

## Regulation of Adenosine-activated GIRK Channels by Gq-coupled Receptors in Mouse Atrial Myocytes

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Adenosine (Ado) is an important mediator of the endogenous defense against ischemia-induced injury in the heart. The action of Ado is mediated by activation of G protein-gated inwardly rectifying K<sup>+</sup> (GIRK) channels. In turn, GIRK channels are inhibited by reducing phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) through Gq protein-coupled receptors (GqPCRs). We previously found that GIRK channels activated by acetylcholine, a muscarinic M2 acetylcholine receptor agonist, are inhibited by GqPCRs in a receptor-specific manner. However, it is not known whether GIRK channels activated by Ado signaling are also regulated by GqPCRs. Presently, this was investigated in mouse atrial myocytes using the patch clamp technique. GIRK channels were activated by 100  $\mu$ M Ado. When Ado was repetitively applied at intervals of 5–6 min, the amplitude of second Ado-activated GIRK currents ( $I_{K(Ado)}$ ) was  $88.3 \pm 3.7\%$  of the first  $I_{K(Ado)}$  in the control. Pretreatment of atrial myocytes with phenylephrine, endothelin-1, or bradykinin prior to a second application of Ado reduced the amplitude of the second  $I_{K(Ado)}$  to  $25.5 \pm 11.6\%$ ,  $30.5 \pm 5.6\%$ , and  $96.0 \pm 2.7\%$ , respectively. The potency of  $I_{K(Ado)}$  inhibition by GqPCRs was different with that observed in acetylcholine-activated GIRK currents ( $I_{K(ACh)}$ ) (endothelin-1 > phenylephrine > bradykinin).  $I_{K(Ado)}$  was almost completely inhibited by 500  $\mu$ M of the PIP<sub>2</sub> scavenger neomycin, suggesting low PIP<sub>2</sub> affinity of  $I_{K(Ado)}$ . Taken together, these results suggest that the crosstalk between GqPCRs and the Ado-induced signaling pathway is receptor-specific. The differential change in PIP<sub>2</sub> affinity of GIRK channels activated by Ado and ACh may underlie, at least in part, their differential responses to GqPCR agonists.

**Key Words:** GIRK channel, Adenosine, Acetylcholine, PIP<sub>2</sub>, Gq protein-coupled receptors

### INTRODUCTION

Adenosine (Ado) is released during cardiac ischemia and can mediate important protective functions in the heart [1-6]. Previous studies have shown that Ado or Ado receptor agonists can cause a reduction in the infarct size or an improvement in left ventricular function when given during reperfusion [1,6] or during both low-flow ischemia and reperfusion in the isolated and perfused heart [7,8]. The protective effect of Ado in the intact heart is mediated by Ado receptors. Transgenic mouse hearts overexpressing the Ado A<sub>1</sub> receptor (A1AdoR) exhibit an enhanced resistance to the deleterious effect of ischemia [9], demonstrating that overexpression of the Ado receptor subtypes can lead to increased cardioprotection.

The mechanism of action of A1AdoR on cardiac myocytes has been intensively studied. It was demonstrated that A1AdoR hyperpolarizes the membrane potential and decreases the action potential duration [10,11]. These effects were known to be mediated by activation of G protein-gated

inwardly rectifying K (GIRK) channels [12-14]. In atrial myocytes, the GIRK channel current also can be activated by the parasympathetic muscarinic M2 acetylcholine receptor (M2AChR) [15-17]. Thus, they are activated by G<sub>i/o</sub>-coupled receptors and represent important mediators of vagally induced bradycardia and cardioprotection during myocardial ischemia [18,19].

