A G Protein \( \gamma \) Subunit-specific Peptide Inhibits Muscarinic Receptor Signaling*

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Muscarinic acetylcholine receptors modulate the function of a variety of effectors through heterotrimeric G proteins. A prenylated peptide specific to the G protein \( \gamma \)5 subunit type inhibits G protein activation by the M2 muscarinic receptor in a reconstitution assay. Scrambling the amino acid sequence of the peptide significantly reduces the efficacy of the peptide. The peptide does not disrupt the G protein heterotrimer. In cultured sympathetic neurons, the \( \gamma \)5 pentapeptide inhibits modulation of Ca\(^{2+}\) current by the M4 receptor. Peptide activity is specific, the scrambled peptide and peptides specific to two other members of the G protein \( \gamma \) subunit family are significantly less effective. The \( \gamma \)5 pentapeptide has no effect on Ca\(^{2+}\) current modulation by the \( \alpha \)-adrenergic and somatostatin receptors. In addition, the \( \gamma \)5 pentapeptide inhibits muscarinic receptor signaling in spinal cord slices with specificity. These results support a specific role for G protein \( \gamma \) subunit types in signal transduction, most likely at the receptor-G protein interface.

The G protein \( \gamma \) subunits are a family of 11 proteins with varying levels of homology to each other and different patterns of expression in mammalian tissues (1). Although the G protein \( \beta \)y complex has been shown to directly modulate effector function and is required for receptor interaction of the G protein, the individual functions of these \( \gamma \) subunits are still unclear. Reconstitution assays with rhodopsin and Gt indicated that G protein coupling with a receptor involves specific contact of the \( \gamma \)1 subunit COOH terminal with the receptor (2, 3). To test whether the COOH-terminal domains of other \( \gamma \) subunits are involved in receptor interaction we have examined the effect of a peptide from the \( \gamma \)5 subunit type on muscarinic receptor signaling. \( \gamma \)5 is expressed abundantly in the heart similar to the muscarinic receptor, M2 (4, 5). We examined the effect of the \( \gamma \)5 COOH-terminal peptide on the activation of \( \alpha \)12 reconstituted with the M2 receptor. To examine the effect of the peptide in cells, we injected a peptide specific to the \( \gamma \)5 COOH terminus into superior cervical ganglion (SCG)3 neurons and measured receptor modulation of N-type Ca\(^{2+}\) current (\( I_{\text{Ca}} \)). SCG neurons contain the M1 and M4 muscarinic receptors which inhibit N-type Ca\(^{2+}\) channels through \( \alpha \)s and \( \alpha \)o, respectively (6, 7). SCG neurons also contain \( \alpha \)-adrenergic and somatostatin receptors that inhibit \( I_{\text{Ca}} \) through \( \alpha \)s (6). This variety of receptors modulating the activity of a common effector allowed us to assess the specificity of the \( \gamma \)5 pentapeptide. The effect of \( \gamma \)5 pentapeptide as well as peptides from \( \gamma \)7 and \( \gamma \)12 on these pathways was examined. Finally, to test the effect of the \( \gamma \)5 peptide on the central nervous system, we introduced the \( \gamma \)5 pentapeptide into postsynaptic neurons in a spinal cord slice and measured the modulation of glutamate receptor mediated synaptic current by muscarinic and \( \alpha \)-adrenergic receptors (8).

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MATERIALS AND METHODS

Cells and Reagents—[\(^{3}H\)N-methylscopolamine and \([\(^{35}S\)]GTP\(_{S}\) were from NEN Life Science Products. Somatostatin was from Peninsula. Geranylgeranyl bromide was from American Radiolabeled Chemicals. Oxtremoreline methiodide (o xo-M), carbachol, and clonidine were from Research Biochemicals. BAPTA and dextran-fluorescein were from Molecular Probes. Leupetin, ATP, and GTP were from Roche Molecular Biochemicals. Dulbecco's modified Eagle's medium and heat-inactivated horse serum were from Life Technologies, Inc. All other chemicals were from Sigma. Purification of recombinant \( \alpha \)s, \( \beta \)His-\( \gamma \), and \( \beta \) complex from bovine brain were as described before (9, 10). A CHO cell line stably transfected with a vector expressing the M2 receptor has also been described before (11) and was provided by the late Dr. E. G. Peralta (Harvard University). Solid peptide synthesis, mass spectrometry, and amino acid analysis were performed at the Protein and Nucleic Acid Chemistry Laboratory, Washington University School of Medicine. Geranylgeranylation was performed and checked as described (12). Peptide sequences were as follows: \( \gamma \)5 pentapeptide, VSSST-NPFRRPKVC or a shorter version, STNFPRPKVC; \( \gamma \)5 scrambled peptide, PSRTPVNSQVSCK; \( \gamma \)7, SENPFKD KKPC; and \( \gamma \)12, SENPFKDKKTC. The shorter \( \gamma \)5 wild type peptide was used in all electro-physiological assays.

