Activation of G protein-coupled inward rectifier K^+ channels in brain neurons requires association of G protein βγ subunits with cell membrane

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Received 2 May 1996; revised version received 8 June 1996

Abstract In cultured noradrenergic neurons from the rat locus coeruleus, application of recombinant G protein $\beta_1\gamma_2$ subunits (30 nM) to the cytoplasmic side induced single channel activity similar to the somatostatin-induced single channel activity of G protein-coupled inward rectifier potassium channels (K_{ir} (G)). In contrast, recombinant GTPγS-activated, myristoylated α_{i2} (100 nM) did not activate this brain K_{ir} (G). Application of $\beta_1\gamma_2$C68S (30 nM or 150 nM), in which the cysteine residue fourth from the carboxyl terminus of γ_2 was replaced by serine, failed to activate the brain K_{ir}(G). This mutant lacks prenylation which is required for the association of βγ subunit with the cell membrane. Thus, our results suggest that the association of βγ subunit with the cell membrane is a prerequisite for activating K_{ir}(G) channels.

Key words: Inward rectifier K^+ channel; G protein subunit; Prenylation; Brain noradrenergic neuron; Locus coeruleus

1. Introduction

G protein-coupled inward rectifier K^+ channels (K_{ir}(G)) have been studied intensively in recent years. Most of the investigations have been conducted on K_{ir}(G) of cardiac origin. Logothetis et al. [1] and Ito et al. [2] observed that the endogenous atrial K_{ir}(G) was activated by purified βγ subunit, but not by α_i subunit. This observation was confirmed by experiments using recombinant G protein subunits [3]. Compared to the cardiac K_{ir}(G), far less is known about the properties of endogenous K_{ir}(G) in brain neurons.

We developed dissociated cultures of noradrenergic neurons from the locus coeruleus; these neurons responded well to somatostatin and met-enkephalin with an increase in K_{ir}(G) conductance [4–6]. The single channel properties of this endogenous brain K_{ir}(G) were recently analyzed, and it was found that this brain K_{ir}(G) differed from the cardiac K_{ir}(G) in several respects (for instance, the degree of inward rectification of the brain K_{ir}(G) is milder than that of the cardiac K_{ir}(G)) [7]. Here, we will show that the brain K_{ir}(G), like its cardiac counterpart, is also activated by βγ subunit but not by α_{i2} subunit.

The carboxyl terminus of γ subunits undergoes prenylation followed by proteolysis and methylation. The prenylation of γ subunit is a prerequisite for the βγ subunit to be anchored to the cell membrane [8,9], and to regulate the activity of adenylyl cyclases [10]. In this report, we will show that prenylation of γ subunit is also necessary for the βγ subunit to be able to activate the brain K_{ir}(G) channel. The data were presented at a meeting [11].

2. Materials and methods

2.1. Materials

2.1.1. G protein subunits Recombinant wild-type $\beta_1\gamma_2$, $\beta_1\gamma_2$C68S, and α_{i2} were purified using the Sf9-baculovirus expression system as described by Kozasa and Gilman [12] and Iñiguez-Lluhi et al. [10]. Recombinant G protein subunits (wild-type $\beta_1\gamma_2$, $\beta_1\gamma_2$C68S, and α_{i2}) were dissolved into the bathing solution (see Section 2.2.2) containing 100 μM GDP. Final G protein subunit solutions contained 8–42 μM CHAPS (3-[(3-chlolamidopropyl)dimethylammonio]-1-propanesulfonate). However, 100 μM CHAPS, without G protein subunits, did not induce channel activity.

2.1.2. Chemicals The sources of the chemicals were: somatostatin (Peninsula); GDP and GTP (Sigma); guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and ATP (Boehringer-Mannheim); CHAPS (Calbiochem).

2.2. Methods

2.2.1. Neuron cultures Locus coeruleus neurons were cultured from 2–4-day-old postnatal Long-Evans rats (Charles River Breeding Laboratories, Willmington MA). The culture method described by Masuko et al. [4] was used with some modifications. Briefly, rats were anesthetized with ether, and brain slices were sectioned from the excised brainstems. Under visual observation with a dissecting microscope, the locus coeruleus was isolated from brain slices, dissociated after treatment with papain (12 units/ml), and plated on a feeder layer consisting of glia cells. Neurons were cultured in a medium which contained a minimum essential medium with Earle's salts (Gibco, cat no. 11430-030) modified by the addition of 0.292 mg/ml L-glutamine, 3.7 mg/ml $NaHCO_3$, and 5 mg/ml D-glucose, and supplemented with 2% heat-inactivated rat serum (prepared in our laboratory), 10 μg/ml L-ascorbic acid, 50 U/ml penicillin and 50 μg/ml streptomycin. Neurons were cultured for 1–2 weeks at ≈37°C in 10% CO_2-90% air with saturated humidity.

