Rat group I Metabotropic Glutamate Receptors Inhibit Neuronal Ca\(^{2+}\) Channels via Multiple Signal Transduction Pathways in HEK 293 Cells

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Abstract

Glutamate receptors are among the most abundant and diverse receptors expressed in the central nervous system (CNS). However, the mechanisms by which they couple to diverse signal transduction pathways and modulate neuronal function are incompletely understood. We report here that group I metabotropic glutamate receptors (mGluRs) inhibit neuronal Ca\(^{2+}\) channels (N-type and P/Q-type) in HEK 293 cells in a voltage-dependent fashion. Using whole-cell current clamp, we observed a decrease in the peak amplitude of evoked Ca\(^{2+}\) currents and a decrease in the Ca\(^{2+}\) current transient. Thus, mGluRs inhibit the rise in intracellular Ca\(^{2+}\) and are not modulated by N-ethylmaleimide-sensitive G proteins. Group I mGluRs couple to G-protein signaling pathways that activate phospholipase C, which leads to the stimulation of inositol phosphate metabolism. We also show that group I mGluRs inhibit the Ca\(^{2+}\) influx in dendritic processes. These findings provide insights into the complex mechanisms of Ca\(^{2+}\) channel regulation by mGluRs and have implications for synaptic plasticity and Ca\(^{2+}\) homeostasis in the CNS.

Introduction

The regulation of intracellular calcium is a cornerstone for many cellular responses to extracellular signals in the CNS. The best examples of this are in presynaptic terminals, where the influx of calcium through voltage-gated calcium channels is absolutely required for active release of neurotransmitter or in dendritic processes where the rising Ca\(^{2+}\) underlies the signal transduction events leading to long-term alterations of synaptic efficacy. These essential second messenger roles for Ca\(^{2+}\) may be contrasted with the apparent neurotoxic effects of failed Ca\(^{2+}\) homeostasis during ischemic insult in many brain regions. A knowledge of Ca\(^{2+}\) influx/release in the CNS therefore has served not only to further our understanding of the physiology underlying proper neuronal function but to better comprehend the role of Ca\(^{2+}\) in the pathophysiology of the CNS.

The voltage-gated calcium channel provides a significant path through which Ca\(^{2+}\) enters many diverse cell types. In the CNS, such channels generally are believed to be composed of at least three distinct subunits (\(\alpha_1\), \(\alpha_2\), \(\beta\), and \(\delta\)). Although the function of the \(\alpha_2\delta\) subunit is not well understood (but see Brust et al. 1993), the \(\beta\) subunit is an essential ancillary subunit required for high expression levels of neuronal calcium channels and alters the biophysical properties of the calcium current (Oclese et al. 1994). The \(\alpha_1\) subunit forms the ion conducting pore and provides the binding sites for pharmacological agents that have been used to identify, along with the channel’s biophysical properties, each channel subtype. For the studies presented below, the \(\alpha_1B\) (or \(\omega\)-conotoxin GIVIA-sensitive) and the \(\alpha_1A\) (or the \(\omega\)-agatoxin IVA-sensitive) containing channels were chosen for examination because they mediate the vast majority of excitation/secretion coupling in the CNS (Lovinger et al. 1994; Takahashi and Momiyama 1993). These channel subtypes also are modulated by a large diversity of G-protein-coupled receptors in an ever-expanding list of central and peripheral nervous tissues (Beech et al. 1992; Choi and Lovinger 1996; Swartz and Bean 1992).

Glutamate exerts its effects in nervous tissue by acting as an agonist for both ion-conducting ligand-gated receptors as well as a family of heterotrimeric G-protein–coupled receptors. The latter receptors, metabotropic glutamate receptors (mGluRs), contain seven transmembrane spanning segments and mediate diverse inhibitory and excitatory responses. Like other G-protein–coupled receptors, the mGluRs can be classified based on the signal transduction pathways to which they couple in native and heterologous systems. Group I mGluRs couple to the stimulation of inositol phosphate me-
tabolism/mobilization of intracellular calcium in heterologous systems by activation of primarily pertussis toxin (PTX)-resistant G proteins (Aramori and Nakanishi 1992), whereas group II and III mGluRs inhibit adenyl cyclase via activation of PTX-sensitive G proteins (Tanabe et al. 1992).

In the CNS, physiological responses mediated by group I mGluR activation include the stimulation of phosphoinosit metabolism (Schoepp and Johnson 1989), modulation of resting membrane currents (Guérineau et al. 1994; Zheng and Gallagher 1995), inhibition of voltage-gated calcium channels via activation of G$_{i/o}$-like G proteins (Choi and Lovinger 1996; Hay and Kunze 1994), and modulation of synaptic transmission via presynaptic autoreceptors (Gereau and Conn 1995). These physiological responses underlie the apparent role of group I mGluRs in the regulation of neuronal excitability (Desai and Conn 1991; McBain et al. 1994) and in both long- and short-term changes of synaptic efficacy in various brain regions (Bashir et al. 1993; Glaum et al. 1992).

The ability of a single class of receptors to couple to these widely divergent signal transduction pathways is a poorly understood aspect of group I mGluR function in the CNS.

Group I receptors can modulate voltage-gated calcium channels and these effectors potentially underlie the effects of group I agonists on synaptic transmission, we have examined the ability of specific group I mGluRs to modulate voltage-gated N-type and P/Q-type calcium channels. Because pharmacological tools are not yet available to allow us to distinguish readily between the various group I mGluRs (i.e., mGluR1a–d and mGluR5a–b) in native tissue, we have used a heterologous expression approach consisting of HEK 293 cell lines stably expressing human neuronal calcium channels into which we have transiently expressed various rat group I mGluR cDNAs. This system therefore allows us to precisely control at least two aspects of the signal transduction pathway, specifically the receptor and calcium channel subtypes. Although the exact compliment of G proteins expressed in HEK 293 cells has not been explicitly defined, mRNAs representing all four major families (i.e., $\alpha_{i/o}$, $\alpha_5$, $\alpha_6$, and $\alpha_1$) have been detected (Toth et al. 1996). Our results show that, unlike group II receptors expressed in this HEK 293 cell heterologous system (McCool et al. 1996), group I receptors can use several distinct signal transduction pathways to inhibit voltage-gated calcium channel function. These studies may help to further define the multifunctional nature group I mGluRs in the CNS.

**Methods**

**Cell culture and transfection**

Isolation and characterization of the HEK 293 cell line stably expressing the human $\alpha_{1b}$, $\alpha_5$, $\beta_2$, $\beta_1$ calcium channel subunits has been described previously (McCool et al. 1996). Stable cell lines expressing the human $\alpha_{1b}$-subunit in place of the $\alpha_{1b}$-subunit were isolated using similar techniques. Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco/BRL, Grand Island, NY) supplemented with bovine calf serum (5.5%, Hyclone Labs, Salt Lake City, UT), 100 U/ml penicillin G/100 mg/ml streptomycin (Gibco/BRL), and 0.5 mg/ml G418 (Sigma Chemical, St. Louis, MO, or CellGro) using a 37°C/5% CO$_2$ incubator.