Recently, it was shown that activation of GIRK channels by G<sub>i/o</sub> protein depends on the presence of PIP<sub>2</sub> [20-23]. Since PIP<sub>2</sub> serves as a substrate for phospholipase C (PLC) that is activated by the Gq protein [24,25], we questioned whether PIP<sub>2</sub> depletion induced by the stimulation of Gq protein-coupled receptors (GqPCRs) could inhibit GIRK currents in physiological conditions. This was previously investigated in acetylcholine (ACh)-activated GIRK channels; stimulation of the  $\alpha$ 1-adrenergic receptor resulted in the inhibition of ACh-activated GIRK currents ( $I_{K(ACh)}$ ) [26]. Subsequent experiments on the specificity of action of GqPCRs demonstrated that prostaglandin F<sub>2</sub> $\alpha$  and endothelin-1 are potent inhibitors of  $I_{K(ACh)}$ , but that bradykinin

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**ABBREVIATIONS:** Ado, Adenosine; GIRK, G protein-gated inwardly rectifying K<sup>+</sup>; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; GqPCRs, Gq protein-coupled receptors;  $I_{K(Ado)}$ , Ado-activated GIRK currents;  $I_{K(ACh)}$ , acetylcholine-activated GIRK currents; A1AdoR, Ado A<sub>1</sub> receptor; M2AChR, M2 acetylcholine receptor; PLC, phospholipase C; ACh, acetylcholine; PKC, protein kinase; PDBu, phorbol-12,13-dibutyrate; Kir, inwardly rectifying K<sup>+</sup>; PMA, phorbol 12-myristate 13-acetate.

has little or no effect [27]. These results imply that cross-talk between GqPCRs and GIRK channels is specifically regulated and that the results obtained in ACh-activated GIRK channels cannot be extrapolated to other receptor and channel types. However, there has been limited research conducted on the regulation of Ado-activated GIRK channels, and the role of GqPCRs in Ado-induced signaling pathway in cardiac myocytes is poorly understood.

The present study investigated the effect of various GqPCR agonists, including phenylephrine, endothelin-1, and bradykinin, on Ado-induced GIRK currents ( $I_{K(Ado)}$ ). It is well-known that activation of these GqPCRs stimulates hydrolysis of  $PIP_2$  in cardiac myocytes [28]. Presently, Ado-activated GIRK channels were inhibited by GqPCRs in a receptor-specific manner and the characteristic feature of GqPCR-induced  $I_{K(Ado)}$  inhibition for each GqPCR agonist was different from that observed in  $I_{K(ACh)}$  inhibition. The differential responses of Ado- and ACh-activated GIRK channels to the stimulation of GqPCRs might be mediated by the difference in  $PIP_2$  affinity.

## METHODS

### Cell isolation

Mouse atrial myocytes were isolated by perfusing a  $Ca^{2+}$  free normal Tyrode solution containing collagenase (0.14 mg/ml; Yakult Pharmaceutical, Tokyo) on a Langendorff column at  $37^\circ C$  as previously described [29]. Isolated atrial myocytes were kept in a high  $K^+$ , low  $Cl^-$  solution at  $4^\circ C$  until used.

### Electrophysiology

Membrane currents were recorded from single isolated myocytes in a perforated patch configuration by using nystatin (200  $\mu g/ml$ ; ICN Biomedical, Irvine, CA) or ruptured whole-cell patch clamp configuration at  $35 \pm 1^\circ C$ . Voltage-clamp was performed using an EPS-8 amplifier (HEKA Instruments, Bellmore, NY) and filtered at 5 kHz. The patch pipettes (World Precision Instruments, Sarasota, FL) were made of a PP-830 Narishige puller (Narishige, Tokyo). The patch pipettes had a resistance of 2–3  $M\Omega$  when filled with the pipette solutions. Normal Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 0.5 mM  $MgCl_2$ , 1.8 mM  $CaCl_2$ , 10 mM glucose, and 5 mM HEPES, titrated to pH 7.4 with NaOH. The  $Ca^{2+}$ -free solution contained 140 mM NaCl, 5.4 mM KCl, 0.5 mM  $MgCl_2$ , 10 mM glucose, and 5 mM HEPES, titrated to pH 7.4 with NaOH. The high  $K^+$  and low  $Cl^-$  solution contained 70 mM KOH, 40 mM KCl, 50 mM L-glutamic acid, 20 mM taurine, 20 mM  $KH_2PO_4$ , 3 mM  $MgCl_2$ , 10 mM glucose, 10 mM HEPESs, and 0.5 mM EGTA. The pipette solution for perforated patches contained 140 mM KCl, 10 mM HEPES, 1 mM  $MgCl_2$ , and 5 mM EGTA, titrated to pH 7.2 with KOH. To ensure a rapid solution turn-over, the rate of superfusion was kept  $>5$  ml/min, which corresponded to 50 times bath volume (100  $\mu l/min$ ).