2.2.2. Single channel recording The inside-out patch clamp method was used for recording single channel events [7,13]. Experiments were conducted on large neurons (soma diameter 28 ± 3 μM; mean ± S.D.), which were very likely noradrenergic [4]. The patch pipette solution (external solution) contained: 156 mM KCl; 2.4 mM $CaCl_2$; 1.3 mM $MgCl_2$; 5 mM HEPES-NaOH; and 0.5 μM tetrodotoxin (pH 7.4) with or without 0.5 μM somatostatin. The bathing solution (the cytoplasmic side solution) contained: 141 mM potassium d-gluconate (or 141 mM potassium aspartate), 8.7 mM NaCl; 5 mM HEPES-KOH; 5 mM EGTA-KOH; 1 mM $MgCl_2$; 2 mM Na_2ATP; 0.1 mM GDP (or 0.1 mM GTP); and ≈5.5 mM KOH to adjust the pH to 7.2. Values of membrane potentials were corrected for the liquid junction potential between the internal solution and the external (patch pipette) solution. Single channel currents were recorded with a List EPC-7 amplifier. The exchange of the bathing (internal) solution was made by superfusing the bath (≈0.1 ml in volume) with the new solution (>0.5 ml). Experiments were performed at ≈23°C.

2.2.3. Single channel data analysis Data analysis was performed as described by Grigg et al. [7]. The data were stored on video cassette tapes through a pulse code modulator (VR-10A, Instrutech Corp.). The records were later analyzed with pCLAMP programs (version

