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Coupling of metabotropic glutamate receptor 8 to N-type Ca^{2+} channels in rat sympathetic neurons

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Running title: Cloning and functional expression of mmGluR8a

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ABBREVIATION: mGluR, metabotropic glutamate receptor; mmGluR, mouse mGluR; rmGluR, rat mGluR; L-Glu, L-glutamate; L-AP4, L-2-amino-4-phosphonobutyric acid; CPPG, *(R,S)*-α-cyclopropyl-4-phosphonophenylglycine; PTX, *Bordetalla pertussis* toxin; NE, norepinephrine; GIRK, G-protein coupled inwardly rectifying potassium channels; SCG, superior cervical ganglion

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ABSTRACT

Group III metabotropic glutamate receptors (mGluRs; mGluR4, 6, 7, and 8) couple to the $G\alpha_{i/o}$ containing G-protein heterotrimers and act as autoreceptors to regulate glutamate release, probably by inhibiting voltage-gated Ca^{2+} channels. Although most mGluRs have been functionally expressed in a variety of systems, few studies have demonstrated robust coupling of mGluR8 to downstream effectors. We therefore tested whether activation of mGluR8 inhibited Ca^{2+} channels. Both L-glutamate (L-Glu) and L-AP4, a selective agonist for group III mGluRs, inhibited N-type Ca^{2+} current in rat SCG neurons previously injected with a cDNA encoding mGluR8a/b. L-AP4 was ~ 100 fold more potent (IC_{50} = 0.1 μM) than L-Glu (~10 μM), but had a similar efficacy as L-Glu (ca. 50% maximal inhibition). The potency and efficacy of L-AP4 and L-Glu were similar for both splice variants. Agonist-induced inhibition was abolished by pretreatment with CPPG, a selective group III mGluR antagonist, and *Pertussis* toxin. Deletion of either a calmodulin (CaM) binding motif in the C-terminus or the entire C-terminus of mGluR8 did not affect mGluR8-mediated response. Our studies indicate that both mGluR8a and 8b are capable of inhibiting N-type Ca^{2+} channel, suggesting a role as presynaptic autoreceptors to regulate neuronal excitability. The studies also imply that the potential CaM binding domain is not required for the mGluR8-mediated Ca^{2+} channel inhibition and the C-terminus of mGluR8a is dispensable for receptor coupling to N-type Ca^{2+} channels.

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INTRODUCTION

As a major excitatory neurotransmitter, glutamate functions through ligand-gated ionotropic and G protein-coupled metabotropic receptors (mGluRs). Genes coding for eight mGluRs (denoted mGluR1-8) with different splice variants have been identified and classified into three groups based on sequence homology, pharmacology, and signaling pathways (Nakanishi, 1994; Pin and Duvoisin, 1995; Conn and Pin, 1997). Group I mGluRs (mGluR1, 5) mainly couple to $G\alpha_{q/11}$ containing G protein heterotrimer to activate phospholipase C, but also couple to other G protein families, including $G\alpha_s$ and $\alpha_{i/o}$ containing G proteins. Group II (mGluR2, 3) and group III mGluRs (mGluR4, 6, 7, 8) couple to $G\alpha_{i/o}$ containing G protein family to inhibit adenylyl cyclase. With the exception of mGluR6, which is postsynpatic and exclusively expressed in the retina, group III mGluRs primarily serve as presynaptic autoreceptors to mediate feedback inhibition at glutamatergic synapses. However, at some synapses these receptors may also be localized at postsynaptic sites (Bradley et al., 1996; Kinoshita et al., 1996; Shigemoto et al., 1997).

mGluR8 was the last cloned subtype amongst the known mGluRs (Duvoisin et al., 1995). Its mRNA has been detected in the olfactory bulb, thalamus, pontine gray, cerebral cortex, hippocampus, cerebellum and retina (Duvoisin et al., 1995; Saugstad et al., 1997). The mGluR8 receptor protein has been found in the dentate gyrus (Shigemoto et al., 1997), olfactory bulb and the olfactory tubercle (Kinoshita et al., 1996; Wada et al., 1998). Electrophysiological studies suggested that mGluR8 functions as a presynaptic autoreceptor to regulate glutamate release from the lateral perforant path terminals in the mouse dentate gyrus (Zhai et al., 2002). It is suggested that mGluR8, like other group III

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mGluRs, controls glutamate release by inhibiting voltage-gated Ca^{2+} channels. However, unlike mGluR4 and mGluR7, which have been functionally expressed in a variety of heterologous system, few studies have reconstituted the coupling of mGluR8 to its downstream effectors. mGluR8 was originally cloned from a mouse retina cDNA library, however, the characterization of mGluR8 was hindered by the fact that mouse mGluR8 coupled very weakly to the inhibition of adenylyl cyclase, with a maximal inhibition of ~ 20% (Duvoisin et al., 1995). On the other hand, glutamate elicited pertussis toxin (PTX) -sensitive potassium currents in *Xenopus laevis* oocytes coexpressing rat mGluR8 and G protein-coupled inwardly rectifying potassium (GIRK) channels (Saugstad et al., 1997). Therefore, as a rationale for the involvement of mGluR8 in regulating glutamate release, it is important to first establish whether voltage-gated Ca^{2+} channels involved in presynaptic inhibition are modulated by mGluR8.

mGluR7 was the first group III mGluR found to be highly localized at presynaptic active zones of hippocampal neurons (Shigemoto et al., 1997). Recently it has been reported that G protein βγ subunits and the Ca^{2+} sensor, calmodulin (CaM), interact in a mutually exclusive way within the proximal region of the C-terminal tail of mGluR7 (O'Connor et al., 1999). Based on this finding, it was proposed that CaM binds to the C-terminus of mGluR7 to promote the dissociation of Gβγ from the receptors thereby making the "released" Gβγ available for inhibiting voltage-dependent Ca^{2+} channels (O'Connor et al., 1999). In support of this notion, CaM inhibitors were shown to block group III mGluR-mediated inhibition of glutamate release in primary hippocampal neurons. Furthermore, deletion of the CaM binding motif from mGluR7 was shown to abolish βγ subunit mediated signaling (O'Connor et al., 1999; El Far et al., 2001). Since

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the CaM binding motif is also present within the first 25 amino acid of the mGluR8 C-terminus (El Far et al., 2001), the CaM binding motif in the mGluR8 C- terminal tail might participate in Ca^{2+} channel inhibition.

Therefore, in our present studies, we first examined whether N-type Ca^{2+} channels could be modulated by activating mGluR8. We then tested if the CaM binding motif in the C-terminus of mGluR8 was involved in the Ca^{2+} channel modulation. We found that mGluR8 significantly inhibited N-type Ca^{2+} channels. However, the CaM binding domain in the C-terminus was not required for the mGluR8-mediated Ca^{2+} channel inhibition. Furthermore, we determined that the entire mGluR8a C-terminus was not crucial for receptor membrane expression and G protein coupling.