### Materials

ACh (Sigma-Aldrich, St Louis, MO) was dissolved in de-ionized water to make a 10 mM stock solution and was stored at  $-20^\circ C$ . On the day of the experiments, one aliquot

was thawed and used. Phenylephrine, endothelin-1, and bradykinin were from Sigma-Aldrich.

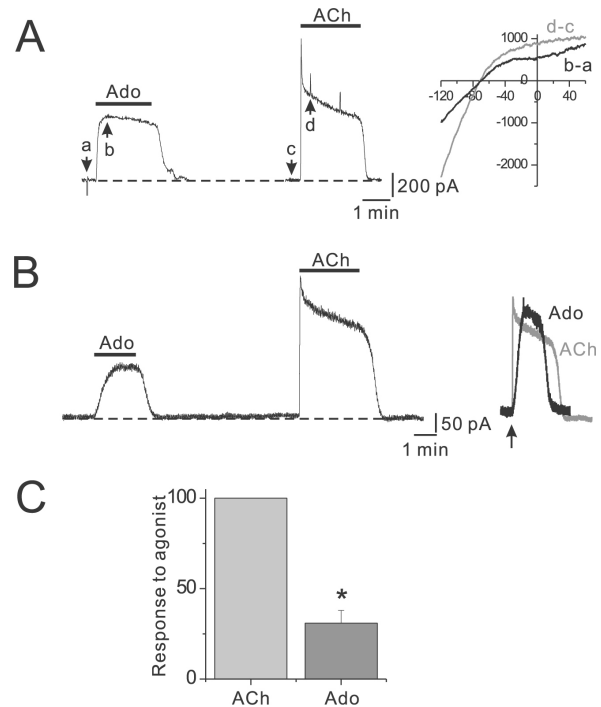
### Statistics and presentation of data

Results in the text and the figures are presented as mean  $\pm$  SEM. (n=number of cells tested). Statistical analyses were performed by using Student's t test. The difference between two groups was considered to be significant when  $p < 0.01$ .

## RESULTS

### Characterization of $I_{K(Ado)}$ in mouse atrial myocytes

$I_{K(Ado)}$  was well-characterized in rat and guinea-pig atrial myocytes [12–14]. However, in mouse preparations,  $I_{K(Ado)}$  has not yet studied in great detail. To investigate the regulation of  $I_{K(Ado)}$  by GqPCRs,  $I_{K(Ado)}$  was first characterized in mouse atrial myocytes.  $I_{K(Ado)}$  was activated by adding 100  $\mu M$  Ado to the bath solution, while the cell was voltage-clamped at the holding potential of  $-40$  mV (Fig. 1A).



**Fig. 1.** Differential activation of atrial GIRK currents by ACh and Ado. (A) Outward current activated by rapid exposure to Ado and ACh. The rapid deflections in this figure represent changes in membrane current due to ramp pulse from  $-120$  to  $+60$  mV, which were applied to obtain I-V curves of  $I_{K(Ado)}$  and  $I_{K(ACh)}$ . Inset, the I-V curves for net  $I_{K(Ado)}$  (black)  $I_{K(ACh)}$  (gray). Data were calculated from data in left. The reversal potential was  $-70$  mV. (B) Ado (100  $\mu M$ ) and ACh (100  $\mu M$ ) were applied for 2 min sequentially at a 6 min interval, as indicated by the horizontal lines above the trace. Inset, response to 100  $\mu M$  Ado (black) and onset of response to 100  $\mu M$  ACh (gray) on an expanded time scale. The response to 100  $\mu M$  Ado was scaled up to match the amplitude of the response to 100  $\mu M$  ACh. The arrow indicates the beginning of agonist exposure. (C) Summarized data for normalized peak amplitude of  $I_{K(Ado)}$  to that of  $I_{K(ACh)}$ .