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PII S0014-5793(96)00661-8

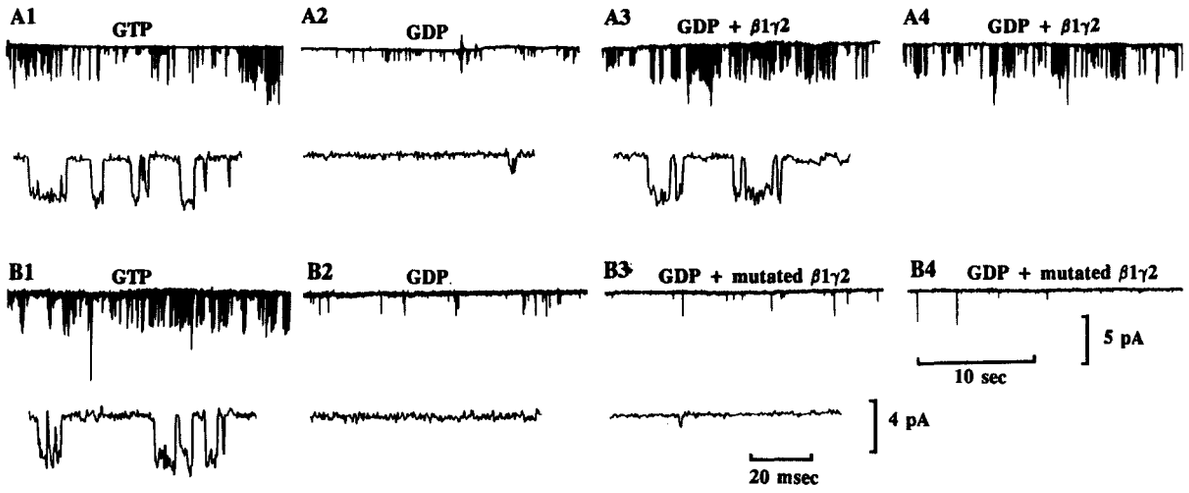

Fig. 1. Applications of recombinant wild-type $\beta_1\gamma_2$ subunits and the mutant protein $\beta_1\gamma_2$C68S to inside-out patches from cultured locus coeruleus neurons. Inward currents (downward direction) represent channel openings. The potential was held at −97 mV (inside negative), and the patch-pipette contained 156 mM K^+ with 0.5 μM somatostatin. The cytoplasmic solution contained 159 mM K^+. (A) Wild-type $\beta_1\gamma_2$ subunits. First, the cytoplasmic solution containing 100 μM GDP did not activate ≈30 pS channels (not shown in this figure). Then, application of 100 μM GTP-containing cytoplasmic solution activated the channels (A1). The channel activity disappeared in 100 μM GDP (exchanged with the GTP-containing solution) (A2). The $\beta_1\gamma_2$ subunits (30 nM) were then applied in the presence of 100 μM GDP (without GTP); this increased the channel activity (A3). This record (A3) was taken 1 min after the start of the $\beta_1\gamma_2$ application. The record in A4 was taken 2 min after the start of the $\beta_1\gamma_2$ subunit application. (B) Experiment with 30 nM $\beta_1\gamma_2$C68S (mutated $\beta_1\gamma_2$). The same protocol as described for the records in (A) was used. (B3) 3 min after the start of the application of $\beta_1\gamma_2$C68S. (B4) 5 min after the start of the application of $\beta_1\gamma_2$C68S; still there was no increase in the channel activity. A fast time-base record for each condition is displayed underneath the main record. During the application of GDP, the activity of small channels was observed (for example, A2). According to our previous analysis [7], the activity of this small channel was not influenced by the GDP-GTP exchange, suggesting that it is not G protein regulated (hence, termed the small background channel). The ionic mechanism of this channel is not clear.

5.5.1, Axon Instruments). The overall frequency response was set at 2 kHz (−3 db by 8-pole Bessel filter) and digitized at 20 kHz.

3. Results

3.1. Channels activated by wild-type $\beta_1\gamma_2$

Previously we investigated, with the inside-out configuration, single channel properties of the K_{ir}(G) in noradrenergic neurons cultured from the locus coeruleus [7]. The K_{ir}(G) was activated by somatostatin and met-enkephalin, and its unitary conductance was ≈30 pS [7]. We examined whether this single channel activity could be induced by βγ subunit. Recombinant $\beta_1\gamma_2$ was used in this study; $\beta_1\gamma_2$ is a major βγ subtype in the brain [14]. In the experiments shown in Fig. 1, we used the inside-out patch method with somatostatin (0.5 μM) in the patch pipette combined with GDP-GTP exchange on the cytoplasmic side [7]. Success at obtaining an inside-out patch was confirmed by observing that single channel activity was induced by GTP (100 μM) (Fig. 1A1) but not by GDP (100 μM) (Fig. 1A2), as reported previously [7]. After this, wild-type $\beta_1\gamma_2$ (30 nM) in the GDP containing solution was applied to the cytoplasmic side of the patch. This application induced single channel activity (Fig. 1A3, A4) very similar to that caused by GTP (Fig. 1A1) ($n = 7$). There were no significant differences in the unitary conductance (≈30 pS) and the open time between the GTP-induced and the $\beta_1\gamma_2$-induced single channel activities ($p = 0.899$, $n = 5$) (Table 1). The $\beta_1\gamma_2$-induced single channel activity began 11–36 s after starting application, and continued during $\beta_1\gamma_2$ application as shown in Fig. 1A3,A4. The channel activity continued even after changing the bath to the GDP-containing solution without $\beta_1\gamma_2$, lasting until the end of the experiments (in 4 patches we obtained continuous activity for 5–11 min after $\beta_1\gamma_2$ was washed away; in one case for 34 min). Thus, the effects of $\beta_1\gamma_2$ were practically irreversible.

3.2. Application of the $\beta_1\gamma_2$C68S mutant

The effect of $\beta_1\gamma_2$C68S (30 μM) was examined, using the same method as described above (Fig. 1B). After inside-out patch formation was confirmed by applying the GTP (100 μM) solution to the cytoplasmic side of the patch (Fig. 1B1), the bath was changed to the GDP (100 μM) solution (Fig. 1B2), and then to $\beta_1\gamma_2$C68S in GDP solution. In contrast to wild-type $\beta_1\gamma_2$ (Fig. 1A3, A4), application of $\beta_1\gamma_2$C68S did not induce single channel activity with ≈30 pS unitary conductance (Fig. 1B3,B4) (observed for 6–8 min after starting

Table 1
Properties of single channels induced by GTP and by wild-type $\beta_1\gamma_2$ subunits

	Open time (ms)	Unitary conductance (pS)	n
GTP (100 μM)	0.611 ± 0.070	30.9 ± 0.75	5
$\beta_1\gamma_2$ (30 nM)	0.619 ± 0.082	31.7 ± 1.35	5

Values are mean ± S.E.M. Open time was calculated from the weighted average of 2 time constants. Unitary conductance (pS) was measured with $[K]_o = 156$ mM, $[K]_i = 159$ mM, at −97 mV and ≈23°C. Patch pipettes contained somatostatin (0.5 μM). $\beta_1\gamma_2$ solution contained GDP (100 μM).