Methods

Preparation of dissociated sympathetic neurons

Sympathetic cervical ganglion (SCG) neurons from adult male Wistar rats (150–300 g) were enzymatically dissociated as previously described (Ikeda, 2004). Briefly, rats were decapitated after anesthesia by CO_2 inhalation as approved by the Institutional Animal Care and Use Committee. Bilateral SCG were dissected and desheathed, cut into small pieces, and then transferred into 6 ml of modified Earle's Balanced Salt Solution (EBSS) containing 0.7 mg/ml collagenase D (Roche, Indianapolis, IN), 0.3 mg/ml trypsin (Worthington Biochemical Corporation, Freehold, NJ) and 0.05 mg/ml DNase I (Sigma, St. Louis, MO). After incubation for 1 h in a shaking water bath at 36 ^{o}C under an atmosphere of 5% CO_2 - 95% O_2, SCG fragments were shaken vigorously to release the neuronal somata. The dissociated neurons were washed twice, resuspended in Minimum Essential Medium (MEM) containing 10% fetal calf sera and 1% penicillin-streptomycin,

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plated onto poly-L-lysine-coated tissue culture dishes (35 mm), and placed in an incubator (95% air and 5% CO_2; 100% humidity) at 37 °C. Following cDNA injection, the neurons were incubated overnight at 37 °C, and current recordings were performed the following day. As appropriate, neurons were incubated overnight with 500 ng/ml PTX (List Biological Laboratories, Campbell, CA).

DNA cloning

Mouse mGluR8a (denoted mmGluR8a) cDNAs was cloned from mouse whole brain cDNA (Clontech, Palo Alto, CA) using standard PCR techniques. To generate a mmGluR8a-EGFP fusion construct, full length mmGluR8a was amplified using two primers: forward 5'-GATCGATCCTCGAGCACCATGGTTTGTGAGGGAAAGCGCTCAACC TCT- 3' containing a X*ho*I restriction site, reverse 5'-GATCCCCGGGATCCGATTGAATGATTA CTGTAGCTGATGTA-3' containing a X*ma*I restriction site. The amplification was carried out using *Pfu*Ultra high-fidelity DNA polymerase (Stratagene, La Jolla, CA) according to the following protocol: 95ºC for 30 sec, 60 ºC for 30 sec, 72 ºC for 3 min (35 cycles). The PCR fragment was analyzed on a 1% agarose gel, subcloned into pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced (CEQ^{TM}8000, Beckman Coulter, Fullerton, CA). The mmGluR8a full length cDNA was then subcloned into the X*ho*I and X*ma*I sites of expression vector pEGFP-N1 (Clontech). QuikChange® mutagenesis (Stratagene) was used to generate wild type mmGluR8a (i.e., non EGFP fusion) by changing the first linker amino acid glycine (GGA) to a stop codon (TGA). Similarly, wild type rat mGluR8b (rmGluR8b; Genbank accession number NM022202 and Y11153) cDNA was amplified from rat whole brain

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cDNA library (Clontech) using two primers: forward 5'-GATCAAGCTTCACCATGGTATGCGAGGGAAAGCGATCAGCC-3' (containing a H*ind*III site), reverse 5'-GATCTCTAGATTAGGAAGTGCTCCCGCTCTTGACCATCGGAAA-3' (containing a X*ba*I site). The PCR fragment was subcloned into the H*ind*III and X*ba*I site of the pcDNA3.1 vector (Invitrogen) and sequenced. Since the amino acid sequence of C-terminus of mouse mGluR8a and that of rat mGluR8a is identical, rat mGluR8a (rmGluR8a; Genbank accession number NM022202) cDNA was generated using QuikChange® mutagenesis (Stratagene) by replacing the C-terminus of rmGlu8b with that of mmGluR8a. The clone was confirmed by sequencing. Constructs with different deletions of the C-terminus of mmGluR8a were generated using QuikChange® mutagenesis (Stratagene). The deletions were confirmed by sequencing.

Cell culture, transfection and imaging.

HEK 293 cells were cultured in MEM supplemented with 10% fetal calf serum under an atmosphere containing 5% CO_2. The cells were transfected with the mGluR8-EGFP cDNA as follows. A mixture of 1 µg of mGluR8-EGFP and 2 µl of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was made in 100 µl of Opti-MEM and preincubated for 20 min. The mixture was then applied to cell culture wells containing HEK 293 cells at ~ 95% confluence. After 24 h incubation, the cells were plated on glass-bottom chambers and examined using an Olympus IX-71 inverted fluorescence microscope equipped with a 60x 1.45 NA objective. Images were captured using a cooled CCD camera (Orca ERG, Hamamatsu, Hamamatsu City, Japan) and Openlab software (Improvision Inc., Lexington, MA).

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cDNA injection

As previously described (Ikeda, 2004; Ikeda & Jeong, 2004), microinjection of cDNA into neuronal nuclei was performed with an Eppendorf FemtoJet microjector and 5171 micromanipulator (Eppendorf, Madison, WI) using custom designed software. Plasmids (pEGFP-N1, Clontech; pcDNA3.1, Invitrogen) containing inserts coding for mmGluR8a, rmGluR8a, and rmGluR8bwere stored at –20 °C as 0.3–1 µg/µl stock solution in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). cDNA was injected at a pipette concentration of 100–200 ng/µl. When EGFP-fusion constructs were not used, neurons were coinjected with EGFP cDNA (pEGFP-N1; 5ng/µl) to facilitate the identification of neurons receiving a successful intranuclear injection.

Electrophysiology

Rat SCG neurons were voltage-clamped using the whole-cell patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments, Foster city, CA). Electrodes were made from borosilicate glass capillaries (Warner Instrument Corp., Hamden, CT, # G85165T-4), coated with Sylgard (Dow Corning, Midland, MI), and fire-polished to final resistances of ~2 MΩ when filled with internal solutions. Uncompensated series resistance was < 6 MΩ and generally electronically compensated ~ 80%. Custom designed software (S5) was use for voltage protocol generation and data acquisition on a Macintosh G4 computer (Apple Computer, Cupertino, CA) equipped with an ITC-18 data acquisition interface (Instrutech, Port Washington, NY). Currents traces were filtered at 1 kHz (-3dB) using a four-pole low-pass Bessel filter and digitized at 10 kHz with the 16-bit analog-to-digital converter in the ITC-18 data acquisition interface. All experiments were carried out at room temperature (22-26 °C).

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Solutions and chemicals

For recording Ca^{2+} currents, the external solution consisted of (in mM): 140 methanesulphonic acid, 145 tetraethylammonium hydroxide (TEA-OH), 10 HEPES, 10 glucose, 10 $CaCl_2$; and 0.0003 tetrodotoxin (TTX), pH 7.4 with TEA-OH. The internal solution contained (in mM): 120 N-methyl-D-glucamine, 20 TEA-OH, 11 EGTA, 10 HEPES, 10 sucrose, 1 $CaCl_2$, 4 MgATP, 0.3 Na_2GTP, and 14 Tris creatine phosphate, pH 7.2 with methanesulphonic acid. The osmolalities of the bath and pipette solutions were adjusted with sucrose to 325 and 300 mosmol/kg, respectively.

A gravity-driven perfusion system positioned ~100 µm from neurons was used for application of all drugs and control solutions. At the end of the perfusion system, a silica gas chromatography column was connected to six parallel columns of the same diameter in series. The column containing normal external solution was kept flowing to avoid flow-induced artifact until the desired solution was applied.