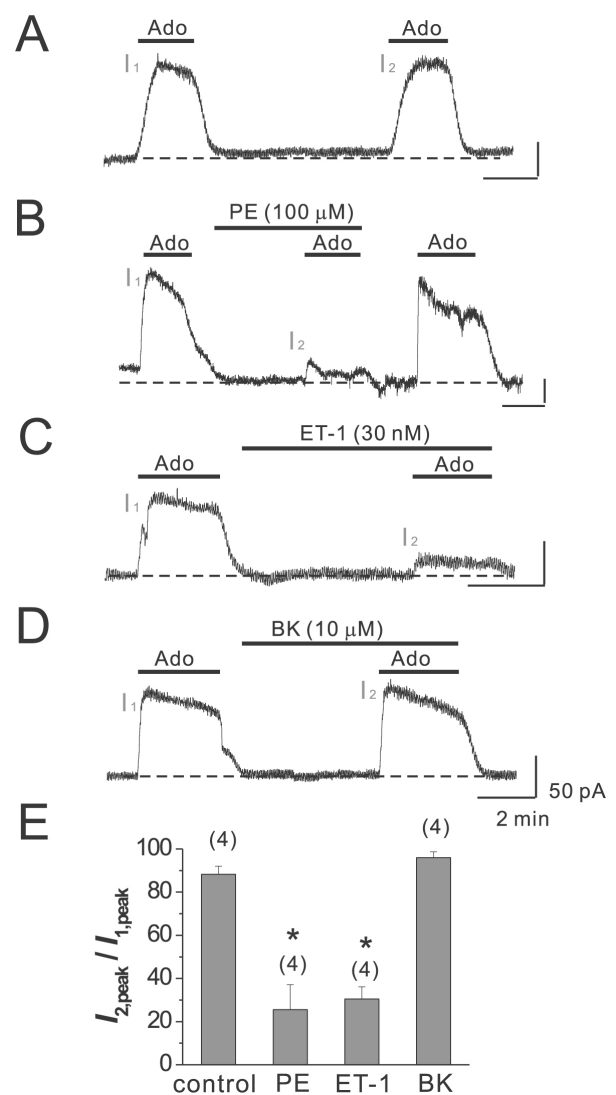
Upon the application of Ado, an increase in outward current was observed. To ensure that the Ado-induced outward currents were GIRK currents, I-V relationships were examined by applying voltage-ramp pulses from  $-120$  mV to  $+60$  mV (at a speed of  $\pm 0.6$  Vs $^{-1}$ ) before and during the application of Ado, as indicated in Fig. 1A. The I-V curves for net  $I_{K(Ado)}$  were obtained by subtracting the control curve (a) from the I-V curves in the presence of Ado (b) (Fig. 1A, inset). This shows a typical inward rectification known for GIRK current with a reversal potential of around  $-70$  mV, which is close to the equilibrium potential of the potassium ion as calculated using the Nernst equation [26]. To compare  $I_{K(Ado)}$  with  $I_{K(ACh)}$ , the I-V curves in the presence of ACh was also obtained. The shape of inward rectification and the reversal potential of two curves were not different, indicating that the current induced by both Ado and ACh were same GIRK currents. However, the current that was activated by Ado was significantly smaller than the total current activated upon stimulation of M<sub>2</sub>AChR if saturating concentrations of ACh ( $\geq 10$   $\mu$ M) and Ado ( $\geq 10$   $\mu$ M) were compared. This is illustrated by the representative current recording in Fig. 1B. On average, the amplitude of  $I_{K(Ado)}$  was  $30.9 \pm 7.0\%$  of  $I_{K(ACh)}$  ( $n=5$ , Fig. 1C). This was consistent with the previous finding that the current activated by saturating concentrations of Ado in atrial myocytes from rat and guinea-pig is about 30% of maximum current activated upon stimulation of M<sub>2</sub>AChR [12-14]. In addition, whereas  $I_{K(ACh)}$  was characterized by rapid activation, activation of  $I_{K(Ado)}$  was slow (Fig. 1B, inset).

