Selective Modulation of Neuronal Nicotinic Acetylcholine Receptor Channel Subunits by $G_\alpha$-Protein Subunits

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G-protein modulation of neuronal nicotinic acetylcholine receptor (nAChR) channels in rat intrinsic cardiac ganglia was examined using diazylated whole-cell and excised membrane patch-recording configurations. Cell dialysis with GTPγS increased the agonist affinity of nAChRs, resulting in a potentiation of nicotine-evoked whole-cell currents at low concentrations. ACh- and nicotine-evoked current amplitudes were increased approximately twofold in the presence of GTPγS. In inside-out membrane patches, the open probability (NPo) of nAChR-mediated unitary currents was reversibly increased fourfold after bath application of 0.2 mM GTP. The open probability of ACh-activated single-channel currents was increased approximately twofold in the presence of GTPγS. In inside-out membrane patches and in glutathione S-transferase pull-down and communoprecipitation experiments. Bath application of 50 nM Gβγ increased the open probability of ACh-activated single-channel currents fivefold, whereas Gα5 (50 nM) produced no significant increase in NPo. Neuronal nAChR subunits α3–α5 and β2 exhibited a positive interaction with Gα5 and Gβγ, whereas β4 and α7 failed to interact with either of the G-protein subunits. These results provide evidence for a direct interaction between nAChR and G-protein subunits, underlying the increased open probability of ACh-activated single-channel currents and potentiation of nAChR-mediated whole-cell currents in parasympathetic neurons of rat intrinsic cardiac ganglia.

Key words: cholinergic; nicotinic receptor; G-protein subunits; parasympathetic; intracardiac ganglia; patch clamp

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ion channels composed of $\alpha$ and $\beta$ subunits and play an important role in signal transmission in the mammalian CNS and peripheral nervous system. To date, eight $\alpha$ (α2–α7, α9, and α10) and three $\beta$ (β2–β4) subunits have been identified in the mammalian nervous system. Neuronal nAChRs on intrinsic cardiac ganglion (ICG) neurons mediate ganglionic transmission and are composed of α3, α5, α7, β2, and β4 subunits (Poth et al., 1997; Bibevski et al., 2000). In contrast to the metabotropic muscarinic AChR (mAChR), the nAChR does not require the activation of guanine nucleotide-binding proteins (G-proteins) to open its associated ion channel. However, nAChRs have been reported to be regulated by protein kinases (Swope et al., 1995); for example, inhibition of Src family tyrosine kinases has been shown recently to reduce the amplitude of nicotine-induced currents in chromaffin cells and recombinant α3β4α5 nAChRs expressed in human embryonic kidney 293 (HEK293) cells (Wang et al., 2004).

G-proteins consist of Gaβγ heterotrimers (Clapham and Neer, 1997), and the conformational change in the G-protein-coupled receptor (GPCR) induced by ligand binding leads to the exchange of GDP for GTP on the Ga subunit and the dissociation or rearrangement of Ga-GTP from its Gβγ dimer (Bunemann et al., 2003). Free Gβγ dimers have been shown to modulate the activity of an array of effector proteins, including voltage-gated Ca$^{2+}$ and K$^+$ channels, by direct interaction with intracellular domains of these channels (Reuveny et al., 1994; Krapivinsky et al., 1995; Herlitze et al., 1996; De Waard et al., 1997). A direct interaction of the Gβγ dimer has been shown recently with native and recombinant glycine receptor channels (Yevenes et al., 2003). The response of glycine, but not GABAA, receptor channels could be enhanced by the activation of GPCRs or activation with the Gβγ dimer itself. In the present study, we show direct modulation of neuronal nAChR channels by the nonhydrolyzable GTP analog GTPγS and the Gβγ subunit, consistent with the idea of direct G-protein activation of neuronal nAChR subtypes.

