK channels with respect to the PLC-linked receptor is involved in channel regulation via PIP_2.

It is worth noting that the result of the present study is not consistent with the results of previous studies performed in

Downloaded from http://www.jbc.org/ by guest on January 1, 2018

expression systems. When an M_1 (14, 28, 29) or M_3 receptor (29) was coexpressed with GIRK channels, channel activity was inhibited by muscarinic receptor agonists. These results indicate that unlike in native cells, GIRK channels can interact with either the M_1 or the M_3 receptor when they are co-expressed. This discrepancy implies that there exists a special mechanism in native cells that results in a specific topological arrangement of receptors, and this mechanism is disrupted when receptors are artificially over-expressed. Such a possibility was suggested by Wellner-Kienitz *et al.* (30), who showed that the transient transfection of the β-adrenergic receptor in atrial myocytes results in isoproterenol-activated pertussis toxin-insensitive GIRK current, whereas in native cells β-adrenergic stimulation did not induce GIRK current. It is thus implied that experiments in native cells are required in studies investigating the specificity of signaling mechanisms and cross-talk between different signal pathways.

The conclusion of the present study that PIP_2 depletion is not related to desensitization of I_{KACh} appears to be contradictory to that of Kobrinsky *et al.* (14). However, if we look carefully, the experimental results are not contradictory to each other. Both studies showed that the ratio of I_{KACh} at steady state (I_{ss}) after several minutes of desensitization to I_{KACh} at peak (I_{peak}), I_{ss}/I_{peak}, was significantly greater in the presence of the M_3 receptor antagonist 4-DAMP. Kobrinsky *et al.* (14) considered this result as evidence for the contribution of M_3 receptor to the mechanism of desensitization. However, it is important to realize that I_{ss}/I_{peak} can be an index for the change in desensitization only when I_{peak} is unchanged. It is well known that fast desensitization of I_{KACh} is dependent on the amplitude and activation kinetics of I_{KACh} (10, 20, 31) and that it is abolished under various experimental conditions where I_{peak} is reduced by M_2 blockade or in the presence of low density of available receptor. Kobrinsky *et al.* (14) failed to recognize the effect of 4-DAMP on I_{peak}, because they only monitored the change in I_{ss}/I_{peak} without monitoring the change in I_{peak} from the same cell. In the present study, we have demonstrated that the inhibition of desensitization (increase in I_{ss}/I_{peak}) by 4-DAMP occurred in parallel with a slowing of current activation and the inhibition of I_{peak} (Fig. 2, *b* and *d*). A similar result was also reported in recent studies performed in rat atrial myocytes (7, 15). These results may suggest that the increase in I_{ss}/I_{peak} by 4-DAMP is due for the most part to the slowing of activation and the reduction of peak current, although the effect of 4-DAMP on the desensitization phase is not entirely excluded.

Kobrinsky *et al.* (14) showed that there is an increase in I_{ss}/I_{peak} in the presence of U73122, one of the most widely used PLC inhibitors. This result is not consistent with our experiments reported previously (16), in that U73122 directly inhibits I_{KACh}. We showed that the ED_{50} of U73122 for I_{KACh} blockade is 0.12 μM, but Kobrinsky *et al.* (14) recorded I_{KACh} in the presence of 1 μM. Meyer *et al.* (7) also observed the inhibition of I_{KACh} by U73122. We do not know the reason for the discrepancy among different studies, but differences in experimental conditions may possibly to be involved. Kobrinsky *et al.* (14) and Meyer *et al.* (7) used whole cell recording, whereas we used the nystatin-perforated patch experiment. Kobrinsky *et al.* (14) used cultured myocytes from newborn rats, whereas we and Meyer *et al.* (7) used adult myocytes from mice and rats, respectively. So, the possibility that differences in preparation of atrial myocytes caused the discrepancy may need to be considered. The previous report showing that inositol phosphate content and sensitivity to U73122 in cardiomyocytes are altered by the cell isolation procedure and vary depending on time in culture (32) may support this possibility.

In the present study, we only showed that PIP_2 depletion is not related to I_{KACh} desensitization, without pursuing what the mechanism of desensitization is. It is known that the short-term desensitization is made up of several components with different kinetics of onset and recovery. Among them, slow components seem to represent receptor desensitization, presumably mediated by phosphorylation via receptor kinase(s) and subsequent events such as receptor internalization (8, 9). In contrast to the slow component, a fast phase of desensitization, 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.* (11) suggested channel dephosphorylation as the underlying mechanism, whereas Hong *et al.* (12) reported the contribution of an unidentified cytosolic protein. The major properties of fast desensitization, such as the rate of current decay and its membrane-delimited nature, can be accounted for by a model relating fast desensitization, analogous to activation, to the nucleotide exchange and hydrolysis cycle of the G protein (10). However, the above-mentioned mechanisms do not describe all of the properties of I_{KACh} in a native myocyte. Therefore further experimental work will be required to clarify the mechanism of desensitization.