Stock solutions of L-glutamate (Sigma), L (+)-2-amino-4-phosphonobutanoic acid (L-AP4) and (*R*,*S*)-R-cyclopropyl-4-phosphonophenylglycine (CPPG; Tocris, Ellisville, MO) were prepared in 100 mM NaOH at a stock concentration of 100 mM, 1 mM, and 10 mM, respectively. PTX (List Biological Laboratories) was prepared in H_2O at a stock concentration of 100 µg/ml. All drugs were diluted in the external solutions from stock solutions to the final concentrations just prior to use.

Data analysis and statistics

Currents were analyzed using Igor Pro software (WaveMetrics, Lake Oswego, OR) on an iMac computer. All data were expressed as mean ± SEM. The Ca^{2+} current percentage inhibition (%) was determined as $(I_{con} - I_{drug})/I_{con}$ x 100, where I_{con} and I_{drug}

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are the Ca^{2+} currents before and after drug application. The concentration-response curves were fit to a Hill equation: $B = B_{max} /\{1 + (IC_{50}/[agonist])^{n}\}$, where B, B_{max}, IC_{50}, [agonist] and *n* are percentage inhibition, maximum inhibition, half inhibition concentration, agonist concentration and Hill Coefficient, respectively. Statistical comparisons among groups were determined by ANOVA. $P < 0.05$ was considered significant.

Results

Molecular cloning of mmGluR8a

In preliminary studies, expression of the original mouse mGluR8a cDNA (GenBank accession number, U17252), a gift from Dr. R. Duvoisin, failed to couple to N-type Ca^{2+} channels in rat SCG neurons (data not shown), whereas expression of rat mGluR8b, an alterative splice variant that differs in the last 16 amino acids of the intracellular C-terminus, robustly inhibited N-type Ca^{2+} channels under the same conditions (this report). This finding seemed to indicate that residues in mGluR8 C-terminus were important determinants of mGluR8 coupling to Ca^{2+} channels. Unexpectedly, exchanging the C-termini of each clone failed to transfer the ability to modulate Ca^{2+} channels (data not shown). Rat mGluR8b was used in these studies as a cDNA sequence for the mouse ortholog was not available. Because the cognate residues of mouse and rat mGluR8 are highly conserved, it seemed unlikely that species differences could account for such profound differences in coupling. We thus undertook a detailed examination of the mouse mGluR8a clone (U17252) used in the preliminary studies. Comparing the mouse mGluR8a cDNA sequence, U17252, with the mouse

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genome sequence using BLAT (Kent, 2002; http://genome.ucsc.edu) revealed eight potential amino acid differences in the translated sequence. Among these discrepancies, amino acid differences at position 343 and 589 likely arose from sequencing errors as our sequencing results at these positions (of the same clone) agreed with the genomic sequence. The remaining six amino acid differences seemed unlikely to result from legitimate polymorphisms since these residues are well conserved amongst rat, human and mouse mGluR8 sequences. As U17252 was the only full length mouse mGluR8 cDNA sequence available in GenBank, we cloned mouse mGluR8 *de novo* from mouse brain cDNA. The newly cloned mouse mGluR8 was defined as mouse mGluR8a (mmGluR8a) since, like rat mGluR8a and 8b (rmGluR8a and rmGluR8b, respectively), an orthologous splice variant of mouse mGluR8b (mmGuR8b) likely exists (i.e., the alternatively spliced exon is present in the mouse genome).

Using primers based on a predicted mouse mGluR8a derived from mouse genomic sequence, a 2727 bp PCR fragment was generated. Sequencing of the fragment revealed a 2727 base pair open reading frame, with original stop codon, TGA, replaced with GGA to generate an EGFP fusion construct. The mouse mGluR8a cDNA sequence predicts a protein of 908 amino acids with an estimated molecular mass of 101,820 Daltons. The mmGluR8a sequence (deposited in GenBank as accession number AY673682) was 100% identical with the mouse genomic sequence located on chromosome 6. The deduced amino acid sequence of AY673682 revealed a 99.4% and 98.5% identity with rat and human orthologs, and 99.3% identity with the original mouse mGluR8. The differences between the mmGluR8a and the original mouse mGluR8 are depicted in Fig. 1A. The majority of the mutations (five amino acids) were located in the

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extracellular N-terminus (the ligand-binding domain) of the receptor, and one mutation in the intracellular C-terminus of the receptor. The two sequencing errors at positions 343 and 589 are indicated with boxes.

Expression of the original mouse mGluR8 fused to EGFP in HEK 293 cells and hippocampal neurons revealed that the majority of the protein appeared trapped inside both HEK 293 cells (Fig. 1B) and hippocampal neurons (data not shown). The fine lace-like pattern of fluorescence was consistent with retention in the endoplasmic reticulum. In contrast, expression of the newly cloned mouse mGluR8a-EGFP fusion construct displayed a rim-like fluorescence consistent with appropriate targeting of the protein to the plasma membrane (Fig. 1C). Accordingly, the new mouse mGluR8a construct was used in all subsequent studies.

Functional expression of mmGluR8a in rat SCG neurons

An important function of presynaptic group III mGluRs is to control glutamate release by inhibiting voltage-dependent Ca^{2+} channels via G protein βγ subunits. Functional mGluRs are not endogenously expressed in rat SCG neurons (Ikeda et al., 1995) hence this expression system facilitates the study of the coupling of molecularly defined mGluR subtypes to the effectors, such as Ca^{2+} channels (Kammermeier & Ikeda, 1999). Previously, we have demonstrated that N-type Ca^{2+} channels are inhibited by activating a variety of mGluRs, including mGluR1, mGluR2, mGluR3, mGluR5, and mGluR7, heterologously expressed in rat SCG neurons (Ikeda et al., 1995; Kammermeier & Ikeda, 1999; 2003). To test the function of the newly cloned mmGluR8a, we injected mmGluR8a cDNA into the nucleus of rat SCG neuron and tested whether the mmGluR8a inhibited Ca^{2+} channels. Figure 2 illustrates the effects of L-Glu (100 μM) and L-AP4 (1

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μM), a selective agonist of group III mGluR, on Ca^{2+} channel currents elicited from uninjected and mmGluR8a cDNA injected SCG neurons. Ca^{2+} channel currents were elicited every 10 sec from a holding potential of -80 mV with a double-pulse protocol (Elmslie et al., 1990). This protocol consists of two identical 25 ms test pulses to +10 mV separated by a 50 ms strong depolarizing conditioning pulse to +80 mV (Fig. 2A, B & C). The prepulse and postpulse currents were measured isochronally at 10 ms from the start of the test pulse. It has been demonstrated previously that the major component of Ca^{2+} current elicited from rat SCG neuron under these conditions is ω-conotoxin GVIA sensitive N-type Ca^{2+} current (Ikeda, 1991). Application of 100 μM L-Glu did not have an effect on Ca^{2+} current elicited from uninjected neurons (Fig. 2A) further indicating that functional mGluRs are not expressed on the soma of rat SCG neurons (Ikeda, 1995; Kammermeier and Ikeda, 1996). For mmGluR8a cDNA injected neurons, L-Glu (100 μM) significantly inhibited the Ca^{2+} current by 51 ± 4% (n = 11). Figure 2B shows current traces elicited from a neuron previously injected with mmGluR8a cDNA in the absence or presence of 100 μM L-Glu. As with many $G\alpha_{i/o}$ containing G protein coupled receptors, mmGluR8a inhibited Ca^{2+} current via a membrane-delimited, $G\beta\gamma$ mediated voltage-dependent pathway characterized by slowed activation kinetics in the prepulse and partial relief of inhibition by a large depolarizing conditioning pulse (reviewed by Hille, 1994; Ikeda & Dunlap, 1999; Ikeda, 1996; Herlitze et al., 1996) (Fig. 2B). The ratio of postpulse current amplitude to prepulse current (post/pre), or facilitation ratio, was increased rapidly by mmGluR8a activation. Mean facilitation ratio increased from 1.06 ± 0.02 to 1.85 ± 0.11 (n = 11).