#### Effects of various GqPCR agonists on GIRK currents.

Next, the effects of various GqPCR agonists on  $I_{K(Ado)}$  were examined in mouse atrial myocytes. The protocol of the experiments for investigating the effects of GqPCR activation on  $I_{K(Ado)}$  is shown in Fig. 2. As shown in Fig. 2A, the re-application of Ado at intervals of 5~6 min triggered an  $I_{K(Ado)}$  ( $I_2$ , the second  $I_{K(Ado)}$ ) with a similar amplitude to  $I_1$  (the first  $I_{K(Ado)}$ ). To enable quantitative analysis, the peak amplitude of  $I_2$  was normalized to the peak current amplitude of  $I_1$ . Under control conditions, the peak value of  $I_2$  ( $I_{2, peak}$ ) was calculated to be  $88.3 \pm 3.7\%$  ( $n=4$ ) of that of  $I_1$  ( $I_{1, peak}$ ). In subsequent experiments, such a paired application of Ado was used to investigate the effect of various GqPCR agonists on regulation of Ado-activated GIRK channel, regarding the  $I_{K(Ado)}$  at the first response as the control. As shown in Fig. 2B, 100  $\mu$ M of the  $\alpha_1$  adrenergic receptor agonist phenylephrine was applied 4 min before the second application of Ado, and  $I_2$  in the presence of Ado was compared with  $I_1$ . The amplitude of  $I_2$  was significantly reduced by pretreatment of phenylephrine. The peak current amplitude of  $I_2$  was  $25.58 \pm 11.6\%$  ( $n=4$ ) of the  $I_{1, peak}$ , which was significantly smaller than the  $I_{2, peak}$  in the absence of GqPCR agonists ( $p < 0.01$ ) (Fig. 2E). Phenylephrine-induced inhibition of  $I_{K(Ado)}$  was reversible after a recovery period, and the third exposure to Ado elicited an outward current with a similar peak amplitude to that of  $I_1$  (Fig. 2C).

GqPCRs regulate ACh-activated GIRK channels in a receptor-specific manner. Adrenergic agonists and angiotensin II (acting via the  $\alpha_1$  and AT<sub>1</sub> receptors, respectively) produce a moderate effect, and endothelin-1 and prostaglandin F<sub>2 $\alpha$</sub>  induced strong inhibition of the channels [27]. However, bradykinin was almost ineffective in inducing channel inhibition [27]. So, we examined whether the potency of inhibition of Ado-activated GIRK channels by

GqPCR agonists matched with their potency in inhibition of GIRK channels activated by ACh (endothelin-1 > prostaglandin F<sub>2 $\alpha$</sub>  > phenylephrine > angiotensin II > bradykinin). The effects of endothelin-1 (30 nM) and bradykinin (10  $\mu$ M) on  $I_{K(Ado)}$  were tested using the same experimental protocol (Fig. 2C and 2D). The peak current amplitude of  $I_2$  after pretreatment of endothelin-1 was  $30.5 \pm 5.6\%$  ( $n=4$ ) of the  $I_{1, peak}$  (Fig. 2E,  $p < 0.01$  vs control). However, the effect of bradykinin on  $I_{K(Ado)}$  was negligible. The peak current amplitude of  $I_2$  after pretreatment of bradykinin was  $96.0 \pm 2.7\%$  ( $n=4$ ) of the  $I_{1, peak}$  ( $p < 0.05$ ) (Fig. 2E,  $p > 0.05$  vs control). These results indicate that phenylephrine, which exhibited the medium potency in inhibiting  $I_{K(ACh)}$ , induced strong inhibition of the channels with a similar extent of