Materials and Methods

Preparation of dissociated intrinsic cardiac ganglia neuron cultures. Parasympathetic neurons from neonatal rat ICGs were isolated and placed in tissue culture. The procedures for the isolation of ICG neurons have been described previously (Xu and Adams, 1992) and were in accordance with guidelines of the University of Queensland Animal Experimentation Ethics Committee. Briefly, Wistar rats (2–12 d of age) were killed by stunning and decapitation, and the heart was excised and placed in HBSS. Clusters of ICGs surrounding the pulmonary veins were dissected from the atria and transferred to HBSS containing collagenase (300 U/ml; Worthington Biochemical, Lakewood, NJ). After 1 h of digestion at 37°C, the ganglia were rinsed twice in HBSS and triturated in culture medium (DMEM with 10% v/v fetal calf serum and 1% v/v penicillin–strepto-
myosin; Invitrogen, Mount Waverley, Victoria, Australia) using a fine-bore Pasteur pipette. The dissociated neurons were plated on sterile 12 mm glass cover slips and incubated at 37°C for 12–24 h in a 95% O2–5% CO2 atmosphere.

Electrophysiological recordings from ICG neurons. Membrane currents were recorded from isolated ICG neurons using standard whole-cell patch clamp and inside-out excised membrane patch recording techniques. Eodiodes for both recording techniques (model GF150F-7.5; Harvard Apparatus, Edenbridge, UK) were pulled and fire-polished and had resistances of 1–2 MΩ when filled with intracellular solution. For single-channel experiments, pipettes were coated with dental wax to reduce leak. Amino acids in proteins; http://www.cbs.dtu.dk/services/TMHMM-2.0/). Amino acid sequences were fitted by unweighted nonlinear regression to the Hill equation.

Results

G-protein activation of nAChRs in neurons of the intrinsic cardiac plexus

Previous results from our laboratory have shown that activation of members of the pituitary adenylate cyclase-activating polypeptide (PACAP)/vasoactive intestinal peptide (VIP) receptor family potentiated ACh- and nicotine-evoked currents in parasympathetic neurons dissociated from neonatal rat intracardiac and submandibular ganglia (Cuevas and Adams, 1996; Liu et al., 2000). This modulation was mediated by a pertussis toxin-sensitive Gαi protein pathway, was unaffected by cell dialysis (Cuevas and Adams, 1996), and could be mimicked by intracellular perfusion with the nonhydrolyzable GTP analog GDPyS (Liu et al., 2000). To further examine the mechanism of G-protein modulation of neuronal nAChRs, intracardiac neurons were dialyzed with 0.2 mM GTP (control), GTPγS, or the nonhydrolyzable GDP analog GDPβS. GPCR-induced potentiation of nAChRs could be mimicked by cell dialysis with 0.2 mM GTPγS (Fig. 1). Representative whole-cell membrane currents evoked by 30 μM nicotine in the presence of different GTP/GDP analogs are shown in Figure 1A. Current–voltage relationships obtained for the peak nicotine-evoked current density from neurons dialyzed with different GTP/GDP analogs are shown in Figure 1B. GTPγS potentiated nicotine-evoked current amplitude at all membrane potentials, increasing peak current density more than threefold from −23.8 ± 2.4 to −76.1 ± 7.2 pA/pF (n = 6) in the presence of GTPγS at −50 mV. GDPβS in the pipette had no
signifcant effect on peak current amplitude; the peak current density at $-50$ mV was $-26.2 \pm 6.3$ pA/pF ($n = 6$). G-protein potentiation of nicotine-evoked current amplitude was dependent on nicotine concentration. A normalized plot of the peak nicotine-evoked current density as a function of nicotine concentration is shown in Figure 1C. The data were fit using the following equation: $y = 1 + ([A]/EC_{50})^h$, where $y$ is the normalized response, $[A]$ is the agonist concentration, $EC_{50}$ is the concentration that gives a half-maximal response, and $h$ is the slope factor (Hill coefficient). The fit of the data indicates a shift toward higher affinity of the nAChR with an $EC_{50}$ of 10.0 $\pm$ 1.2 $\mu$M obtained for neurons dialyzed with GTP$\gamma$S compared with 48.6 $\pm$ 1.7 $\mu$M obtained for control. However, dialysis with GDP$\beta$S did not significantly change the $EC_{50}$ for nicotine (43.5 $\pm$ 4.7 $\mu$M).