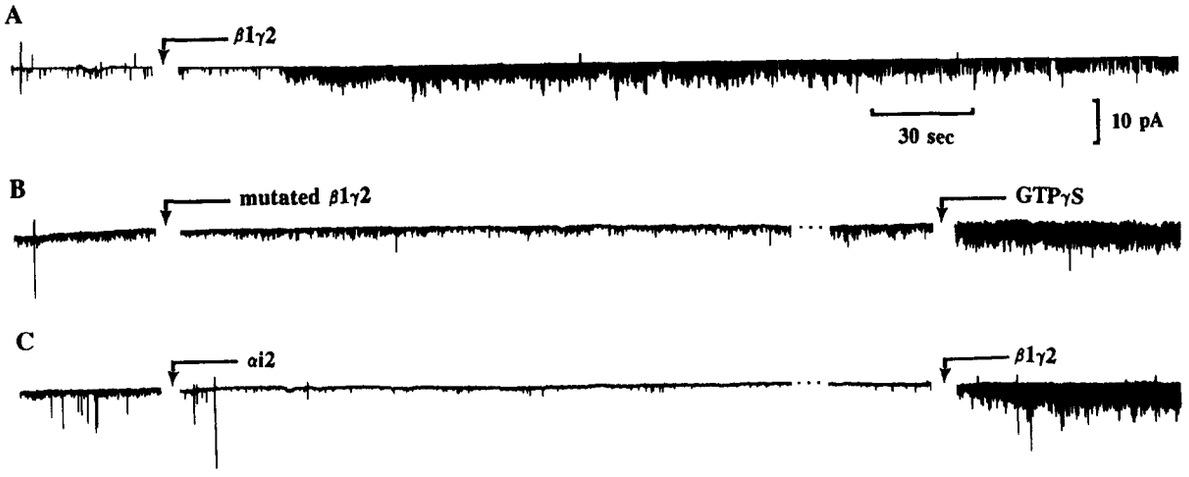

Fig. 2. Applications of recombinant wild-type $\beta_1\gamma_2$, $\beta_1\gamma_2$C68S and GTPγS-activated α_{i2} to inside-out patches from locus coeruleus neurons. The potential was held at −97 mV. Patch pipettes contained 156 mM K^+ and no agonist. The cytoplasmic solution contained 159 mM K^+ and 100 μM GDP. (A) The cytoplasmic solution containing 100 μM GDP did not activate ≈30 pS channels. The succeeding application of 30 nM $\beta\gamma_2$ (arrow) did induce the ≈30 pS channel activity which lasted until the end of the experiment (16 min). (B) The activity of ≈30 pS channels was observed neither during the presence of 100 μM GDP in the cytoplasmic solution nor during the presence of 150 nM $\beta_1\gamma_2$C68S (mutated $\beta_1\gamma_2$) (applied during the gap marked to by the first arrow). However, channel activity was induced by the application of 10 μM GTPγS (the second arrow). The dotted line during $\beta_1\gamma_2$C68S application represents a time period of 213 s. (C) Application of 100 μM GDP as well as the succeeding application of 100 nM recombinant GTPγS-activated α_{i2} subunits (α_{i2}, the first arrow) did not activate ≈30 pS channels. In contrast, the succeeding application of 30 nM wild-type $\beta_1\gamma_2$ (second arrow) did activate these channels. The dotted line during the presence of α_{i2} represents a time period of 212 s.

application ($n=8$)), suggesting that $\beta_1\gamma_2$C68S cannot activate the $K_{ir}(G)$.

The effect of $\beta_1\gamma_2$C68S was also examined using patch pipettes containing no agonist. As shown in Fig. 2A, wild-type $\beta_1\gamma_2$ subunits (30 nM) in a GDP (100 μM) containing bathing solution stimulated ≈30 pS single channel activity ($n=6$). In contrast, $\beta_1\gamma_2$C68S (30 nM, $n=4$ or 150 nM, $n=3$) did not induce such single channel activity (Fig. 2B, first arrow). (In $\beta_1\gamma_2$C68S experiments, the successful formation of inside-out patches was confirmed by applying either a GTPγS (10 or 100 μM) containing bathing solution (Fig. 2B, second arrow) [7] or wild-type $\beta_1\gamma_2$ (30 nM) in a GDP (100 μM) solution.) These results indicate that $\beta_1\gamma_2$C68S cannot activate the $K_{ir}(G)$ regardless of the presence or absence of the external agonist.