**Fig. 2.** Differential regulation of  $I_{K(Ado)}$  by different GqPCRs. (A) Ado (100  $\mu$ M) was applied for 2 min twice at a 6 min interval, as indicated by the horizontal lines above the trace. (B~D) Phenylephrine (PE, 100  $\mu$ M), endothelin-1 (ET-1, 30 nM), or bradykinin (BK, 10  $\mu$ M) was applied prior to the application of Ado as indicated by the horizontal lines above the trace. (E) Summarized data for potency of  $I_{K(Ado)}$  inhibition. The numbers in parentheses indicate numbers of cells. \* $p < 0.01$ . Error bars indicate S.E.M.

inhibition to that obtained following the application of endothelin-1. Thus, it can be suggested that crosstalk between GqPCRs and Ado-activated GIRK channels occurs in a receptor-specific manner and the characteristic feature of GqPCR-induced  $I_{K(Ado)}$  inhibition for each GqPCR agonist differs from that observed in  $I_{K(ACh)}$  inhibition.

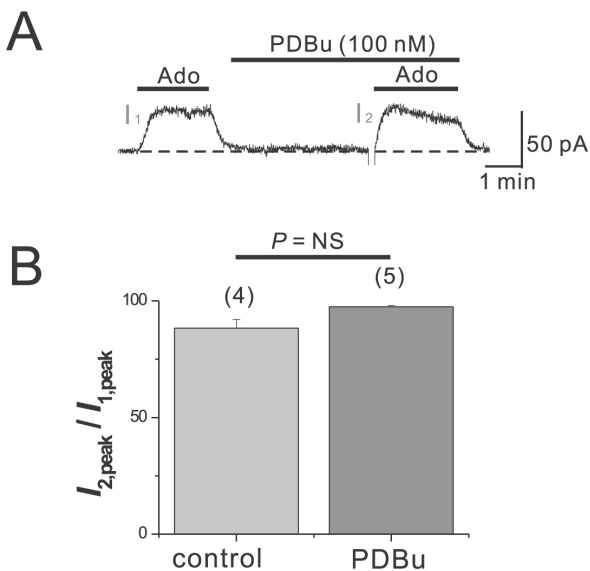
#### Phorbol ester does not affect $I_{K(Ado)}$ in mouse atrial myocytes

Adrenergic and endothelin-1 receptors are known to activate PLC [28] and inhibit ACh-activated GIRK channels via depletion of  $PIP_2$  in cardiac myocytes [26,27]. However, in hippocampal neurons, GIRK channel inhibition by  $G_q$ -coupled muscarinic  $M_1$  receptors is mediated by protein kinase (PKC) activation, even though stimulation of muscarinic  $M_1$  receptors induces robust  $PIP_2$  hydrolysis [30]. To investigate whether PKC activation is required for receptor-induced  $I_{K(Ado)}$  inhibition, we tested if direct activation of PKC by phorbol ester inhibited  $I_{K(Ado)}$ . Phorbol-12,13-dibutyrate (PDBu), a PKC activator, however, had no effects on  $I_{K(Ado)}$  when pretreated for 4 min before second Ado application (Fig. 3A). The  $I_{2, peak}$  in the presence of PDBu was  $97.5 \pm 0.5\%$  of the  $I_{1, peak}$  ( $n=5$ ), which was not significantly different from control condition ( $88.3 \pm 3.7\%$ ,  $n=4$ ,  $P > 0.05$ , Fig. 3B). This result was consistent with the notion that PKC activation did not affect  $I_{K(Ado)}$  and that PKC was not a major mechanism in the inhibition of  $I_{K(Ado)}$  by GqPCRs.