**G-protein activation affects the open probability of nAChRs**

Single-channel recording was performed using excised inside-out membrane patches to examine the mechanism(s) underlying the potentiation of nicotine-evoked whole-cell currents by GTP$\gamma$S in rat intracardiac neurons. The product of the number of functional channels and single-channel open probability ($NP_o$) for the inside-out patches was determined using the following equation: $NP_o = T/T_0$, where $T$ is the total open time for a patch over time $T$. Consecutive recordings of $\pm 30$ s in duration were made in the presence of a control solution containing 0.2 mM GTP, GTP$\gamma$S, or GDP$\beta$S.

Single-channel currents were only evoked when ACh (5 $\mu$M) was present in the pipette. ACh elicited unitary currents occurring as clusters of bursts of openings separated by long closed intervals, whereas bursts within a cluster were separated by closings of intermediate length (Fig. 2A). ACh-evoked channel activity recorded from the same patch increased in the presence of bath-applied 0.2 mM GTP$\gamma$S. This potentiation of single-channel activity could be reversed after washout (data not shown). The unitary nAChR current traces from a patch in the presence of 0.2 mM GDP$\beta$S are similar to those obtained under control conditions (Fig. 2A). The $NP_o$ obtained in the presence of GTP$\gamma$S was 0.86 $\pm$ 0.14, which was four times larger than ACh (5 $\mu$M) alone (0.24 $\pm$ 0.10), whereas bath application of 0.2 mM GTP or GDP$\beta$S did not significantly alter the open probability (0.27 $\pm$ 0.1 and 0.15 $\pm$ 0.04) compared with control conditions (Fig. 2B). The mean unitary current amplitudes were 1.52 $\pm$ 0.02 pA (control) and 1.49 $\pm$ 0.03 pA (GTP$\gamma$S), respectively, and were not statistically different ($p < 0.01$) (Fig. 2C). However, the normalized distribution of channel closings recorded from eight membrane patches in the presence and absence of bath-applied GTP$\gamma$S reflects the significant differences shown for $NP_o$ (Fig. 2D).

**Agonist-specific modulation of nAChR currents**

Pharmacological profiles of nAChR agonists vary among different subunit compositions and provide a useful tool for distinguishing between receptor populations. To determine whether whole-cell currents evoked by particular nicotinic agonists can be differentially modulated by G-proteins, we used 0.2 mM GTP$\gamma$S or GTP (control) in the pipette solution and applied focal pulses (50 ms) of half-maximal concentrations of agonists every 30 s (except for choline, which was applied every 60 s to avoid receptor desensitization). The peak amplitude of each agonist-evoked current was measured and normalized to the initial current obtained after cell dialysis. Currents evoked by the endogenous neurotransmitter ACh (100 $\mu$M) (Fig. 3Ai), nicotine (30 $\mu$M) (Fig. 3Aii), and the synthetic compound 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (100 $\mu$M) could be potentiated by whole-cell dialysis with 0.2 mM GTP$\gamma$S. Currents evoked by cytosine (30 $\mu$M) (Fig. 3Aiii) and choline (10 mM) could not be
modulated by intracellular dialysis with either 0.2 mM GTP or GTPγS. The time course of modulation followed a sigmoidal regression analogous to receptor activation, with the slope coefficient indicating the rate of activation. ACh-evoked peak current amplitude was increased by 199 ± 11% \((n = 10)\) (Fig. 3Bi) and reached a maximum after 5 min with a slope factor of −4.3 ± 0.5. Similarly, a maximum increase of 221 ± 14% \((n = 10)\) was observed for nicotine-evoked currents with a slope factor of −6.15 ± 0.8. The potentiation of DMPP-evoked currents was 145 ± 8% \((n = 8)\), with a slope factor of −5.2 ± 2.2. The relative current amplitudes evoked by application of cytisine and nicotine on the same neuron after break-in (0 s) and after 5 min are shown in Figure 3Bii.