Patch clamp experiments were done on 1-day cultured SCG neurons from 2- to 4-week-old male Harlan Sprague-Dawley rats. Neurons were dissociated, and plated as described (13). Preparation of M2 Receptor-containing Membranes—CHO cell membranes containing M2 were obtained as described (14). To deplete endogenous G protein subunits membranes were washed with 20 mM sodium phosphate buffer, pH 7.4, containing 5 mM MgCl\(_{2}\), 5 mM urea, 100 \( \mu \)M GTP\(_{S}\). Immunoblot analysis with antibodies specific to \( \beta \)1 showed a significant decrease in that subunit after this treatment.

Current Recording—Whole-cell recording of \( I_{\text{Ca}} \) used 50–60% compensation of series resistance. \( I_{\text{Ca}} \) current records were sampled (25 kHz). Voltage-dependent inhibition of \( I_{\text{Ca}} \) was studied with two 10-ms test pulses to +10 mV, from a holding voltage at ~80 mV, one before (P1) and other (P2) after a 25-ms prepulse to +125 mV. Facilitation ratio and amplitude of \( I_{\text{Ca}} \) were measured as described (19). Agonist-mediated inhibition of \( I_{\text{Ca}} \) was quantified only for the P1 test

* The abbreviations used are: SCG, superior cervical ganglion; CHO, Chinese hamster ovary; EPSC, excitatory postsynaptic current; GTP\(_{S}\), 5'-O(3-thiotriphosphate); oxo-M, oxtremoreline methiodide; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; \( \alpha \), ohm(s); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; F, farad(s).
pulse. To avoid one source of systematic bias, control and experimental measurements were alternated in each set of experiments.

**Cytoplasmic Injection—**SCG neurons were pressure-injected with Gγ peptides by using an Eppendorf transjector system. Injection pipettes were filled with a solution containing 50 μM geranylgeranylated γ subunit-peptides and 0.05% dextran-fluorescein (Mw = 10,000) as injection marker. After injection, cells were returned to the incubator and 1–2 h later were transferred to a 50-μl chamber for I_Ca recording. Experiments were done at 25 °C. To block Na+ and I-type Ca2+ currents, 0.5 μM tetrodotoxin and 2 μM nifedipine were added to Ringer’s solution.

External and internal solution compositions were as described (13).

**Experiments in Spinal Cord Slices—**Spinal cord slices were prepared and whole-cell recordings performed as described previously (8). Peptide was included in the solution in the recording pipette (see Ref. 8). Carbachol effect was measured 30 min after first EPSC was recorded. Agonists were applied in bath solution (artificial cerebrospinal fluid) for 10 min and then washed out with bath solution (~10 min). 10 μM bicuculline methiodide and 1 μM strychnine hydrochloride were present in the bath solution throughout the experiment. Statistical comparisons were made with the use of one-way analyses of variance (Dunnett test for post-hoc comparison) or Student’s t test. p < 0.05 was considered significant.

**RESULTS AND DISCUSSION**

γ5 Subunit Type-specific Peptide Inhibits G Protein Activation by M2—Membranes from CHO cells expressing high levels of M2 receptor were depleted of endogenous G protein subunits as described in the methods section and assayed for activity by binding of antagonist. The receptors bound [3H]N-methylscopolamine with a dissociation constant of 0.35 nM. When M2 containing membranes were reconstituted with heterotrimeric G_{i2} (under conditions similar to those in Fig. 1B), GTPγS binding by the G protein was stimulated 5-fold by the agonist, carbachol, compared with the antagonist, atropine (data not shown). Little or no GTPγS was incorporated (i) in the absence of the G protein heterotrimer, (ii) in the presence of the α subunit alone, or (iii) in the presence of the βγ complex alone. These results indicated that the membranes were free of functional G proteins and that the M2 receptors in this preparation were functional with properties similar to those previously reported (17). To examine interaction between the M2 receptor and a γ subunit, γ5, we synthesized a 14-amino acid peptide specific to the COOH-terminal sequence of γ5 and chemically modified it with geranylgeranyl, a C-20 isoprenoid that is posttranslationally added to the COOH-terminal cysteine of most γ subunits (1) (Fig. 1A). The γ5 peptide was then tested for its ability to inhibit G protein activation. If the γ subunit tail of G_{i} interacts with M2, the peptide should compete with the heterotrimer for a site on the receptor. Results in Fig. 1B show that the wild type peptide significantly reduced the rate of agonist-stimulated GTPγS binding by the G protein. A peptide with the same amino acids scrambled was significantly less effective, indicating that this effect was sequence specific (Fig. 1B).

**γ5 Peptide Does Not Disrupt the G Protein Heterotrimer—**Heterotrimerization of a G protein is essential for receptor interaction (16). To rule out the possibility that the inhibition of G protein activation was due to the disruption of the heterotrimer by the γ5 peptide, an experiment was performed under conditions similar to the receptor assays (described in Fig. 1C). The hexahistidine-tagged βγ complex was brought down with resin containing Ni^{2+}. Although aluminum fluoride disrupted the heterotrimer (Fig. 1C, compare lane 2 with lane 3), both in the absence and in the presence of the wild type γ5 peptide, similar amounts of βγ were co-eluted with βHisγγ (Fig. 1C, compare lane 5 with lane 1). This indicated that the heterotrimer was not disrupted by the γ5 peptide.