Transient transfection procedures for mGluR constructs were identical to those reported previously (McCool et al. 1996) and involved a CaPO$_4$-mediated protocol. Expression constructs containing group I receptor cDNAs were under the direction of cyto-megaviruses (CMV)-based promoters and have been described elsewhere (see Joly et al. 1995). Transiently transfected cells were used 36–48 h after the introduction of the expression plasmid. The N-type channel-expressing cell line was used without any special culturing techniques. However, after transfection, the P/Q-type channel-expressing cell line was incubated at 37°C for 10–12 h to allow for receptor expression then shifted to 28°C >6 h before electrophysiology to maximize channel expression. Although we do not fully understand the apparent need for this exposure to lower temperatures to maximize channel expression, the most likely explanation would involve the interaction between nonnative protein folding pathways available to the channel at higher temperatures and overexpression of the channel protein, a potential hazard in any heterologous system.

**Isolation of superior cervical ganglion neurons**

Superior cervical ganglion (SCG) neurons were acutely isolated from adult female Sprague-Dawley (250–275 g) rats using slight modifications of a previously reported procedure (Ikeda et al. 1995). Briefly, ganglia were isolated and incubated in DMEM (Sigma Chemical) containing 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.4 with NaOH), 1 mg/ml collagenase type D (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.3 mg/ml trypsin (Sigma Chemical), and 0.1 mg/ml DNase I (Sigma Chemical) at 37°C for 1 h. Neurons then were dispersed by vigorous shaking and washed twice with plating media, which contained DMEM, 10% fetal bovine serum (FBS; Hyclone Labs), and 1% penicillin-streptomycin solution (GIBCO/BRL). Dispersed neurons then were plated onto poly-L-lysine-coated dishes and used for recordings within 24 h.

**Solutions and drugs**

The internal pipette solution for whole cell recordings consisted of (in mM) 125 N-methylglucamine (NMG), 20 tetraethylammonium (TEA)–OH; 14 Tris–phosphocreatine, 10 HEPES, 11 ethyl ene glycol-bis(β-aminoethyl ether)–N,N,N’-N’-tetraacetic acid, 1 CaCl$_2$, 4 Mg-ATP, and 0.3 Tris-GTP; pH 7.2 with methane sulfonic acid and HCl (final [Cl] was 10 mM); adjust to 300–310 mmol/kg with sucrose. For perfused-patch recordings, the internal pipette solution contained (mM) 140 CsCl, 10 HEPES, and 2 MgCl$_2$; pH 7.2 with CsOH. Amphotericin B (Sigma Chemical) was added from a concentrated stock in dimethyl sulfoxide to the CsCl internal at a final concentration of 50 μg/ml; this solution was sonicated vigorously immediately before use to ensure a relatively homogeneous suspension.

Cells were exposed continuously to a bath-applied solution consisting of (in mM) 150 NaCl, 10 dextrose, 10 HEPES, 2.5 KCl, 2.5 CaCl$_2$, and 1.0 MgCl$_2$; pH 7.4 with NaOH; osmolarity adjusted to 320–340 mmol/kg with sucrose. To isolate currents mediated by the calcium channels, cells were perfused locally with a TEA-Ba$^{2+}$ solution from an array of high-performance liquid chromatography (HPLC) tubing (150 μm ID; Hewlett Packard Analytic Di rect, Wilmington, DE) positioned within 50–100 μm of the cell. This TEA-Ba$^{2+}$ isolation solution entirely encompassed the cell and was composed of the following (in mM): 140 TEA hydroxide, 10 HEPES, 15 dextrose, and 5 BaCl$_2$; pH 7.35 with methane sulfonic acid; osmolarity adjusted to 320–330 mmol/kg with sucrose. 1-L-Glutamate hydrochloride (Research Biochemicals Interna tional, Natick, MA) and N-ethylmaleimide (NEM; Sigma Chemical) were made fresh each day as concentrated stocks in DI,HO; (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (t-ACPД;
Research Biochemicals International), (R,S)-3,5-dihydroxyphenylglycine (DHPG, Tocris Cookson, St. Louis, MO), quisqualate (Tocris Cookson), and 25,1'S,2'S-(carboxycyclopentyl)glycine (L-CCGI; Tocris Cookson) were diluted fresh daily from frozen stocks. PTX and choleragenoid (Research Biochemicals International) were added to final concentrations of 500 ng/ml and 1 µg/ml, respectively, from concentrated stocks made according to the manufacturer’s instructions.

**Electrophysiology**

All recordings were performed at ambient room temperature with standard patch-clamp techniques (Hamill et al. 1981) using the axopatch-1D amplifier (Axon Instruments, Foster City, CA) in the voltage-clamp mode. Gigaohm seals were formed using polished patch pipettes made from borosilicate glass (World Precisions Instruments, Sarasota, FL). For the whole cell patch-clamp recording configuration, patch pipettes typically had input resistances of 0.5–2 MΩ using the NMG/TEA internal solution; whole cell capacitance (typically 10–40 pF) and series resistance (typically <10 MΩ) were compensated manually after opening the cell. For perforated patch recordings, patch pipettes had input resistances of 0.25–1 MΩ using the CsCl/amphotericin B internal; and whole cell capacitance and series resistance were generally 10–25 pF and <20 MΩ, respectively. Whole cell currents were typically on-line leak-subtracted using a p/n protocol; and all currents were low-pass filtered (3-pole Bessel filter) at 1–5 kHz with >70% compensation. Depolarizing test pulses to potentials where inward barium current was maximal were given at 0.25 Hz to prevent prolonged channel inactivation. Data were digitized at 10 kHz with a Labmaster DMA (Axon Instruments), stored on a 486 microprocessor, and analyzed off-line using pClamp software (Axon Instruments). Current amplitudes for whole cell and perforated-patch traces were measured from cursors placed immediately before the initiation of a depolarizing test pulse and at the peak of the calcium current, typically 15 ms after initiation of the test pulse. To calculate percent inhibition, the average of amplitudes before and after agonist application were compared with the average current amplitude during agonist application (typically 30 s or 2 episodes). Numerical analysis was performed using the QuatroPro software package (v 5.00; Borland International, Scotts Valley, CA). Current traces, other graphs, and Hill plots were generated using GraphPad Prism (v 1.03; GraphPad Software, San Diego, CA). Statistical analysis was performed using Instat (v 2.05; GraphPad Software).