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L-AP4 has been extensively used as a selective group III receptor agonist. As expected, L-AP4 (1 μM) produced the characteristic voltage-dependent Ca^{2+} channel inhibition (Fig. 2C) in mmGluR8a-expressing neurons. The mean Ca^{2+} current inhibition by 1 μM L-AP4 was 54 ± 2% (n = 18). The faciliation ratio increased from 1.07 ± 0.03 to 1.90 ± 0.07 (n = 18). Both L-Glu- and L-AP4-induced Ca^{2+} current inhibition was rapid and reversible, reaching a steady-state level within ~10 s, and usually completely reversing within ~20 s upon washing the cell with control external solution (Fig. 2D).

The mmGluR8a-EGFP fusion construct was used in the majority of the present studies. To assess whether EGFP fusion affected mmGluR8a function, wild type mmGluR8a cDNA was injected in the nucleus of rat SCG neuron. 100 μM L-Glu inhibited Ca^{2+} current by 52 ± 5% (n = 4) in the wild type mmGluR8a-expressing neurons. The faciliation ration increased from 1.09 ± 0.02 to 1.97 ± 0.14 (n = 4). There was no significant difference between the EGFP fusion and wild-type mmGluR8a-mediated Ca^{2+} channel inhibition ($P > 0.05$), suggesting that fusion of EGFP to the C-terminus did not alter the coupling of mmGluR8a to N-type Ca^{2+} channels.

Rat mGluR8a has been shown to activate GIRK channels heterologously expressed in *Xenopus* oocytes (Saugstad et al., 1997), however the coupling of rat mGluR8a to the N-type Ca^{2+} channel has not been examined. Like mouse mGluR8a, activation of rat mGluR8a also produced voltage-dependent Ca^{2+} channel inhibition. 100 μM L-Glu inhibited Ca^{2+} current by 50 ± 2% (n = 8) in rmGluR8a-expressing neurons. There was no significant difference between mouse and rat mGluR8a-mediated Ca^{2+} channel inhibition ($P > 0.05$).

Inhibition of Ca^{2+} currents by activation of mGluR8b

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The rat alternative splice variant rmGluR8b differs from rmGluR8a at the last 16 amino acids of the C-terminus (Corti et al., 1998). Although mmGluR8b has not been cloned *de novo*, the alternatively spliced exon 9 responsible for rmGluR8b is found on mouse chromosome 6 and is very similar (98.0% identical) to the rat exon 9. Since the C-terminus is important for coupling of some mGluRs, such as mGluR1 and mGluR7, we sought to examine whether mGluR8b modulated N-type Ca^{2+} current in mGluR8b-expressing neurons. As in mmGluR8a-expressing neurons, L-Glu (100 μm) and L-AP4 (1 μM) inhibited the Ca^{2+} current in all the neurons injected with rat mGluR8b cDNA. Mean Ca^{2+} current inhibition in the presence of 100 μM L-Glu and 1 μM L-AP4 was 50 ± 3% (n = 13; Fig 3A) and 53 ± 2% (n=13; Fig. 3B), respectively. The Ca^{2+} channel inhibition was also voltage-dependent based on the slowing of current activation kinetics and the increased facilitation ratio. The faciliation ratio increased from 0.94 ± 0.02 to 1.59 ± 0.08 by L-Glu (n = 13) and from 1.01 ± 0.04 to 1.76 ± 0.09 by L-AP4 (n = 13).

Concentration-dependent Ca^{2+} current inhibition by L-Glu and L-AP4 **in mGluR8a/b -expressing neurons**

Figure 4A & B show the time courses of concentration-dependent Ca^{2+} current inhibition induced by L-Glu and L-AP4 in two mmGluR8a-expressing SCG neurons. Since both L-Glu and L-AP4 induced Ca^{2+} current inhibition are rapid and reversible, a series concentrations of agonists were tested in a random order for each neuron. Figure 4C depicts the concentration-response curves of Ca^{2+} current inhibition in mmGluR8a- and rmGluR8b-expressing neurons. The data points represent the mean inhibition determined from 4 to 18 cells. For either L-Glu or L-AP4, at all concentrations tested, there was no significant difference between mmGluR8a- and rmGluR8b-expressing

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neurons ($P > 0.05$). A Hill equation was fitted to the data using a nonlinear least squares algorithm. From this analysis, the maximum inhibition, IC_{50} and Hill coefficient for L-Glu in mmGluR8a and rmGluR8b–expressing neurons were 57% and 55%, 11 μM and 14 μM, 0.9 and 0.8, respectively. For L-AP4, the maximum inhibition, IC_{50} and Hill coefficient were 62% and 62%, 0.1 μM and 0.1 μM, 0.9 and 0.6, in mmGluR8a and rmGluR8b-expressing neurons, respectively. Thus, L- AP4 was ~ 100 fold more potent than L-Glu, but had a similar efficacy. The potency and efficacy of L-AP4 and L-Glu was similar for the two splice variants.

CPPG prevented L-AP4 induced Ca^{2+} channel inhibition in mGluR8a/b-expressing neurons

CPPG is a selective group II/III mGluR antagonist. 300 μM CPPG completely abolished L-Glu activated GIRK currents in *Xenopus* oocytes coinjected with rat mGluR8a and GIRK channels (Corti et al., 1998). To ascertain the pharmacological properties of the expressed receptors, CPPG was co-applied with 1 μM L-AP4. Figure 5A & C illustrates the L-AP4 induced Ca^{2+} current inhibition before and during coapplication of 1 μM CPPG in mmGluR8a- (Fig. 5A) and rmGluR8b- (Fig. 5C) expressing neurons. In all cells tested, 1 μM CPPG attenuated Ca^{2+} current inhibition in response to L-AP4 (35 ± 4%, n = 9; 18 ± 4%, n = 8, for mmGluR8a and rmGluR8b, respectively), while 10μM CPPG nearly completely abolished Ca^{2+} current inhibition (6 ± 1%, n = 8 for mmGluR8a; 1 ± 1%, n=6 for rmGluR8b). The effect of CPPG on L-AP4 induced Ca^{2+} current inhibition was reversible as L-AP4 produced ~ 50% Ca^{2+} current inhibition after washout of the antagonist (Fig. 5B). Application of either 1 μM or 10 μM CPPG alone

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has no effect on Ca^{2+} current amplitude in mmGluR8a and rmGluR8b-expressing neurons (Fig. 5C&D).