REFERENCES

1. Huang, C. L., Feng, S., and Hilgemann, D. W. (1998) *Nature* **391,** 803–806
2. Zhang, H., He, C., Yan, X., Mirshahi, T., and Logothetis, D. E. (1999) *Nat. Cell Biol.* **1,** 183–188
3. Sui, J. L., Petit-Jacques, J., and Logothetis, D. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1307–1312
4. Ho, I. H., and Murrell-Lagnado, R. D. (1999) *J. Physiol.* **520,** 645–651
5. Logothetis, D. E., and Zhang, H. (1999) *J. Physiol.* **520,** 630
6. Cho, H., Nam, G. B., Lee, S. H., Earm, Y. E., and Ho, W. K. (2001) *J. Biol. Chem.* **276,** 159–164
7. Meyer, T., Wellner-Kienitz, M. C., Biewald, A., Bender, K., Eickel, A., and Pott, L. (2001) *J. Biol. Chem.* **276,** 5650–5658
8. Bunemann, M., and Hosey, M. M. (1999) *J. Physiol.* **517,** 5–23
9. Shui, Z., Boyett, M. R., Zang, W. J., Haga, T., and Kameyama, K. (1995) *J. Physiol.* **487,** 359–366
10. Chuang, H. H., Yu, M., Jan, Y. N., and Jan, L. Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11727–11732
11. Shui, Z., Boyett, M. R., and Zang, W. J. (1997) *J. Physiol.* **505,** 77–93
12. Hong, S. G., Pleumsamran, A., and Kim, D. (1996) *Am. J. Physiol.* **270,** H526–H537
13. Jan, L. Y., and Jan, Y. N. (2000) *Nature Cell Biol.* **2,** E165-E167
14. Kobrinsky, E., Mirshahi, T., Zhang, H., Jin, T., and Logothetis, D. E. (2000) *Nat. Cell Biol.* **2,** 507–514
15. Shui, Z., Yamanushi, T. T., Boyett, M. R. (2001) *J. Physiol.* **536P,** 14–15
16. Cho, H., Youm, J. B., Ryu, S. Y., Earm, Y. E., and Ho, W. K. (2001) *Br. J. Pharmacol.* **134,** 1066–1072
17. Wang, H., Han, H., Zhang, L., Shi, H., Schram, G., Nattel, S., and Wang, Z. (2001) *Mol. Pharmacol.* **59,** 1029–1036
18. Kim, D., Jun, K. S., Lee, S. B., Kang, N. G., Min, D. S., Kim, Y. H., Ryu, S. H., Suh, P. G., and Shin, H. S. (1997) *Nature* **389,** 290–293
19. Caulfield, M. P., and Birdsall, N. J. (1998) *Pharmacol. Rev.* **50,** 279–290
20. Zang, W. J., Yu, X. J., Honjo, H., Kirby, M. S., and Boyett, M. R. (1993) *J. Physiol.* **464,** 649–679
21. Bunemann, M., Brandts, B., and Pott, L. (1996) *J. Physiol.* **494,** 351–362
22. Willars, G. B., Nahorski, S. R., and Challiss, R. A. (1998) *J. Biol. Chem.* **273,** 5037–5046
23. Botelho, R. J., Teruel, M., Dierckman, R., Anderson, R., Wells, A., York, J. D., Meyer, T., and Grinstein, S. (2000) *J. Cell Biol.* **151,** 1353–1368
24. Lanier, L. M., and Gertler, F. B. (2000) *Curr. Biol.* **10,** R655–R657
25. Hilgemann, D. W., Feng, S., and Nasuhoglu, C. (2001) *Sci. STKE* **111,** RE19
26. Steinberg, S. F., and Brunton, L. L. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 751–773
27. Aprigliano, O., Rybin, V. O., Pak, E., Robinson, R. B., and Steinberg, S. F. (1997) *Am. J. Physiol.* **272,** H2726–H2735
28. Hill, J. J., and Peralta, E. G. (2001) *J. Biol. Chem.* **276,** 5505–5510
29. Leaney, J. L., Dekker, L. V., and Tinker, A. (2001) *J. Physiol.* **534,** 367–379
30. Wellner-Kienitz, M. C., Bender, K., and Pott, L. (2001) *J. Biol. Chem.* **276,** 37347–37354
31. Wellner-Kienitz, M. C., Bender, K., Meyer, T., Bunemann, M., and Pott, L. (2000) *Circ. Res.* **86,** 643–648
32. Woodcock, E. A., and Lambert, K. A. (1997) *J. Mol. Cell Cardiol.* **29,** 3275–3283

Downloaded from http://www.jbc.org/ by guest on January 1, 2018

Acetylcholine-induced Phosphatidylinositol 4,5-Bisphosphate Depletion Does Not Cause Short-term Desensitization of G Protein-gated Inwardly Rectifying K^+ Current in Mouse Atrial Myocytes

Hana Cho, Ji-Young Hwang, Daesoo Kim, Hee-Sup Shin, Yangmi Kim, Yung E. Earm and Won-Kyung Ho

J. Biol. Chem. 2002, 277:27742-27747.
doi: 10.1074/jbc.M203660200 originally published online May 17, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203660200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 11 of which can be accessed free at
http://www.jbc.org/content/277/31/27742.full.html#ref-list-1

Downloaded from http://www.jbc.org/ by guest on January 1, 2018