**RESULTS**

**Voltage-dependent modulation of calcium channels by group I receptors**

α1B-expressing (N-type) cells transiently transfected with mGluR1a and mGluR5a were chosen for study based on cell morphology, with receptor-expressing cells generally being fairly large (30–40 pF) and having a “granular” appearance. Although the specific reasons for this unique appearance are unknown, these characteristics seem independent of the subtype of mGluR cDNA transiently expressed (group I vs. group II) and may be related generally to overexpression as similar results are seen with other kinds of proteins (McCool, unpublished observations). Twenty-six of 111 mGluR1a-transfected cells (50.4 ± 2.6% inhibition; mean ± SE) and 29 of 67 mGluR5a-transfected cells (41.8 ± 2.4% inhibition) responded to application of a saturating concentration of L-glutamate (100 µM) with robust and reversible inhibition of whole cell N-type barium currents. Similar results were obtained for the splice variants of both mGluR1 and mGluR5. Specifically, mGluR1b inhibited whole cell N-type barium currents (data not shown) by 41.4 ± 2.8% in four of eight cells tested, whereas mGluR1c modulated calcium channels (data not shown) in two of eight cells tested with an average of 30.5% inhibition. Likewise, mGluR5b-mediated inhibition (38.6 ± 8.5%; data not shown) was found in 5 of 10 cells tested. With regard to P/Q-type channels, modulation was generally smaller in the α1A-expressing (P/Q-type) stable cell line. Nonetheless, 6 of 28 mGluR1a transfected cells (25.2 ± 5.2% inhibition), and 6 of 14 mGluR5a transfected cells (25.8 ± 3.3% inhibition) α1A-expressing cells responded to 100 µM glutamate with robust and reversible inhibition. Mock transfected (vector alone) or untransfected α1b-expressing cells (McCool et al. 1996) and α1A-expressing cells (data not shown, n = 5) did not respond to application of any mGluR agonist, indicating that these cells do not express endogenous glutamate receptors capable of inhibiting barium currents. Glutamate application to mGluR1a- or mGluR5a-expressing cells did not cause any changes in resting membrane currents when using either whole cell or perforated-patch (see further text) recording configurations. The relatively low percentage of responding cells (20–50% of cells tested) was highly dependent on the specific expression construct but was within the dish-to-dish variability seen during transient expression studies with CMV-driven green fluorescent protein constructs (data not shown).

To assess whether group I receptor modulation of N-type and P/Q-type channels was similar to that observed in neurons, we assessed the voltage dependence of the inhibition using a voltage protocol similar to that in Ikeda (1991). In this protocol, two test pulses to moderately depolarized potentials at which inward barium currents were maximal (approximately −10 mV from a holding potential of −90 mV) are separated by a large membrane depolarization (+80 mV). Voltage dependence is represented by a “relax” from inhibition in the second test pulse relative to the first test pulse. For both mGluR1a and mGluR5a coupled to either N-type or P/Q-type channels, the large intervening depolarization partially relieved inhibition (Fig. 1, A−D), indicating that modulation was indeed voltage dependent. In all cases, modulation in the first test depolarization was characterized by an apparent slowing of macroscopic activation kinetics (see Fig. 1, A−D, left); however, “slowing” appeared more pronounced for modulation of human P/Q-type channels than for human N-type channels. Together, these properties indicate that group I modulation of N-type and P/Q-type calcium channels in HEK 293 cells is mediated by a signal transduction pathway similar to the membrane-delimited, voltage-dependent pathway previously described for group II mGluR modulation of N-type calcium channels in HEK 293 cells (McCool et al. 1996) and for mGluR2 modulation of N-type channels when transiently expressed in sympathetic neurons (Ikeda et al. 1995).

**Receptor pharmacology**

The pharmacological characterization of mGluR1a and mGluR5a coupled to N-type calcium channels in this HEK 293 cell system is depicted in Fig. 1, E and F. For both receptors, inhibition by each agonist was characterized by a
Concentrations (µM) of traces are as follows: For mGluR1a-expressing cells, EC50 values were 0.5 (µM) and partially activated (~70% of maximal) by high concentrations of L-CCGI (10 µM), consistent with group I receptor pharmacology (Conn and Pin 1997).

Modulation by group I mGluRs is mediated primarily by G1/G2-like G proteins

G protein α subunits generally can be classified based on their sensitivity to inactivation and are dependent on naturally occurring bacterial toxins, such as cholera toxin and PTX. Recently, it has been shown that low concentrations of the sulfhydryl alkylating agent, NEM, can preferentially inactivate voltage-dependent calcium channel modulation by G1/G2 α subunit-containing, PTX-sensitive G proteins (Mizuno et al. 1990). As there are no alterations of the biochemical properties of G1/G2 heterotrimers after treatment with NEM (Asano and Ogasawara 1986), G-protein/receptor uncoupling is presumed to be the major effect of NEM, similar to the actions of PTX.

To investigate the nature of the G protein used by group I receptors in this HEK 293 system, individual cells transfected with mGluR1a or mGluR5a were identified based on their response to agonist (100 µM L-glutamate) and subsequently treated with NEM (50 µM, 2 min) during the recording. Exposure to L-glutamate after treatment with NEM indicated that N-type (Fig. 2A) channel modulation was reduced significantly from 59.0 ± 4.5% to 7.1 ± 3.1% (n = 6, P < 0.001, Student’s t-test) and from 47.6 ± 3.5% to 9.5 ± 3.8% (n = 4; P < 0.003) for cells expressing mGluR1a and mGluR5a, respectively. Likewise, inhibition of P/Q-type channels (Fig. 2B) by mGluR1a and mGluR5a was reduced from 25.3 ± 4.7% to 5.1 ± 1.6% (n = 5, P < 0.02) and from 23.3 ± 3.4% to 7.6 ± 2.6% (n = 4, P < 0.02), respectively, after treatment with NEM. Although occasional potentiation and inhibition of whole cell currents was observed during the course of NEM treatment (not shown), these effects were never consistent, similar to the results reported by Shapiro et al. (1994a), and may indicate minimal effects of NEM on the channels themselves.

In isolated SCG neurons, modulation of N-type calcium channels by α2-adrenergic receptors via a voltage-dependent, primarily PTX-sensitive pathway (Beech et al. 1992) was also acutely sensitive to exposure to NEM (Fig. 2C), consistent with previous reports (Shapiro et al. 1994a). Conversely, voltage-dependent modulation of N-type channels in SCG neurons by vasoactive intestinal peptide (VIP) receptors was resistant to NEM (Fig. 2D). These results are consistent with previous
FIG. 2. Group I mGluR modulation of neuronal voltage gated calcium channels in HEK 293 cells is mediated by G\textsubscript{i}/G\textsubscript{o}-like heterotrimeric G proteins. For this and subsequent figures, the numbers in parentheses indicate the number of cells. 