Voltage-dependent inhibition by activation of mmGluR8a and rmGluR8b receptors

The effect of voltage on L-Glu and L-AP4 induced Ca^{2+} current inhibition was characterized by generating current-voltage (I–V) relationships in the absence or presence of either 100 μM L-Glu or 1 μM L-AP4. Ca^{2+} currents were elicited from a holding potential of –80 mV with 70 ms test pulses between –120 and +80 mV. I–V curves (Fig. 6A) were normalized to the maximum Ca^{2+} current in the absence of agonist. The mean inhibition of Ca^{2+} currents produced by L-Glu and L-AP4 were plotted against membrane potential over the voltage range shown in Fig. 6B. L-Glu and L-AP4 induced maximal inhibition at test potentials that generated the largest Ca^{2+} current (i.e., around 0 mV) in both mmGluR8a and rmGluR8b-expressing neurons. The relationship between Ca^{2+} current inhibition and test pulse potentials displayed a "bell-shaped" profile, clearly indicating that the mGluR8a/b-mediated Ca^{2+} current inhibition was highly voltage-dependent.

PTX blocked mGluR8a/b – mediated Ca^{2+} channel inhibition

PTX is a useful tool for elucidating signaling pathways mediated by the $G_{i/o}$ protein. Group I mGluR inhibits Ca^{2+} channel via both $G_{i/o}$ and $G_{q/11}$-mediated pathways (Kammermeier & Ikeda, 1999). We therefore tested whether mGluR8-mediated Ca^{2+} inhibition involves a number of different G protein families as well. Unlike mGluR1, overnight pretreatment with PTX (500 ng/ml) completely abolished the glutamate (100 μM) induced Ca^{2+} current inhibition in both mmGluR8a- and rmGluR8b–expressing neurons (3 ± 1%, n = 5, Fig. 7A&C; 4 ± 1%, n = 4, Fig. 7B & C, respectively),

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suggesting that only a PTX-sensitive G-protein, i.e., $G\alpha_{i/o}$ containing G protein, was involved in mGluR8-mediated Ca^{2+} current inhibition.

Deletion of the intracellular C- terminus of mmGluR8a did not affect Ca^{2+} channel modulation

It is well accepted that N-type Ca^{2+} channel modulation is mediated by G protein βγ subunit released after activation of G protein coupled receptors. Recently O'Connor et al. (1999) proposed a novel mechanism of presynaptic Ca^{2+} channel modulation in which the binding of CaM to the C-terminus of group III mGluRs is required to release preassociated G protein βγ subunits from the mGluRs. Subsequently, the dissociated βγ subunits inhibit voltage-gated Ca^{2+} channels to control glutamate release. This model is supported by the fact that deletion of the CaM binding motif from mGluR7 and CaM antagonists prevent βγ subunit mediated modulation of GIRK channels by mGluR7. Since the same CaM binding motif was identified in the first 25 amino acid of the C-terminus of mGluR8 (El Far et al., 2001), it was anticipated that the CaM binding motif in C-terminus of mGluR8 might play a similar role in regulating Ca^{2+} channel activity. Therefore, we made a construct in which the first 26 amino acids (H844–L869) of the mmGluR8a C-terminus were deleted (defined as mmGluR8aΔC1; Fig. 8A). Unexpectedly, the deletion of the CaM-binding motif did not affect glutamate-induced Ca^{2+} channel inhibition compared with the wild-type receptor ($P > 0.05$; Fig. 8C). Application of L-Glu (100 μM) to neurons expressing mmGluR8aΔC1 produced 51 ± 5% inhibition of Ca^{2+} current ($n = 9$)–a value similar to wild type receptors. Deletion of the middle 19 (I870-S888) mmGluR8aΔC2; Fig. 8A) and the last 20 amino acids (L889-I908) mmGluR8aΔC3; Fig. 8A) of the mmGluR8a C-terminus also failed to impair the

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glutamate-induced Ca^{2+} channel inhibition. 100 μM L-Glu blocked Ca^{2+} current by 47 ± 8% (n = 4) and 53 ± 4% (n = 5) in mmGluR8aΔC2 and mmGluR8aΔC3- expressing neurons, respectively (Fig. 8C). These data imply that the CaM binding domain in mGluR8 is not crucial for mGluR8-mediated Ca^{2+} channel modulation.

The mGluR7 C-terminus has been shown to be necessary for cell surface delivery. Deletion of the mGluR7 C-terminus produces a protein that is restricted to a perinuclear intracellular compartment, probably the Golgi, and does not reach the cell surface in hippocampal neurons (McCarthy et al., 2001). However, deletion of the entire mGluR8a C-terminus (mmGluR8aΔCT; Fig. 8A) did not affect mmGluR8a-mediated Ca^{2+} channel inhibition (Fig. 8B), 100 μM L-Glu inhibited Ca^{2+} current by 58 ± 5% (n =6; Fig. 8C), which was not different from that produced by wild-type mmGluR8a ($P>0.05$), suggesting that the mGluR8a C-terminus is not crucial for the receptor membrane targeting and the coupling of the effectors.

DISCUSSION

All mGluR cDNA were originally cloned from rat, except for mGluR8, which was first cloned from a mouse retina cDNA library. The mouse cDNA clone encoding mGluR8a coupled weakly to the inhibition of adenylyl cyclase (Duvoisin et al., 1995) and failed to inhibit Ca^{2+} current in rat sympathetic neurons heterologously expressed with mouse mGluR8. However, heterologously expressed rat mGluR8a couples to GIRK channels in *Xenopus laevis* oocytes (Saugstad, et al., 1997) and N-type Ca^{2+} channel in rat sympathetic neurons (this report). The functional differences between rat and mouse mGluR8a are unlikely due to species variation since the two clones share 98.6% identity. Our experiments indicate that the reason the original mouse mGluR8a lacks function is

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due to inadequate trafficking of the receptor to the plasma membrane. It is likely that the amino acid discrepancies in the N-terminal domain in the original mouse mGluR8a account for the failure of mGluR8a delivery to the plasma membrane. The single amino acid discrepancy in the C-terminus (N905D) cannot account for the receptor retention since a chimeric receptor, where the rmGluR8b C-terminus was replaced by the C-terminus of the original mouse mGluR8, is strongly plasma membrane targeted and couples to N-type Ca^{2+} channels (data not shown). The origins of the discrepancies found in the U17252 sequence are unclear although errors introduced by the PCR seem a likely possibility. The mmGluR8a cDNA sequence determined in this report (AY673682) has a 100% identity with putative exon genomic sequence thus ruling out post-transcriptional modification.

Ca^{2+} channel inhibition is the proposed mechanism by which mGluR8 acts as autoreceptor to inhibit glutamate release. Like other group III mGluRs, mGluR8s are localized within presynpatic active zones and excluded from the soma of adult neurons. The inaccessibility of most presynaptic terminals to electrophysiological techniques makes it difficult to directly study the coupling of native mGluR8 to Ca^{2+} channels. Therefore, heterologous expression of mGluR8 by intranuclear injection of mGluR8 cDNA in an isolated adult mammalian neuron that has well-studied G protein pathways facilitates *in situ* exploration of mGluR8. In this neuronal expression system, we demonstrated that activation of mouse mGluR8a and rat mGluR8b initiates a PTX-sensitive, voltage-dependent N-type Ca^{2+} channel inhibition.