3.3. Application of GTPγS-activated α_{i2}

To examine if G protein α-subunits can activate the $K_{ir}(G)$, we applied GTPγS-activated recombinant α_{i2}. Our recent experiments using antibodies and antisense oligonucleotides against various subtypes of PTX-sensitive G protein α-subunit (Takano et al., manuscript to be submitted) suggest that the activation of $K_{ir}(G)$ conductance by somatostatin in locus coeruleus neurons is mediated through G_{i2} but not through G_{i1}, G_{i3}, or G_o. The recombinant α_{i2} subunits used in this study were purified from the membrane fraction of Sf9 cells, and were thus myristoylated and palmitoylated [15].

As shown in Fig. 2C, after an inside-out patch was obtained in a bathing solution containing GDP (100 μM), GTPγS-activated α_{i2} (100 nM) in the GDP solution was applied to the cytoplasmic side of the patch. The patch pipettes did not contain somatostatin, and at the end of the experiments, the formation of the inside-out patch was examined by applying $\beta_1\gamma_2$ (30 nM) in the GDP solution (Fig. 2C, second arrow) or GTPγS (10 μM) in a bathing solution without GDP. In all cases ($n=4$), GTPγS-activated α_{i2} did not induce single-channel activity with ≈30 pS unitary conductance (observed for 4.5–8 min after the start of α_{i2} application). These results suggest that the $K_{ir}(G)$ in locus coeruleus neurons is activated by $\beta_1\gamma_2$, but not by α_{i2}.

4. Discussion

4.1. Brain $K_{ir}(G)$ are activated by $\beta_1\gamma_2$ but not by α_{i2}

Previously contradictory data were published on whether the βγ subunit [1,2] or the α_i subunit [16] activates the atrial $K_{ir}(G)$. More recently, the results of Wickman et al. [3], using recombinant G proteins, indicate that βγ subunits but not α_i subunits activate the endogenous atrial $K_{ir}(G)$. The same conclusion was reached by others using the cloned atrial inward rectifier channel (GIRK1) [17,18].

Little is known about whether endogenous $K_{ir}(G)$s in brain neurons are also activated by the βγ subunit. In the present study we have clearly demonstrated that endogenous $K_{ir}(G)$ in noradrenergic neurons in the locus coeruleus are activated by recombinant $\beta_1\gamma_2$ subunits. Oh et al. [19] reported that the $K_{ir}(G)$ in rat hippocampal neurons was activated by transducin βγ purified from rod outer segment membranes.

According to our recent experiments (Takano et al., to be submitted), the somatostatin effect on locus coeruleus neurons is mediated through G_{i2}. In the present study, we have shown that in brain neurons a GTPγS-activated recombinant α_{i2} does not activate the inward rectifier K^+ channels. These results suggest that when somatostatin reacts with somatostatin receptors in locus coeruleus neurons, a heterotrimeric G protein, $\alpha_{i2}\beta\gamma$, couples to a somatostatin receptor and then dissociates into βγ subunit and GTP-bound α_{i2} subunit. The endogenous $K_{ir}(G)$ is then activated by the βγ subunit, but not by the GTP-bound α_{i2} subunit. Wickman et al. [3] reported that different subtypes of recombinant βγ subunits can activate the cardiac $K_{ir}(G)$. Thus, the specificity of the G protein appears to reside in the coupling of the receptor and the G

protein, and not in the coupling between the βγ subunit and the channel.

4.2. $\beta_1\gamma_2$C68S does not activate the $K_{ir}(G)$

The post-translational modification of G protein γ subunit consists of three steps: prenylation, proteolysis, and methylation. In the case of γ_2, a non-retinal γ subunit, prenylation is geranylgeranylation [20–23]. Point mutation of γ_2 subunits at the fourth position from the carboxyl terminus, from cysteine to serine (γ_2C68S), blocks prenylation of γ subunits, but does not prevent the formation of a βγ complex [9]. In the present study we observed that contrary to the wild-type $\beta_1\gamma_2$, the mutated $\beta_1\gamma_2$ ($\beta_1\gamma_2$C68S) did not activate the $K_{ir}(G)$, suggesting that prenylation is necessary for the interaction of the βγ subunits with the $K_{ir}(G)$. It is also likely that the anchoring of βγ subunits to the cell membrane is a prerequisite for channel activation since $\beta_1\gamma_2$C68S does not associate with the cell membrane, and does not affect the activity of adenylyl cyclase type I and type II [8–10]. The fact that activation of the $K_{ir}(G)$ by $\beta_1\gamma_2$ subunits was very difficult to reverse by washing is consistent with the idea that $\beta_1\gamma_2$ is anchored to the cell membrane.

Acknowledgements: We are grateful to Dr. Ronald H. Farkas for critical reading of the manuscript, and to Ms. Linda Johnston for technical and editorial assistance. This work was supported by National Institutes of Health Grant AG06093.

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