A and B: after identification of a cell as expressing either mGluR1a or mGluR5a (based on an inhibitory response to agonist), the cell was exposed to minimal concentrations of the sulphydryl alkylating agent, N-ethylmaleimide (NEM; 50 μM for 2 min), during the recording and subsequently retested with agonist (100 μM L-glutamate). For both receptors coupled to either N-type (A) or P/Q-type (B) channels, modulation was almost entirely NEM sensitive, indicating that group I mGluRs use G\textsubscript{i}/G\textsubscript{o} to modulate neuronal calcium channels in HEK 293 cells. For N-type channels (A), mGluR1a inhibition was reduced from 59.0 ± 4.0% to 7.1 ± 3.0% after NEM treatment, and mGluR5a was reduced from 47.6 ± 3.5% to 9.5 ± 3.8%. Likewise, for P/Q-type channels (B), mGluR1a inhibition fell from 25.3 ± 4.7% to 5.1 ± 1.6% after exposure to NEM, whereas mGluR5a inhibition fell from 25.8 ± 3.3% to 8.6 ± 2.0%. 

C and D: NEM selectively interferes with modulation of N-type calcium channels by G\textsubscript{i}/G\textsubscript{o}-coupled (α\textsubscript{2}-adrenergic) (Shapiro et al. 1994a) but not G\textsubscript{s}-coupled [vasoactive intestinal peptide (VIP)] (Zhu and Ikeda 1994), receptors in acutely isolated superior cervical ganglia neurons. Modulation of whole cell barium currents by the α\textsubscript{2}-adrenergic receptor agonist UK14304 (C) was reduced from 33.6 ± 6.3% to 9.6 ± 2.5% after exposure to NEM. Conversely, inhibition mediated by VIP (D) is insensitive to NEM, with inhibition being 44.2 ± 3.0% before exposure and 40.7 ± 3.9% after exposure.

reports that VIP inhibition of N-type channels in SCG neurons is mediated by a cholera toxin (CTX)-sensitive G protein (Zhu and Ikeda 1994) (see also Fig. 3). Additionally, it has been shown previously that muscarinic receptor modulation of M-type potassium channels in SCG neurons is also resistant to NEM (Choi et al. 1995), consistent with this modulation being mediated by the G\textsubscript{q/11}-class of G proteins (Caulfield et al. 1994). Together, these results indicate that inhibition mediated by mGluR1a and mGluR5a in both the HEK 293 cell lines was mediated almost entirely by G\textsubscript{i}/G\textsubscript{o}-like G proteins and, as exemplified by the insensitivity of VIP N-type calcium channel modulation to NEM, N-ethylmaleimide most likely does not disrupt steps in the voltage-dependent pathway downstream of the G protein.

PTX treatment reveals an additional signal transduction pathway for N-type channel modulation

To confirm the signal transduction pathway used by group I mGluRs to inhibit N-type calcium channels involves G\textsubscript{i}/G\textsubscript{o}-like G proteins, we treated transiently transfected cells overnight with PTX (0.5 μg/ml; 12–18 h), a treatment paradigm that completely eliminates modulation of calcium channels by group II mGluRs in HEK 293 cells (McCool et al. 1996) and in neuronal expression systems (Ikeda et al. 1995). PTX treatment of mGluR1a- (Fig. 3A) and mGluR5a (Fig. 3B)-transfected cells failed to remove all of the group I modulation of N-type channels, in apparent contradiction to the results obtained using NEM. Specifically, using data taken from three to five independent experiments, inhibition was reduced from 54.1 ± 3.8% (n = 11) and 44.2 ± 2.7% (n = 10) in control mGluR1a- and mGluR5a-expressing cells, respectively, to 22.4 ± 2.1% (n = 8) and 23.8 ± 1.9% (n = 7) after overnight PTX treatment (Fig. 3, A and B). This decrease in inhibition was not due to a shift in maximal efficacy of L-glutamate as the concentration used here (100 μM) was still saturating (not shown). mGluR2-transfected cells tested at the same time using the same concentration of PTX and time of exposure showed complete loss of glutamate inhibition after PTX treatment (not shown, see McCool et al. 1996). Because expression of the P/Q-type channel required incubation of cells at nonphysiological temperatures (see METHODS) and such temperatures may seriously effect the actions of PTX and other cellular processes, we have
FIG. 3. Treatment of group I-expressing cells with pertussis toxin (PTX) reveals an additional signal transduction pathway for inhibition of N-type channels. A and B: modulation by mGluR1a (A) or mGluR5a (B) is only partially sensitive to PTX, with inhibition being reduced from 54.1 ± 3.8% to 22.4 ± 2.1% for mGluR1a and from 44.2 ± 2.7% to 23.8 ± 1.9% for mGluR5a using a saturating concentration of L-glutamate (100 μM). This PTX-resistant modulation is also resistant to NEM (25.6 ± 2.9% for mGluR1a and 24.3 ± 3.5% for mGluR5a) and to cholera toxin (CTX; 21.5% for mGluR1a and 23.5 ± 5.0% for mGluR5a). C: a comparison of NEM-resistant modulation without PTX treatment (see Fig. 2A) to that after PTX treatment (see A and B) demonstrates the significantly larger amount of NEM-resistant inhibition remaining after PTX treatment when compared with control modulation. For this comparison, the mean “control” values (100 μM L-glutamate columns, untreated cells) from the N-type channel experiments in Fig. 2A and from A and B were used. For mGluR1a, NEM-resistant modulation was 12.1 ± 5.3% of the total control (i.e., untreated cells) inhibition without PTX treatment (see Fig. 2A) and increased to 47.3 ± 5.4% of control inhibition after PTX treatment (see A). Likewise, NEM-resistant modulation by mGluR5a increased from 19.9 ± 7.9% of control (Fig. 2A) to 55.0 ± 6.4% of control (see B) after treatment with PTX. D: CTX used in A and B inactivates VIP-mediated modulation (see Zhu and Ikeda 1994) of N-type channels in superior cervical ganglion (SCG) neurons (decrease from 49.7 ± 2.1% to 6.4 ± 1.7% inhibition) while having very little effect on modulation by the α2-adrenergic agonist, UK14304 (57.5 ± 6.3% inhibition for controls, 49.6 ± 5.1% inhibition for CTX-treated cells).

not yet used this cell line to characterize any additional signal transduction pathways.