Both group I and III mGluRs have splice variants that result from the use of alternative exons coding for different C-termini (Conn & Pin, 1997). Although no

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functional differences between two variants of mGluR5 were reported (Joly et al., 1995; Minakami et al., 1994), functional differences between the variants of mGluR1 (mGluR1a and mGluR1b) were observed (Pin et al., 1992) suggesting that the long C-terminal domain of mGluR1a plays a role in receptor coupling efficiency. In our experiments, activation of either mGluR8a or mGluR8b produced a very similar Ca^{2+} channel inhibition, which is in agreement with the notion that the different C-terminal tails of the mGluR8 splice variants have minimal influence on G protein coupling efficacy (Corti et al., 1998).

L-AP4 is a potent group III receptor agonist. In *Xenopus laevis* oocytes coexpressing rat mGluR8 and GIRK, both L-AP4 and L-Glu evoked inward potassium currents, where L-AP is ~ 4 fold more potent than L-Glu (Saugstad et al., 1997). The EC_{50} values for L-AP4 and L-Glu were 0.67 μM and 2.5 μM respectively (Saugstad et al., 1997). However, in our experiments, L-AP4 is ~ 100 fold more potent than L-Glu in mGluR8-mediated Ca^{2+} channel inhibition. IC_{50} values for L-AP4 and L-Glu are 0.1 μM and 11 μM, respectively. In studies of Saugstad et al. (1997), the Hill coefficient for L-AP4 and L-Glu was different, ~1 for L-AP4 and ~2 for L-Glu, while the Hill coefficients for L-AP4 and L-Glu were similar (~1) in the present study. The discrepancy between the two studies is not clear but may due to differences in the expression conditions and channels studied.

We previously have demonstrated that group I mGluRs heterologously expressed in SCG neurons modulate both N-type Ca^{2+} and M-type K^+ channels through discrete G protein signaling pathways (Kammermeier & Ikeda, 1999). Voltage-dependent Ca^{2+} channel inhibition occurred via a PTX-sensitive $G\alpha_{i/o}$ containing G protein. Voltage-

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independent Ca^{2+} channel inhibition and M-type K^{+} channel inhibition arise from a PTX- and CTX-insensitive G protein, presumably $G\alpha_{q/11}$ containing G protein. In the present study, PTX totally abolished Ca^{2+} channel inhibition, supporting the notion that mGluR8 couples to ion channels exclusively through the $G\alpha_{i/o}$ class of heterotrimeric G-proteins.

Several studies have shown that the C-terminus in mGluR7a is involved in axon targeting and presynaptic clustering as well as binding CaM and $G\beta\gamma$ subunits. $G\beta\gamma$ and Ca^{2+}/CaM interact in a mutually exclusive way within the highly conserved first 25 amino acids of the C-terminus of mGluR7. It is proposed that activated CaM displaces pre-bound $G\beta\gamma$ from mGluR7, the "released" $G\beta\gamma$ is then available for downstream signaling, such as inhibiting N-type Ca^{2+} channel thus inhibiting glutamate release. Since the CaM binding motif is found in the C-termini of most group III mGluRs, including mGluR4a, 7a, 7b, 8a and 8b (El Far et al., 2001), one should expect that deletion of this CaM-binding motif would abolish mGluR8-mediated Ca^{2+} channel inhibition. Unexpectedly, deletion of this conserved CaM binding motif in the C-terminus of mGluR8a did not affect the glutamate-induced Ca^{2+} channel inhibition. Deletion of the middle 19 and the distal 20 amino acids of the C-terminus had no effect on the mGluR8a-mediated Ca^{2+} channel inhibition. mGluR8 may use the second intracellular loop cooperating with the other intracellular domains to couple to its effectors, like Group I mGluRs do (Gomeza et al., 1996). Since the homology of the middle and the distal portion of the C terminus of mGluR7 and mGluR8 is not very high compared with the proximal portion of the C-terminus where CaM binding site is located, the two type receptors may use two different mechanism to fulfill their function. On the other hand, although CaM binding site in the mGluR7 C-terminus is required for coupling of GIRK

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channels in HEK 293 cells, the involvement of the mGluR7 CaM binding site in N-type Ca^{2+} channel modulation remains to be determined. Without the C-terminus, mGluR7 is trapped in perinuclear compartment in cultured hippocampal neurons and cannot be delivered to the membrane (McCarthy et al., 2001), however the C-terminus of mGluR8a may not be necessary for plasma membrane targeting, at least in our mammalian neuronal expression system, as mGluR8aΔCT still coupled to G proteins and induced Ca^{2+} channel inhibition. Further experiments to quantify the expression level of mGluR8a with different deletions of C-terminus will help determine the role of mGluR8a C-terminus in the membrane trafficking.

In summary, our results show both mGluR8a and mGluR8b are capable of eliciting voltage-dependent, PTX-sensitive N-type Ca^{2+} channel inhibition, suggesting a role as autoreceptors in the presynaptic site to regulate neuronal excitability. Despite the differences in the C-terminal tails, mGluR8a and mGluR8b have very similar pharmacological profiles in terms of Ca^{2+} channel modulation. Finally neither CaM binding domain in the C-terminus nor the entire C-terminus of mGluR8a is required for receptor coupling to N-type Ca^{2+} channels.

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Figure legends

Fig. 1. Newly cloned mouse mGluR8a (AY673682) differs in sequence and expression pattern from U17252. A, the alignment predicted translation products from nucleotide sequences AY673682 with U17252 shows eight amino acid differences (marked as red). The two residues affected by sequencing errors in U17252 are enclosed with boxes. The amino acid sequence of mouse mGluR8a (AY673682) shows a high identity with rat mGluR8a (NM022202), the differences were marked as blue. The putative signal sequence and the seven transmembrane domains are indicated with bars (Saugstad et al., 1997; Malherbe et al., 1999). B & C, expression of the EGFP-tagged constructs of U17252 (*B*) and AY67382 (*C*) in HEK 293 cells shows different patterns of expression . Expression of the original mouse clone (U17252) revealed a lace-like pattern consistent with retention of protein in the endoplasmic reticulum. Conversely, expression of AY673682 revealed a rim-like fluorescence pattern consistent with plasma membrane targeting.

Fig. 2. Both L-Glu and L-AP4, a selective agonist for group III mGluRs, inhibits Ca^{2+} current in rat SCG neurons expressing mmGluR8a. A , B & C, superimposed Ca^{2+} current traces evoked with the double-pulse voltage protocol (*bottom of A*) in the absence and presence of 100 μM L-Glu and 1 μM L-AP4 from a control (*A*) and mmGluR8a expressing (*B & C*) neurons. Currents were evoked every 10 sec. The dashed lines indicate the zero current level. D, time courses of the Ca^{2+} current amplitudes (*upper panel*) and facilitation ratio (*lower panel*) for a mmGluR8a expressing neuron. Ca^{2+} current was measured 10 ms after initiation of the test pulse (+10 mV). Facilitation ratio (post/pre) was calculated as the ratio of Ca^{2+} current amplitude determined from the test

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pulse (+10 mV) occurring after (postpulse, open circles) and before (prepulse, filled circles) the +80 mV conditioning pulse. The filled bars indicate drug applications.

Fig. 3. Ca^{2+} current inhibition in SCG neurons expressing rat mGluR8b. A, superimposed Ca^{2+} current traces evoked from a neuron expressing rmGluR8b in the absence or presence of 100 μM L-Glu. The dashed lines indicate the zero current level. B, superimposed Ca^{2+} current traces evoked from a neuron expressing rmGluR8b in the absence or presence of 1 μM L-AP4. The dashed lines indicate the zero current. The measurement of Ca^{2+} current is illustrated in Figure 1A.