Geranylgeranylated γ5 Peptide Selectively Disrupts Muscarinic Modulation of N-type Ca2+ Currents—To test the effect of peptides specific to the COOH-terminal region of the γ5 subunit on signaling in cells, cultured SCG neurons were injected with a wild type γ5 peptide or the γ5 peptide with the amino acid sequence scrambled (γ5 s) (described under "Materials and Methods"). The effect of maximal concentration of the muscarinic agonist oxo-M was measured on I_Ca amplitude and on the facilitation ratio. As indicated in Fig. 2A, voltage-dependent facilitation were revealed by inserting a depolarizing prepulse. In the cells injected with the γ5-scrambled peptide, I_Ca amplitude was little affected by the prepulse in the absence of agonist (C1 compared with C2 and open circles compared with filled circles on plot). Oxo-M produced a large inhibition of I_Ca that could be partially relieved by the prepulse, thereby increasing the facilitation ratio from 1.11 to 2.22. In un.injected cells and in cells

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**Fig. 1. G protein activation by M2 is inhibited by γ subunit-specific peptides. A, chromatographic traces of γ5 peptide with and without the prenyl moiety. γ5 peptides were purified by fast protein liquid chromatography using a PrepRPC HR16/10 column. Peptides were eluted with a gradient of water/acetonitrile. The unprenylated peptide (γ5) was eluted at a concentration of ~25% acetonitrile and the prenylated peptide (γ5-gg) at ~50% acetonitrile.**

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**Fig. 2A. GTPγS binds to βγ subunits.**

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**Fig. 2B. GTPγS binds to βγ subunits.**

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**Fig. 2C. GTPγS binds to βγ subunits.**
injected with the γ5-scrambled peptide, oxo-M increased the facilitation ratio and inhibited \( I_{Ca} \) similarly (Fig. 2, C and D). Thus, cytoplasmic injection by itself did not disrupt muscarinic signaling. In contrast, muscarinic modulation of \( I_{Ca} \) was substantially different in the γ5 peptide-injected cell; first, inhibition was smaller (compare C1 and O1 records in Fig. 2B); second, the +125 mV prepulse was less effective in relieving \( I_{Ca} \) suppression (compare O1 and O2 records and open and filled triangles in Fig. 2B). Indeed, in 13 neurons injected with the γ5 peptide, oxo-M inhibited \( I_{Ca} \) by only 31.8% (Fig. 2D) and increased facilitation ratio from 1.08 to only 1.33 (Fig. 2C). Thus the γ5 peptide blocked the voltage-dependent inhibition of \( I_{Ca} \) mediated by M4 receptors.

γ5 Peptide Does Not Disrupt α2-Adrenergic, Somatostatin, or M1 Muscarinic Signaling in Sympathetic Neurons—We wanted to assess in the same neurons whether the γ5 peptide disrupted modulation of \( I_{Ca} \) by other G protein-coupled receptors, namely α2-adrenergic or somatostatin receptors. Therefore, after \( I_{Ca} \) recovered upon oxo-M and Cd2+ treatment, neurons were challenged with norepinephrine or somatostatin. Table I summarizes the results. Here, because there were no statistically significant differences between un.injected cells and cells injected with γ5 scrambled peptide, we pooled together both samples (control) to facilitate comparison with the γ5 peptide-injected cells. Neither voltage-dependent inhibition of \( I_{Ca} \) by norepinephrine nor by somatostatin were affected by the γ5 peptide. In SCG neurons \( I_{Ca} \) is also suppressed by M1 muscarinic peptide.
G Protein Peptide Inhibits Muscarinic Receptor Signaling

Among members of the muscarinic receptor family it is known that M2 and M4 share similar properties in terms of G protein coupling (21, 22). The ability of the γ5 peptide to inhibit M2 activation of a G protein in a reconstituted system and also inhibit signaling from M4 receptors in intact cells implies that the γ5 peptide interacts with this class of muscarinic receptors.

The inability of the γ7 and γ12 peptides to affect signaling from M4 in combination with the inability of γ5 to affect signaling from receptors other than M4 indicate a high degree of specificity in the action of the peptide. Past findings where antisense oligonucleotides specific to two different γ subunits inhibited the action of the muscarinic and somatostatin receptor signaling indicated that G protein γ subunit types may have a specific role in signaling (23). Results from the analysis of rhodopsin coupling to Gt with different γ subunit types indicated specificity between γ subunit types and a receptor at the protein level (24). The results here indicate that there may be selectivity in the interaction between γ subunit types and receptors. Furthermore, the indication of such specificity in intact cells raises the possibility that peptides from the γ subunits and their more potent analogues can be used to selectively disrupt individual pathways in a signaling network.

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