Interestingly, again contrasting with the modulation of N-type channels seen in control cells, the inhibition remaining after overnight PTX treatment was also entirely resistant to NEM (Fig. 3, A and B) and was 25.6 ± 2.9% (n = 5) and 24.3 ± 3.5% (n = 3) for mGluR1a and mGluR5a, respectively, after exposure to NEM (compare with 22.4 ± 2.1% and 23.8 ± 1.9% inhibition before NEM treatment, see preceding text). To more thoroughly compare NEM-resistant modulation in experiments with and without previous PTX exposure, mean “control” values (not significantly different, unpaired Student’s t-test) in the data shown in Figs. 2, A and B, and 3, A and B, were combined, and the NEM-resistant modulation remaining before and after overnight PTX treatment of group I-expressing cells was normalized to control inhibition. The NEM-resistant inhibition seen without PTX treatment was significantly (P < 0.002 for mGluR1a and P < 0.03 for mGluR5a) smaller than the NEM-resistant inhibition seen after PTX treatment (Fig. 3C). For mGluR1a, NEM-resistant inhibition was 12.1 ± 5.3% of control (e.g., untreated) inhibition before PTX exposure and increased to 47.3 ± 5.4% of control after PTX treatment. Similarly, NEM-resistant modulation by mGluR5a was 19.9 ± 7.9% of control before PTX treatment and 55.0 ± 6.4% of control after PTX. These results suggest that PTX treatment causes an apparent switch in the G proteins used by mGluR1a and mGluR5a from a NEM-sensitive to a NEM-resistant form.

The NEM-resistant pathway was not mediated by Gs-containing G proteins as modulation by mGluR1a and mGluR5a in both control (not shown) and PTX-treated cells was not sensitive to cholera toxin (Fig. 3, A and B). The CTX preparation used in these experiments was active as it removed modulation of N-type calcium channels by VIP in sympathetic neurons from superior cervical ganglia without sig-
Significantly affecting \( \alpha_2 \)-adrenergic receptor-mediated modulation in these same cells (Fig. 3D), confirming previous observations by Zhu and Ikeda (1994).

**Characterization of the PTX-resistant pathway**

Modulation by mGluR1a and mGluR5a using this PTX/NEM-resistant pathway did not appear to be the result of some mechanistically novel signal transduction pathway. The modulation still was relieved partially after a large, depolarizing prepulse, indicating that the PTX-resistant pathway was voltage dependent (Fig. 4, A and B). In fact, the relative contribution of voltage dependent \& independent inhibition was similar regardless of the types of G proteins (NEM sensitive vs. NEM resistant) or the types of mGluR (group I vs. group II) coupled to channel inhibition. For the data shown in Fig. 4C, ‘‘voltage-independent’’ modulation was defined as the inhibition remaining after a depolarizing prepulse, whereas ‘‘voltage-dependent’’ modulation was calculated as the difference between total percent inhibition and voltage-independent inhibition. In mGluR5a-expressing cells, voltage-independent modulation represented 49% of the total inhibition. Similarly, for mGluR5a-expressing cells treated 18–24 h with PTX (0.5 \( \mu \)g/ml), it is 48% of the total inhibition. Likewise, for the strictly PTX-sensitive coupling between mGluR2 and these channels (McCool et al. 1996), voltage-independent inhibition is 43% of the total modulation. This is the first demonstration of a voltage-dependent modulation of N-type calcium channels by PTX/CTX/NEM-insensitive G proteins.

A time course for the loss of the NEM-sensitive component and development of the NEM-resistant component was also examined (Fig. 5, A and B). For both mGluR1a and mGluR5a, PTX treatment (0.5 \( \mu \)g/ml, 37°C) caused a rapid loss of the NEM-sensitive modulation that was complete after 5 h PTX exposure, with half-maximal loss after ~2 h of PTX treatment. Conversely, the NEM-resistant component appeared to develop with a considerably slower time course, with the percent inhibition values ~5 h being approximately half of those seen for overnight PTX treatment.

The relatively slow accumulation of the NEM-resistant modulation by mGluR1a and mGluR5a after PTX treatment is reminiscent of metabolic processes such as translation. To address this possibility, we treated both control and PTX-treated cells with the translation inhibitor cycloheximide (CHX, 50 \( \mu \)g/ml, 12–18 h). This concentration of CHX is 2.5-fold higher than that required to inhibit 94% of \(^3\)H-leu.
cine incorporation in extracts from HEK 293 cells (Schmidt et al. 1994). Incubation with CHX modestly and nonsignificantly \( P > 0.05 \), analysis of variance (ANOVA) \] decreased the amount of modulation by mGluR5a in cells treated with CHX alone compared with controls (percent inhibition: control, 45.6 ± 6.4%, \( n = 8 \); +CHX, 35.2 ± 4.6%, \( n = 5 \); 23% reduction in modulation). In contrast, the accumulation of PTX-resistant modulation (Fig. 5C, open bars) was prevented by incubation with cycloheximide (percent inhibition: PTX treated, 26.3 ± 5.3%, \( n = 6 \); CHX and PTX treated, 9.5 ± 1.9%, \( n = 5 \); 64% reduction in modulation; significance at \( P < 0.05 \), ANOVA). The effects of CHX are even more apparent when one compares the relative levels of NEM-resistant modulation (Fig. 5C, closed bars) \] with percent inhibition values dropping significantly (\( P < 0.01 \), ANOVA) from 27.2 ± 6.2% in cells treated with PTX alone to 7.9 ± 1.0% in PTX + CHX-treated cells, a level of modulation not significantly different from NEM-resistant modulation in control (6.3 ± 1.1%) and CHX-only (7.8 ± 2.1%) cells. These results strongly suggest that development of the NEM-resistant modulation after PTX treatment requires new protein synthesis and more generally that PTX treatment may itself alter signal transduction components.