Fig. 4. Dose-dependent Ca^{2+} current inhibition in mmGluR8a or rmGluR8b expressing neurons. A & B, time courses of the Ca^{2+} current amplitudes (*upper panel, A &B*) and facilitation ratio (*lower panel, A &B*) for two mmGluR8a expressing neurons. Ca^{2+} current was measured 10 ms after initiation of the test pulse (+10 mV). Facilitation ratio (post/pre) was calculated as the ratio of Ca^{2+} current amplitude determined from the test pulse (+10 mV) occurring after (postpulse, open circles) and before (prepulse, filled circles) the +80 mV conditioning pulse. The filled bars indicate drug applications. C. The smooth curves were obtained by fitting data to a Hill equation. Data are plotted as the mean ± SEM. Each point on the dose–response curves represents the mean inhibition from 4 to 18 cells.

Fig. 5. CPPG, a selective group II/III mGluRs antagonist, significantly reduces Ca^{2+} channel inhibition mediated by mmGluR8a and rmGluR8b. A & C, superimposed Ca^{2+} current traces evoked from neurons expressing mmGluR8a (*A*) or rmGluR8b (*C*) in the absence or presence of 1μM L-AP4 , and in the presence of both 1 μM L-AP4 and 1 μM CPPG. B, time course of the Ca^{2+} current amplitudes (*upper panel*) and facilitation ratio

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(*lower panel*) for a mmGluR8a expressing neuron. Ca^{2+} current was measured 10 ms after initiation of the test pulse (+10 mV). Facilitation ratio (post/pre) was calculated as the ratio of Ca^{2+} current amplitude determined from the test pulse (+10 mV) occurring after (postpulse, open circles) and before (prepulse, filled circles) the +80 mV conditioning pulse. The solid bars indicate drug applications. D, summary graph of mean ± SEM Ca^{2+} current inhibition by 1 μM L-AP4, 1 μM L-AP4 + 1 μM CPPG, 1 μM CPPG, 1 μM L-AP4 + 10 μM CPPG, 10 μM CPPG from neurons expressing mmGluR8a or rmGluR8b. Ca^{2+} current inhibition was measured isochronally 10 ms after initiation of the test pulse (+10 mV) in the absence or presence of drugs.

Fig. 6. Voltage-dependence of mGluR8a/b–mediated Ca^{2+} channel inhibition by L-Glu and L-AP4. A, normalized current-voltage (I-V) relationship derived from currents of mmGluR8a- or rmGluR8b-expressing neurons in the absence or presence of 100 μM L-Glu or 1 μM L-AP4. Ca^{2+} currents were elicited with 70-ms voltage steps in 5 or 10 mV increments from –120 mV to + 80 mV from a holding potential of –80 mV. The Ca^{2+} current at each voltage step was normalized to the Ca^{2+} current elicited by the depolarizing step to +5 mV in the absence of drugs. B, the mean percentage inhibition of Ca^{2+} currents was plotted against each voltage step to demonstrate the voltage-dependent Ca^{2+} channel inhibition by 100 μM L-Glu or 1 μM L-AP4.

Fig. 7. PTX blocked mGluR8a/b–mediated Ca^{2+} current inhibition. A & B, superimposed Ca^{2+} current traces evoked from mmGluR8a- (*A*) or rmGluR8b- (*B*) expressing neurons pretreated with PTX in the absence or presence of 1 μM L-AP4. C, summary graph of mean ± SEM Ca^{2+} current inhibition by 1 μM L-AP4 from mGluR8a/b expressing neurons with or without treatment of PTX. Ca^{2+} current inhibition was measured

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isochronally 10 ms after initiation of the test pulse (+10 mV) in the absence or presence of drugs.

Fig. 8. Deletion of the intracellular C-terminal tail of mmGluR8a did not affect Ca^{2+} channel modulation. A, schematic illustrating the portions of the mmGluR8a C-terminus deleted in different constructs as labeled. B, superimposed Ca^{2+} current traces evoked from a neuron expressing the mmGluR8aΔCT construct in the absence or presence of 100 μM L-Glu. C, the summary bar graph shows the L-Glu (100 μM) induced Ca^{2+} channel inhibition for neurons injected with wild-type mmGluR8a, mmGluR8aΔC1, mmGluR8aΔC2, mmGluR8aΔC3, or mmGluR8aΔCT constructs. Ca^{2+} current inhibition was calculated as described in Figure 2A. Numbers in parentheses indicate the number of neurons tested.

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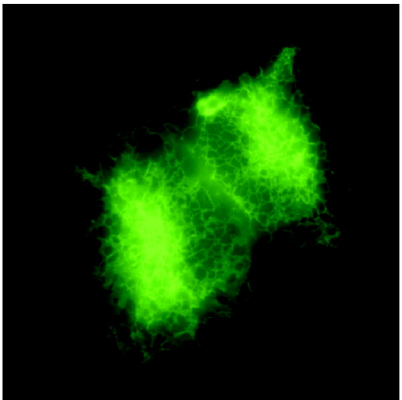
A

```
mouse(AY673682)  1  MVCEGKRSTS CPCFFLLTAK FYWILTMMQR THSQEYAHSI RLDGDIILGG
mouse(U17252)          ST                                       L
rat   (UM022202)       LA                                       V
                51  LFPVHAKGER GVPCGDLKKE KGIHRLEAML YAIDQINKDP DLLSNITLGV
                                   D                    T
                                   E                    I
               101  RILDTCSRDT YALEQSLTFV QALIEKDASD VKCANGDPPI FTKPDKISGV

               151  IGAAASSVSI MVANILRLFK IPQISYASTA PELSDNTRYD FFSRVVPPDS

               201  YQAQAMVDIV TALGWNYVST LASEGNYGES GVEAFTQISR EIGGVCIAQS

               251  QKIPREPRPG EFEKIIKRLL ETPNARAVIM FANEDDIRRI LEAAKKLNQS
                                                    G
                                                    R
               301  GHFLWIGSDS WGSKIAPVYQ QEEIAEGAVT ILPKRASIDG FDRYFRSRTL

               351  ANNRRNVWFA EFWEENFGCK LGSHGKRNSH IKKCTGLERI ARDSSYEQEG
                               S G     S
                               W E     L
               401  KVQFVIDAVY SMAYALHNMH KELCPGYIGL CPRMVTIDGK ELLGYIRAVN
                                        L
                                        R
               451  FNGSAGTPVT FNENGDAPGR YDIFQYQINN KSTEYKIIGH WTNQLHLKVE

               501  DMQWANREHT HPASVCSLPC KPGERKKTVK GVPCCWHCER CEGYNYQVDE
                                                    G
                                                    E
               551  LSCELCPLDQ RPNINRTGCQ RIPIIKLEWH SPWAVVPVFI AILGIIATTF
                                                      L
                                                      F
               601  VIVTFVRYND TPIVRASGRE LSYVLLTGIF LCYSITFLMI AAPDTIICSF

               651  RRIFLGLGMC FSYAALLTKT NRIHRIFEQG KKSVTAPKFI SPASQLVITF

               701  SLISVQLLGV FVWFVVDPPH TIIDYGEQRT LDPENARGVL KCDISDLSLI

               751  CSLGYSILLM VTCTVYAIKT RGVPETFNEA KPIGFTMYTT CIIWLAFIPI

               801  FFGTAQSAEK MYIQTTTLTV SMSLSASVSL GMLYMPKVYI IIFHPEQNVQ

               851  KRKRSFKAVV TAATMQSKLI QKGNDRPNGE VKSELCESLE TNTSSTKTTY

               901  ISYSNHSI*
                    D
                    N
```