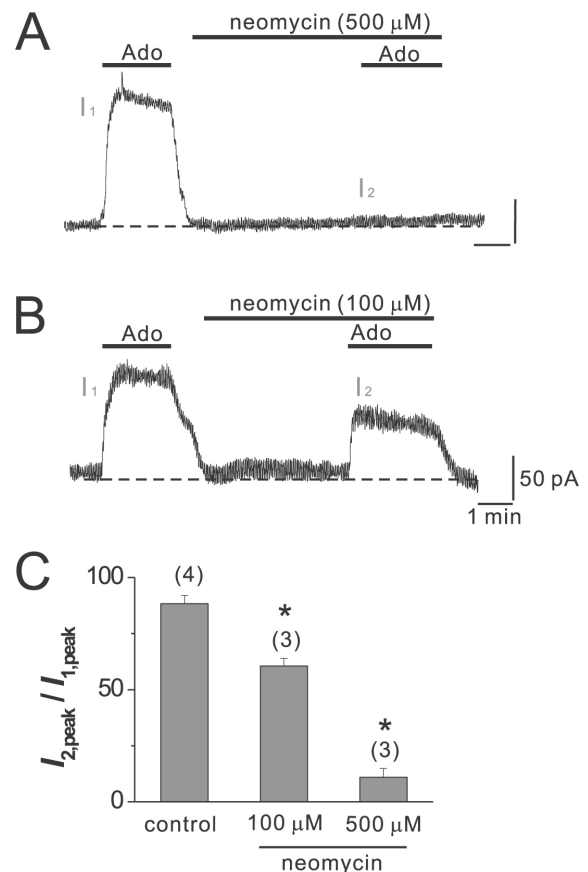
#### Neomycin inhibition as an assay for $PIP_2$ affinity in GIRK channels

Differences in the affinity of inwardly rectifying  $K^+$  (Kir) channels for  $PIP_2$  may be responsible for differences among

Kir channels in their specific regulation by a given modulator [31]. Thus, we hypothesized that the differential sensitivity of  $I_{K(Ado)}$  and  $I_{K(ACh)}$  to GqPCR agonists might reflect their different affinities for  $PIP_2$ . Given that  $\beta\gamma$  subunits of  $G_{i/o}$  proteins activate GIRK channels by directly binding to the GIRK channel, increasing the channels' affinity for  $PIP_2$  [21], it is plausible that A1AdoR and M2AChR increase the affinity of GIRK channels for  $PIP_2$  to different extents. The  $PIP_2$  affinity of ACh- and Ado-activated GIRK channels can be measured using neomycin. Neomycin is a polycation that binds specifically to  $PIP_2$  [32]. In electrophysiological experiments, the neomycin sensitivity of a Kir channel has been used as a measure of its  $PIP_2$  affinity; channels with a high  $PIP_2$  affinity are less sensitive to neomycin than those with low  $PIP_2$  affinity [33]. In previous studies, we showed that neomycin does not significantly affect the activation of  $I_{K(ACh)}$  even at  $500 \mu M$  [26]. Presently, an experiment was devised to examine whether  $I_{K(Ado)}$  was affected by  $500 \mu M$  neomycin. As shown in Fig. 4A, neomycin pretreatment completely inhibited the  $I_{K(Ado)}$ . On average,  $500 \mu M$  neomycin caused a reduction of  $I_{K(Ado)}$  to  $11.0 \pm 3.9\%$  ( $n=3$ , Fig. 4C). The effect of neomycin on  $I_{K(Ado)}$  was concentration-dependent. When  $100 \mu M$  neomycin was pretreated before second Ado application,  $I_{2, peak}$  was re-



**Fig. 3.** PDBu has little effect on  $I_{K(Ado)}$  in mouse atrial myocytes. (A) PDBu (100 nM), a PKC activator, was applied as a pretreatment before the activation of  $I_2$ . (B) the  $I_{2, peak}$  in the absence and presence of PDBu was  $88.3 \pm 3.7\%$  ( $n=4$ ) and  $97.5 \pm 0.5\%$  ( $n=5$ ) of  $I_{1, peak}$ , respectively. The numbers in parentheses indicate numbers of cells. Error bars indicate S.E.M.