**Group I receptors initiate irreversible inhibition of N-type channels in perforated patch recordings**

Group I receptors have been shown previously to couple to signal transduction pathways that stimulate phosphoinositol metabolism and release of intracellular calcium in both native (Schoepf et al. 1990) and heterologous systems (Kinston et al. 1995; Pin et al. 1992), including HEK 293 cells (Flor et al. 1996). We examined the effect of group I receptor activation on N-type calcium channels using a recording configuration, the perforated patch, that should interfere only minimally with subtle changes in the intracellular environment. After forming a gigaohm seal, depolarizing test voltages were initiated only after maximal electrical access to the cell interior had been achieved as evidenced by saturation of the capacitive transients during small depolarizing steps. Inward barium currents through N-type channels recorded using the perforated-patch configuration were similar to those in normal rat hippocampal slice recordings. This sensitivity to CHX \] for 2 min \) . For mGluR1a (A), the percent NEM-sensitive inhibition was 52.3 ± 4.0%, \( 11.0 ± 2.3%; 0 ± 5.2\), and \( 0 ± 2.4% \) at 0, 2, 5 h, and overnight (ON), respectively; whereas the percent NEM-resistant inhibition was 7.7 ± 2.7% (see Fig. 2), 12.3 ± 2.5%, 16.3 ± 6.0%, and 25.6 ± 2.9% at 0, 2, 5 h, and ON, respectively. Similarly for mGluR5a (B), percent NEM-sensitive inhibition was 34.8 ± 4.8%, \( 21.3 ± 6.4% ; 2.6 ± 2.4% \), and \( 0 ± 3.1% \) at 0, 2, 5 h, and ON; whereas percent NEM-resistant inhibition was 8.5 ± 3.0%, 10.3 ± 2.5%, 15.4 ± 3.0%, and 24.3 ± 3.5% at these same times. C: development of the NEM/PTX-resistant modulation by mGluR5a is dependent on protein synthesis. Total inhibition (○) was compared with NEM-resistant modulation (■) after PTX treatment (0.5 μM/ml overnight) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX; 50 μM/ml). Total inhibition values are 45.6 ± 6.4% for control; 35.2 ± 4.6% for CHX alone; 26.3 ± 5.3% for PTX alone; and 9.5 ± 1.9% for PTX + CHX. NEM-resistant inhibition values are 6.3 ± 1.1% for control; 7.8 ± 2.4% for CHX alone; 27.2 ± 6.2% for PTX alone; and 7.9 ± 1.0% for PTX + CHX. Although PTX only partially removes inhibition by 100 μM L-glutamate (PTX-alone percent inhibition is ~58% of control inhibition; □), coincubation with CHX makes the modulation by mGluR5a relatively more PTX sensitive (PTX + CHX percent inhibition is only ~27% of the inhibition after CHX treatment alone; □). This sensitivity to CHX is most evident when comparing the percent NEM-resistant inhibition (■) after PTX treatment with and without cotreatment with CHX.

**FIG. 5.** A and B: a time course of PTX treatment indicates that the relative contributions of NEM-sensitive (denoted NEM sensitive) and NEM-resistant (NEM resistant) components to N-type channel modulation are differentially affected. The NEM-sensitive component was calculated by subtracting the NEM-resistant modulation from the total amount of inhibition; the NEM-resistant component is the amount of inhibition after treatment with NEM (50 μM for 2 min). For mGluR1a (A), the percent NEM-sensitive inhibition was 52.3 ± 4.0%, \( 11.0 ± 2.3% ; 0 ± 5.2\), and \( 0 ± 2.4% \) at 0, 2, 5 h, and overnight (ON), respectively; whereas the percent NEM-resistant inhibition was 7.7 ± 2.7% (see Fig. 2), 12.3 ± 2.5%, 16.3 ± 6.0%, and 25.6 ± 2.9% at 0, 2, 5 h, and ON, respectively. Similarly for mGluR5a (B), percent NEM-sensitive inhibition was 34.8 ± 4.8%, \( 21.3 ± 6.4% ; 2.6 ± 2.4% \), and \( 0 ± 3.1% \) at 0, 2, 5 h, and ON; whereas percent NEM-resistant inhibition was 8.5 ± 3.0%, 10.3 ± 2.5%, 15.4 ± 3.0%, and 24.3 ± 3.5% at these same times. C: development of the NEM/PTX-resistant modulation by mGluR5a is dependent on protein synthesis. Total inhibition (○) was compared with NEM-resistant modulation (■) after PTX treatment (0.5 μM/ml overnight) in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX; 50 μM/ml). Total inhibition values are 45.6 ± 6.4% for control; 35.2 ± 4.6% for CHX alone; 26.3 ± 5.3% for PTX alone; and 9.5 ± 1.9% for PTX + CHX. NEM-resistant inhibition values are 6.3 ± 1.1% for control; 7.8 ± 2.4% for CHX alone; 27.2 ± 6.2% for PTX alone; and 7.9 ± 1.0% for PTX + CHX. Although PTX only partially removes inhibition by 100 μM L-glutamate (PTX-alone percent inhibition is ~58% of control inhibition; □), coincubation with CHX makes the modulation by mGluR5a relatively more PTX sensitive (PTX + CHX percent inhibition is only ~27% of the inhibition after CHX treatment alone; □). This sensitivity to CHX is most evident when comparing the percent NEM-resistant inhibition (■) after PTX treatment with and without cotreatment with CHX.
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corded in the perforated-patch configuration rapidly inhib-
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stable for many minutes (Fig. 6B). A distinct modulatory events differed considerably with regard to
stimulate a rise of intracellular calcium from the HEK 293
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min (Fig. 6B). A, b, c =
7 min after initiation of the recording. Calibration bars are
10% during the recording;
A: stability of this recording configuration is
exemplified by the sample control traces in which current amplitude varied
by <10% during the recording; a = 1 min, b = 2 min, c = 4 min, and
d = 7 min after initiation of the recording. Calibration bars are x = 10 ms and y = 0.25 nA. B: in contrast, agonist application to a mGluR5a-expressing
cell caused a rapid, reversible modulation (compare trace a (or control),
b (or 100 μM L-glutamate, 24% inhibition), and c (or wash; 90% of control trace amplitude)] superimposed on a slowly developing and long-lasting inhibition [d (74% of control trace), e (30% of control), and f (21% of control)]. Traces shown are 2.25 min (a), 2.5 min (b), 3.0 min (c), 3.5 min (d), 6.5 min (e), and 9.5 min (f) after initiation of the recording. Calibration bars are x = 10 ms and y = 0.75 nA.

FIG. 6. Perforated-patch recordings indicate that group I receptors can modulate N-type channels using multiple signal transduction pathways that differ in time to onset and duration. Traces were not leak subtracted; – - - , zero current level. A: stability of this recording configuration is exemplified by the sample control traces in which current amplitude varied by <10% during the recording; a = 1 min, b = 2 min, c = 4 min, and
d = 7 min after initiation of the recording. Calibration bars are x = 10 ms and y = 0.25 nA. B: in contrast, agonist application to a mGluR5a-expressing
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those observed in the whole cell configuration and were stable for many minutes (Fig. 6A). Conversely, in cells transfected with mGluR1a (not shown) or mGluR5a (Fig. 6B), barium currents were modulated by two distinct processes on exposure to agonist (100 μM L-glutamate). These distinct modulatory events differed considerably with regard to the time to onset after agonist exposure and the duration of inhibition.

Application of agonist to group I-expressing cells recorded in the perforated-patch configuration rapidly inhibited barium currents (Fig. 6B, a and b); removal of agonist and application of wash resulted in an initial, almost complete, reversal of channel modulation (Fig. 6B, a and c). At no time during agonist application was there a change in resting membrane currents. The ‘‘fast’’ modulation was maximal as soon as the current was initiated, usually 10–15 s after agonist application, and was superimposed on a slowly developing inhibition that became obvious only 15–30 s after agonist removal/initiation of wash (Fig. 6B, c – f). Interestingly, this slowly developing inhibition took 5–6 min to reach maximal levels and did not reverse for the duration of the recording (10–15 min, Fig. 7A). Importantly, agonist stimulation in mGluR2-expressing cells did not initiate this slowly developing, ‘‘irreversible’’ inhibition while leaving the rapid/reversible modulation intact (Fig. 7B).