\[ \text{Slope factor} \]

**Gα-protein subunits differentially interact with nACHR subunits**

Given the agonist-dependent potentiation of nACHR-mediated currents, the molecular specificity of G-protein modulation of particular nACHR subunits was examined. We had shown previously that VIP- and PACAP-mediated potentiation of ACh-evoked currents in rat parasympathetic neurons is mediated only by G\(_{\alpha}\) and G\(\beta\gamma\) subunits (Liu et al., 2000). Bath application of either G\(_{\alpha}\) or G\(\beta\gamma\) to the excised inside-out membrane patch increased the single-channel open probability. Representative single-channel recordings obtained after bath application of either G\(_{\alpha}\) or G\(\beta\gamma\) to the excised membrane patch are shown in Figure 4A. G\(\beta\gamma\) (50 mM) significantly increased \(N_P\), fivefold from 0.23 ± 0.12 to 1.15 ± 0.12 \((n = 4)\) by reducing the distribution of channel closings (Fig. 4B). Application of G\(_{\alpha}\) increased \(N_P\), but it was not statistically significant \((0.41 ± 0.13, n = 4)\) from control.

To examine the direct interaction of G-protein subunits with nACHR subunits, in vitro GST pull-down experiments were performed. The intracellular loop region of the rat nACHR subunits of α3 (321–471), α4 (331–603), α5 (321–411), α7 (300–472), β2 (321–458), β3 (325–433), and β4 (320–463) were tested for their ability to bind either to the G\(_{\alpha}\) subunit or to the G\(\beta\gamma\) dimer. We found a positive interaction with G\(_{\alpha}\) for the nACHR subunits α3–α5, β2, and β3. All of the nACHR subunits tested, except for β3, also exhibited a positive interaction with the G\(\beta\gamma\) dimer. The β4 and α7 subunits failed to interact with any of the G-protein subunits tested, and the control GST alone failed to bind the G-protein subunits (Fig. 5A).

Given that nACHR GST-α4 was consistently shown to give the strongest signals in in vitro pull-down assays with G\(_{\alpha}\) and G\(\beta\gamma\), we tested the ability of this fusion protein to bind other proteins. Therefore, we incubated GST-α4 (331–603) and GST-α7 (300–472) with rat ICG lysate (~2 mg) and tested for their ability to bind to the G\(\beta\gamma\) dimer or the GABA\(_A\) receptor subunit α1. We found a positive interaction of α4, but not α7, with endogenous G\(\beta\gamma\) in the lysate, but both nACHR subunits failed to bind to the α1 subunit of the GABA\(_A\) receptor (Fig. 5B). Coimmunoprecipitation of ICG lysate with G-protein subunits and selective nACHR antibodies showed that both G\(_{\alpha}\) and G\(\beta\gamma\) bind α3/α5- and β2-containing native nACHRs in rat ICG neurons (Fig. 5C). Reverse experiments using the same antibodies were negative and thus excluded cross-reactivity between the antibodies. Furthermore, failure of coimmunoprecipitation using a rabbit preimmune control IgG also excluded unspecific binding of the Ig-heavy chain (data not shown).

**Figure 2.** G-protein activation increases the single-channel open probability of nACHRs. A. Direct activation of G-proteins on nACHR single-channel activity recorded from an excised membrane patch in the inside-out configuration (6 s trace). Single-channel currents were evoked in the presence, but not the absence, of ACh (5 μM) and bath application of GTPγS and GDPβS application. B. Bar graph of the effect of G-protein activation on \(N_P\), of nACHR channels \((n = 8)\). Holding potential, −60 mV. C, D, Unitary current amplitudes (C) and closed-time distributions (D) from eight inside-out patches exposed to control buffer and 0.2 mM GTPγS. Frequency of events was normalized (Norm. frequency) to the total number of events for each condition. Data points represent mean ± SEM. **p < 0.01. 10−2.
Discussion