**Fig. 4.** Neomycin inhibition of  $I_{K(Ado)}$ . (A, B) Neomycin at concentrations of  $500 \mu M$  (A) or  $100 \mu M$  (B) was applied prior to the application of Ado as indicated by the horizontal lines above the trace. (C) Summarized data for the neomycin inhibition of  $I_{K(Ado)}$ . The numbers in parentheses indicate numbers of cells. \* $p < 0.01$ . Error bars indicate S.E.M.

duced to  $60.5 \pm 3.5\%$  ( $n=3$ , Fig. 4B and 4C). Taken together, these data indicate that  $I_{K(Ado)}$  is more sensitive to neomycin than  $I_{K(ACh)}$ , suggesting that  $I_{K(Ado)}$  has a lower  $PIP_2$  affinity.

## DISCUSSION

This study demonstrates that A1AdoR-activated GIRK channels are inhibited by GqPCR agonists in mouse atrial myocytes, and that the potency of inhibition is GqPCR-specific. Adrenergic and endothelin receptor stimulation reduced the  $I_{K(Ado)}$  by about 70%, whereas bradykinin receptor stimulation had little effect on  $I_{K(Ado)}$ . Such a specific regulation of  $I_{K(Ado)}$  was not attributable to the difference in the potency of  $PIP_2$  hydrolysis by GqPCRs, since all GqPCRs tested in this study could induce  $PIP_2$  hydrolysis in cardiac myocytes [28]. This discrepancy might support the view that there is coupling specificity of GqPCR and GIRK channels [27]. The sensitivity of A1AdoR-activated GIRK channels to GqPCRs is different from that observed in ACh-activated GIRK channels (endothelin-1 > phenylephrine > bradykinin, [27]). When  $PIP_2$  affinity in GIRK channels was estimated by neomycin inhibition, the  $PIP_2$  affinity of GIRK channels activated by Ado was lower than that of ACh-activated GIRK channels. Considering that the channels with low  $PIP_2$  affinity were more susceptible to  $PIP_2$  depletion, it can be suggested that differential change in  $PIP_2$  affinity of GIRK channels activated by Ado and ACh may underlie, at least in part, their different responses to the stimulation of GqPCRs.

It is well-known that the  $PIP_2$  affinity of Kir channels varies widely among different types of Kir channels [21,23], and that the differences in their affinity are responsible for differences among Kir channels in their specific regulation by a given modulator [31]. For example, Kir 3.4 or Kir 2.3 channels, which have low affinity for  $PIP_2$ , are inhibited by ACh or by the PKC activator phorbol 12-myristate 13-acetate (PMA), whereas Kir 2.1 or Kir 3.4 channels in the presence of  $G\beta\gamma$ , which have high affinity for  $PIP_2$ , are only marginally affected. Here, we showed that the GIRK channel- $PIP_2$  interactions in the presence of  $G_{i/o}$ -coupled receptor agonists became different depending on the type of  $G_{i/o}$ -coupled receptors. Thus, the magnitude and kinetics of GIRK inhibition by GqPCRs might differ widely among different  $G_{i/o}$ -coupled receptor agonists. This hypothesis is supported by the previous finding that ACh-activated GIRK channels in native myocytes are not affected by PDBu [34] whereas baclofen-activated GIRK channels in hippocampal neurons are inhibited by PDBu [35]. This discrepancy may reflect the fact that GIRK channel- $PIP_2$  interactions in the presence of  $G_{i/o}$ -coupled receptor agonists in hippocampal neurons are not as strong as those in cardiac myocytes. The present study confirmed that the Ado-induced GIRK channels are not inhibited by PDBu in mouse atrial myocytes (Fig. 3).

The present data represents the first evidence that Ado-activated GIRK channels are regulated by GqPCRs. The functional consequence of inhibition of  $I_{K(Ado)}$  by the  $\alpha 1$ -adrenergic and endothelin receptors may be an early cessation of the protective effect of Ado during cardiac ischemia. This discovery may be of particular importance, since it provides a novel pathway for the interaction of GqPCRs with Ado-induced signaling. The clinical importance of this interaction needs to be investigated in future studies.

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