The rapidly reversible modulation by group I receptors in the perforated-patch recording configuration was also voltage-dependent as determined by the two-pulse protocol outlined in the preceding text (not shown). Furthermore, this modulation was not substantially affected after the ‘‘irreversible’’ had become maximal. For example, inhibition mediated by the rapid pathway after saturation of the irreversible inhibition was 28.8 ± 6.2% for mGluR1a (compare with 32.3 ± 2.4% inhibition for 1st agonist application, n = 4) and 23.2 ± 5.8% for mGluR5a (37.2 ± 9.2% inhibition for 1st agonist application, n = 5). Similarly, a second agonist challenge of mGluR2-expressing cells caused 64.2 ± 6.6% inhibition (compare at 64.9 ± 5.3% inhibition, n = 5, for the 1st exposure to agonist).

To more quantitatively measure this receptor-initiated irreversible inhibition, the data in Fig. 7, A and B, were expressed in the following manner: once a stable recording was obtained (usually 1–2 min after achieving a stable opening), current amplitudes during initial episodes before agonist application were used to extrapolate (via linear regression, Fig. 7, A and B, − − − ) ‘‘expected’’ current amplitudes at later (ca. 10 min) times during the recording. These expected values then were compared with the ‘‘observed’’ current amplitudes at the end of the recording by an expected/observed ratio. Note that receptors incapable of initiating irreversible inhibition would theoretically have an expected/observed ratio around unity. As shown in Fig. 7C, both mGluR1a and mGluR5a robustly induced irreversible N-type channel inhibition as evidenced by their expected/observed ratios significantly greater than unity (2.5 ± 0.5, n = 4 for mGluR1a, P < 0.03; and 3.5 ± 0.6, n = 5 for mGluR5a, P < 0.01), whereas mGluR2 did not (ratio = 0.9 ± 0.1, n = 5, P = 0.3), consistent with the notion that only group I receptors can initiate this type of modulation.

The most plausible explanation for the irreversible inhibition is related to the poor buffering of intracellular calcium in this recording configuration. Indeed, receptor-mediated changes in intracellular calcium can influence significantly the physiology of voltage-gated calcium channels in native systems (Kramer et al. 1991). Furthermore, group I mGluRs stimulate a rise of intracellular calcium from the HEK 293 cells stably expressing N-type calcium channels, even in the absence of extracellular Ca2+ (McCool, unpublished observations). To test whether the irreversible inhibition initiated by group I receptors was indeed related to a rise intracellular calcium, mGluR5a-expressing cells were treated with the cell-permeant analogue of bis-(α-aminophenoxyl)−N,N′,N″,N‴-tetraacetic acid (BAPTA), BAPTA-AM (500 μM, 37°C for 20 min) and subjected to perforated-patch recordings. BAPTA-loaded cells expressing mGluR5a exhibited sig-
significantly less irreversible inhibition ($P < 0.05$, 2-tailed Student’s $t$-test) than control cells (ratios were $\sim 3.5$ for controls and 1.6 for BAPTA-AM–treated cells). These results imply that the release of intracellular calcium plays some critical role in the slowly developing, irreversible modulatory pathway.

**Discussion**

Group I metabotropic glutamate receptors have been long thought of as the ‘‘PI-coupled’’/‘‘$G_i$-coupled’’ branch of the mGluR family. However, group I-like receptors in various preparations initiate physiological responses not classically associated with PTX-resistant $G$ proteins, like the voltage-dependent inhibition of voltage-gated calcium channels (Choi and Lovinger 1996) and the inhibition of synaptic transmission (Gereau and Conn 1995). These findings led us to the possibilities that either additional receptors with a pharmacology similar to group I receptors but signal transduction properties more like group II or III receptors remained to be isolated or the currently identified group I receptors are actually quite ‘‘promiscuous’’ in their $G$ protein selectivity. Hints of the latter phenomenon were revealed by findings that group I receptors may use PTX-sensitive $G$ proteins to stimulate phosphatidylinositol metabolism and arachidonic acid release in various mammalian heterologous systems (Aramori and Nakanishi 1992; Pickeing et al. 1993) and to stimulate phosphatidylinositol metabolism (Suzdak et al. 1993) and $\mathrm{Ca}^{2+}$ release (Milani et al. 1993) in cerebellar granule cells. We have shown clearly that the currently known group I receptor clones, when transiently expressed in HEK 293 cells, may use $G_i/G_o$-mediated pathways to modulate rapidly both N-type and P/Q-type calcium channels in a voltage-dependent fashion. However, previous studies using heterologous expression of mGluR1a in SCG neurons (Ikeda et al. 1995) did not indicate a strong coupling between this receptor, PTX-sensitive $G$ proteins, and N-type calcium channels. Nonetheless, the modulation by group I receptors in HEK 293 cells is essentially identical to that seen for quisqualate-mediated inhibition in acutely isolated deep-layer cortical (Choi and Lovinger 1996) and CA3 hippocampal (Swartz and Bean 1992) neurons. Furthermore, the pharmacology of mGluR1a and mGluR5a are consistent with that reported for these receptors in other heterologous and native systems (reviewed by Conn and Pin 1997). Although our result appear to conflict somewhat with the results of Ikeda et al. (1995), coupling of group I receptors to N-type calcium channels in SCG neurons has been accomplished recently using methodologies that allow for consistently high levels of receptor expression (S. Ikeda, personal communication). Together, these observations support the notion that group I receptors can couple to numerous signal transduction pathways in a variety of heterologous as well as native systems using a variety of $G$ proteins.