G-protein subunits have been reported recently to directly modulate the glycine receptor, a member of the pentameric ligand-gated ion channel superfamily (Yevenes et al., 2003). The results presented in this study demonstrate another member of this family that is regulated by $G_{\alpha}$ and $G_{\beta\gamma}$ subunits. Previous experiments demonstrated that $nAChR$-mediated currents can be potentiated by coactivation of GPCRs by VIP and PACAP (Liu et al., 2000) and that this modulation is mediated by a pertussis toxin-sensitive G-protein pathway. We now show that this potentiation is attributable to direct binding of G-protein subunits to intracellular regions of specific $nAChR$ subunits. Using rat intracardiac ganglion neurons, activation of GPCRs and the release of G-protein subunits have been shown to be fast enough to increase ACh- and nicotine-evoked currents during coactivation of $nAChRs$ (Liu et al., 2000). Therefore, simultaneous activation of $G_{\alpha}$-coupled receptors during cholinergic transmission is likely to increase the open

![Figure 3](image3.png)

![Figure 4](image4.png)
composed mainly of nAChRs of rat intrinsic cardiac ganglia have been shown to be ACh binding to the receptor.

The cellular region of specific nAChR subunits to increase the affinity of the nAChR and G-protein interaction required neither auxiliary protein expression at the cell surface; the coimmunoprecipitation of native nAChRs with Go\(_3\alpha\) and Go\(_\beta\gamma\) subunits clearly demonstrates direct protein interaction.

In contrast to the present findings, alternative pathways for the modulation of nAChRs by activation of GPCRs in chick ciliary ganglia have been reported previously (Pardi and Margiotta, 1999). Activation of PACAP receptors in chick ciliary ganglion neurons has been shown to modulate nicotine-evoked currents using two different pathways: the first inhibits nAChRs via an unknown G-protein activation that involves phospholipase C, IP3, and mobilization of Ca\(^{2+}\) from internal stores, and a second pathway enhances currents by the activation of stimulatory G-protein and involves the generation of cAMP. A different signaling pathway involving Src family kinases (SFKs) has been described recently, whereby the inhibition of SFKs or the expression of kinase-defective c-Src reduced the peak amplitude of nicotine-induced currents in chromaffin cells or in HEK293 cells expressing the \(\alpha_3\beta_2\delta_5\) subunit combination (Wang et al., 2004). In rat parasympathetic neurons, intracellular dialysis failed to antagonize nAChR potentiation (Liu et al., 2000). The rapid onset observed for GPCR-induced potentiation of nAChR-mediated currents and the fact that specific nAChR subunits bind to Go\(_3\alpha\)-protein subunits demonstrates the direct activation of nAChRs by G-proteins without involvement of any diffusible factor. The amplitude of glycine-activated Cl\(^-\) currents in mouse spinal neurons was similarly enhanced after the activation of a GPCR and after application of purified Go\(_\beta\gamma\) (Yevenes et al., 2003). In contrast to glycine receptors, nAChRs are composed of a variety of \(\alpha\) and \(\beta\) subunits providing distinct molecular and pharmacological properties. Given the diversity of nAChR subtypes and their importance in both the CNS and peripheral nervous system, the subtype-specific modulation by G-protein subunits may have a significant effect on cholinergic transmission.

The present findings also indicate another important effector target for Go\(_3\) and Go\(_\beta\gamma\) subunits that bind and affect a variety of enzymes and ion channels such as the inward rectifying potassium channels, some voltage-gated N- and P/Q-type calcium channels (for review, see Dascal, 2001), and more recently, the ionotropic glycine receptor (Yevenes et al., 2003). The mechanism has been shown to be fast and reversible and provides a new pathway to describe synaptic processes in neural networks, giving rise to unique receptor cross talk and modulation. Because neuronal nAChRs are found throughout the nervous system, G-protein modulation of specific nAChR channels may serve as an endogenous regulator of ganglionic transmission in autonomic pathways, as well as central presynaptic nAChRs involved in regulation of drug addiction and neurological disorders such as Alzheimer’s disease and epilepsy.
References