**Fig. 7.** Irreversible inhibition in the perforated patch configuration is specific to group I receptors and dependent on the agonist-induced release of intracellular calcium. A: a time course of absolute current amplitudes for the mGluR5a-expressing cell shown in Fig. 6B illustrates both the rapid, reversible inhibition and the slowly developing, irreversible inhibition. B: in contrast, a time course for a mGluR2-expressing cell indicates the presence of only the rapid, reversible pathway. For both A and B, agonist (100 $\mu$M L-glutamate) application is indicated by ---, and the percent inhibition for each application also is included. ···, linear regression line derived using the ‘‘baseline’’ current amplitudes of each recording (i.e., prior to the 1st agonist application); $\times$, ‘‘expected’’ current amplitudes based on predictions from the linear regression. Relative differences between the predicted or expected amplitudes and the observed amplitudes are expressed as an expected/observed ratio in C. Generally, receptors initiating the irreversible pathway should give expected/observed ratios significantly more positive than unity. C: summary of perforated patch experiments for group I (mGluR1a and 5a) and a group II (mGluR2) receptors indicate that only group I receptors have ratios significantly different from unity. Furthermore, treatment of mGluR5a-expressing cells with bis-(o-aminophenoxo)-N,N',N'-tetraacetic acid (BAPTA)-AM (500 $\mu$M) prevented the irreversible inhibition, indicating the ability of group I receptors to release $\mathrm{Ca}^{2+}$ in HEK 293 cells may be an essential component of the ‘‘irreversible’’ inhibition. Ratio values are: $2.67 \pm 0.51$ for mGluR1a; $2.99 \pm 0.59$ for mGluR5a; $1.43 \pm 0.20$ for mGluR5a + BAPTA-AM; $0.99 \pm 0.02$ for mGluR2.
Along these lines, we have demonstrated that group I receptors expressed in HEK 293 cells may inhibit a single effector, the N-type calcium channel, using both Ca\(^{2+}\)-independent and -dependent mechanisms. Interestingly, the Ca\(^{2+}\)-independent, voltage-dependent pathway described above can be divided further into PTX/NEM-sensitive and -insensitive components. In fact, group I modulation of N-type channels can switch from a primarily NEM-sensitive form to an insensitive form by treatment with PTX. This ‘switch’ is in stark contrast to the entirely PTX- and NEM-sensitive modulation by group II receptors (mGluR2–3) (McCool et al. 1996). Although we have not specifically ruled out the possibility that NEM may directly influence mGlu receptors, the fact that the PTX-resistant modulation by group I mGluRs is also NEM-resistant tends to strengthen the idea that NEM does not significantly alter the properties of group I receptors.

Because the NEM-resistant modulation by group I receptors slowly develops relative to the rapid loss of the NEM-sensitive modulation after PTX treatment, NEM-resistant modulation may be the result of contributions either by previously existing receptor pools or by newly synthesized receptors/G proteins/channels. Alternatively, mGluRs may stay associated with calcium channels even after ADP-riboseylation of Gi/Go-α subunits and are replaced only slowly by receptors coupled to PTX-resistant G proteins. Although we have not addressed the latter possibility, our results with calcium channel heterogeneity and PTX-resistant CHX are consistent with the new protein synthesis being an essential component involved in the development of NEM-resistant modulation after PTX treatment. Although not necessary to explain our observations, the contributions of previously existing receptor pools remain to be addressed directly.

The modulation of N-type calcium channels by PTX-insensitive/NEM-resistant G proteins is most likely not an artifact associated with heterologous expression. Group I receptors in central neurons provide clear indications that they also may partially use a NEM-resistant, voltage-dependent pathway to modulate voltage-gated calcium channels (Choi and Lovinger 1996). Additionally, modulation of N-type channels by angiotensin II AT\(_1\) receptors in sympathetic neurons (Shapiro et al. 1994b) is only partially PTX sensitive while being relatively more NEM sensitive. The results of this study therefore may represent a PTX-induced switch in the types of G proteins used by this receptor similar to that reported here. We currently are examining the possibility that PTX may directly influence the kinds of G proteins used by group I mGluR and other receptors in native neuronal systems. More generally, our results indicate that partially PTX-sensitive responses should be reexamined using independent measures (like NEM). Although it is true that NEM is by no means as specific in its effects at the cellular level as PTX, it also should be realized that PTX may not merely be a benign indicator of G, involvement in a given receptor-driven response but itself may alter the signal transduction components used by a given receptor. In addition to our results, neurochemical studies of cultured cerebellar granule cells (Cullen et al. 1994) where PTX treatment ‘induces’ a CHX-sensitive increase in glutamate release also support this notion.

While the NEM-sensitive/-resistant, voltage-dependent pathways are similar in most respects to well characterized signal transduction events in other systems, the BAPTA-sensitive, ‘long-lasting’ modulation of N-type channels initiated by group I receptors in HEK 293 cells when recorded in the perforated-patch configuration is a relatively novel form of receptor-mediated modulation. Although we have not yet addressed the mechanism by which presumed Ca\(^{2+}\) rises potentially may influence N-type channels, the \(\alpha_{\text{m}}\) (N-type) channel subunit is known to be a target for Ca\(^{2+}\)-sensitive kinases, like protein kinase C and Ca\(^{2+}\)/calmodulin-dependent protein kinase II, in vitro (Hell et al. 1994). Furthermore, the phosphorylation state of the N-type channel can dramatically influence its biophysical properties (Werz et al. 1993). The time course of such processes and whether they can be initiated by G protein-coupled receptors in HEK 293 cells has yet to be determined.

It has been demonstrated that group I mGlu receptor activation in native in vitro systems may elicit modulatory processes that inhibit N-type or P/Q-type calcium channels. Similar to our observations in HEK 293 cells, these events are highly dependent on the Ca\(^{2+}\)-buffering capacity of the intracellular electrode solution (Sahara and Westbrook 1993). Furthermore, the irreversible/ Ca\(^{2+}\)-dependent pathway in HEK 293 cells appears to associate with the ability of a given receptor to couple to phospholipase C (PLC) and/or PTX-resistant G proteins because both mGluR1a and mGluR5a are capable of initiating this pathway, whereas mGluR2, a receptor that appears to couple only to PTX-sensitive G proteins in this HEK 293 cell system, does not. Because group I mGluRs can activate the PLC/ Ca\(^{2+}\) signal transduction pathway in HEK 293 cells (Flor et al. 1996) and activation of this pathway is PTX-resistant (Pin, unpublished observations), our results suggest that the activation of phospholipase C may lie somewhere between the group I mGluRs and the releasable pool of intracellular calcium. Additional experiments will be necessary to unequivocally assign a role for a rise in intracellular calcium in the irreversible pathway and to define the components of this modulatory process both upstream and downstream from intracellular calcium release.

We have shown that group I metabotropic glutamate receptors can use multiple signal transduction pathways to modulate the activity of neuronal voltage-gated calcium channels. It is interesting to speculate that the relative contributions of the rapid, voltage-dependent, BAPTA-insensitive and the long-lasting, BAPTA-sensitive, presumably Ca\(^{2+}\)-dependent pathways may govern the apparent diversity of processes activated by group I receptors that encroach on the regulation of synaptic transmission. Presynaptic group I mGluR autoreceptors could modulate the release of neurotransmitter either by the rapidly reversible inhibition of calcium channels leading to more short-term alterations in synaptic efficacy or by the long-lasting irreversible modulation resulting in long-term changes in synaptic strength. Likewise, it is not difficult to imagine the existence of factors that influence the relative activity of each potential pathway to fine-tune the physiological outcome of group I mGluR activation. It will be necessary to use native systems, such as acutely isolated central neurons or brain slices, to compare...
with the HEK 293 signal transduction pathways and to address such issues. Nonetheless, our study has emphasized the diversity of signal transduction pathways potentially available to modify neuronal voltage-gated calcium channel function.

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