B



C

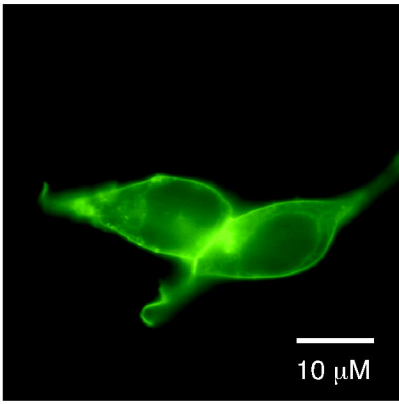


Fig. 2

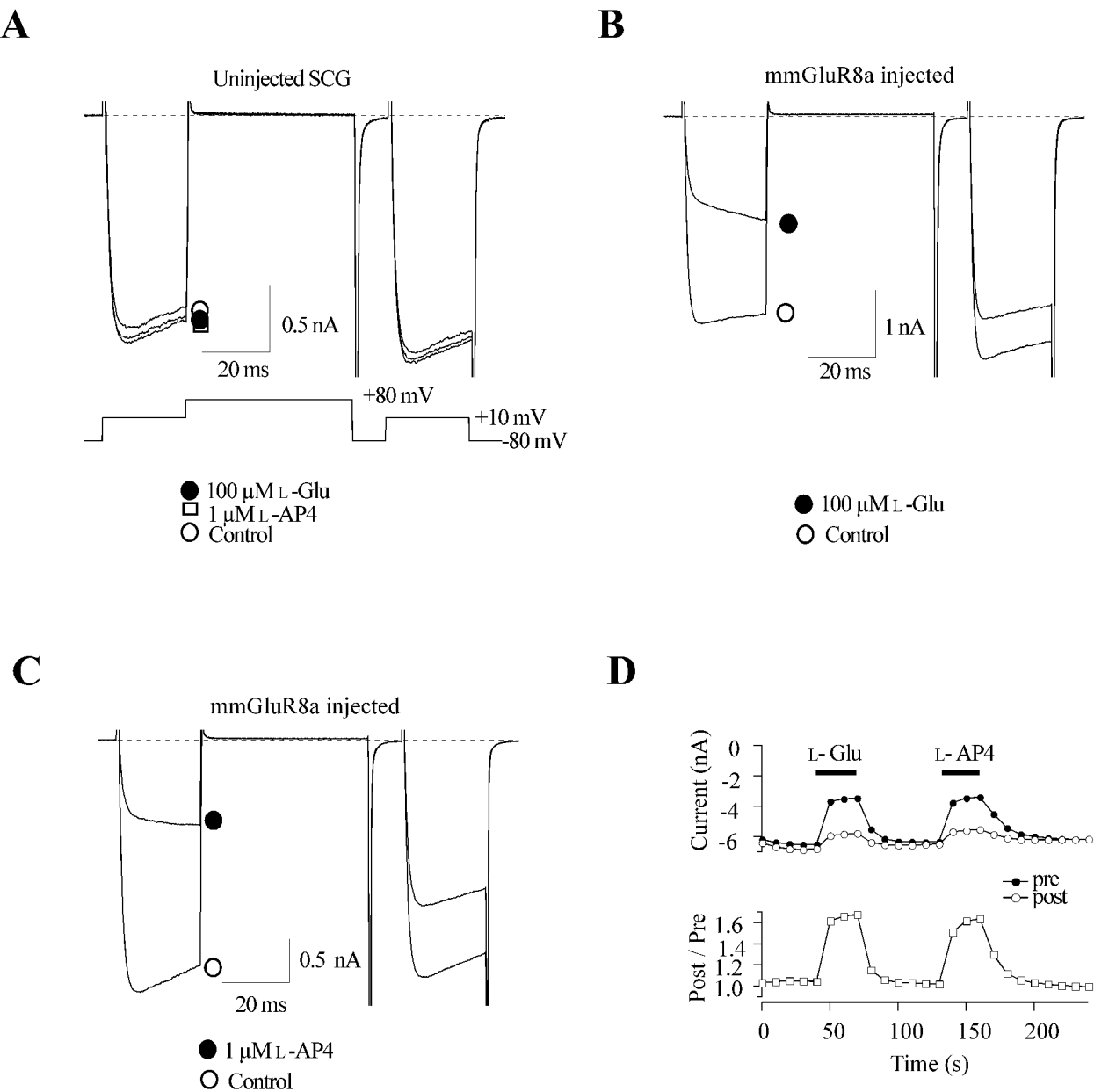
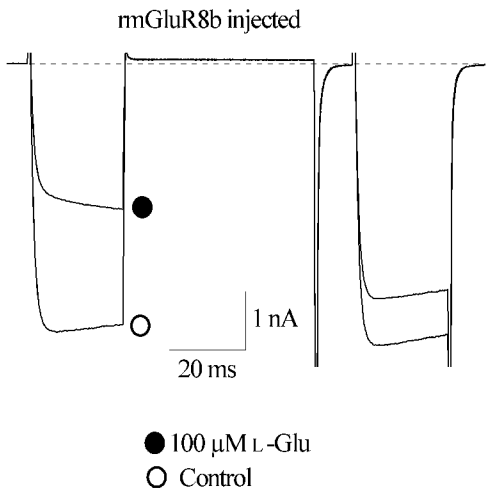

A
Uninjected SCG
0.5 nA
20 ms
+80 mV
+10 mV
-80 mV
100 µM L-Glu
1 µM L-AP4
Control
B
mmGluR8a injected
1 nA
20 ms
100 µM L-Glu
Control
C
mmGluR8a injected
0.5 nA
20 ms
1 µM L-AP4
Control
D
Current (nA)
0
-2
-4
-6
L- Glu
L- AP4
pre
post
Post / Pre
1.6
1.4
1.2
1.0
0
50
100
150
200
Time (s)

Fig. 3

A


rmGluR8b injected
1 nA
20 ms
100 µM L-Glu
Control

B

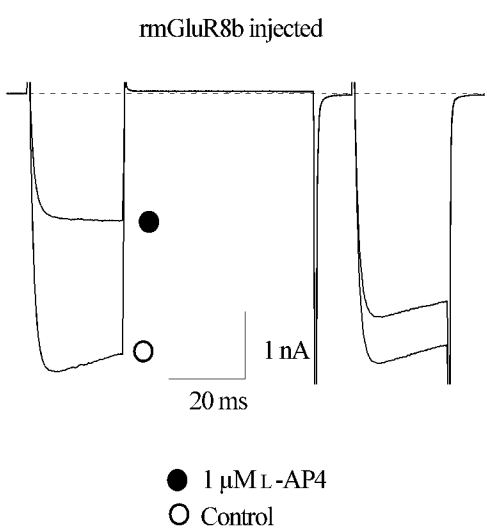
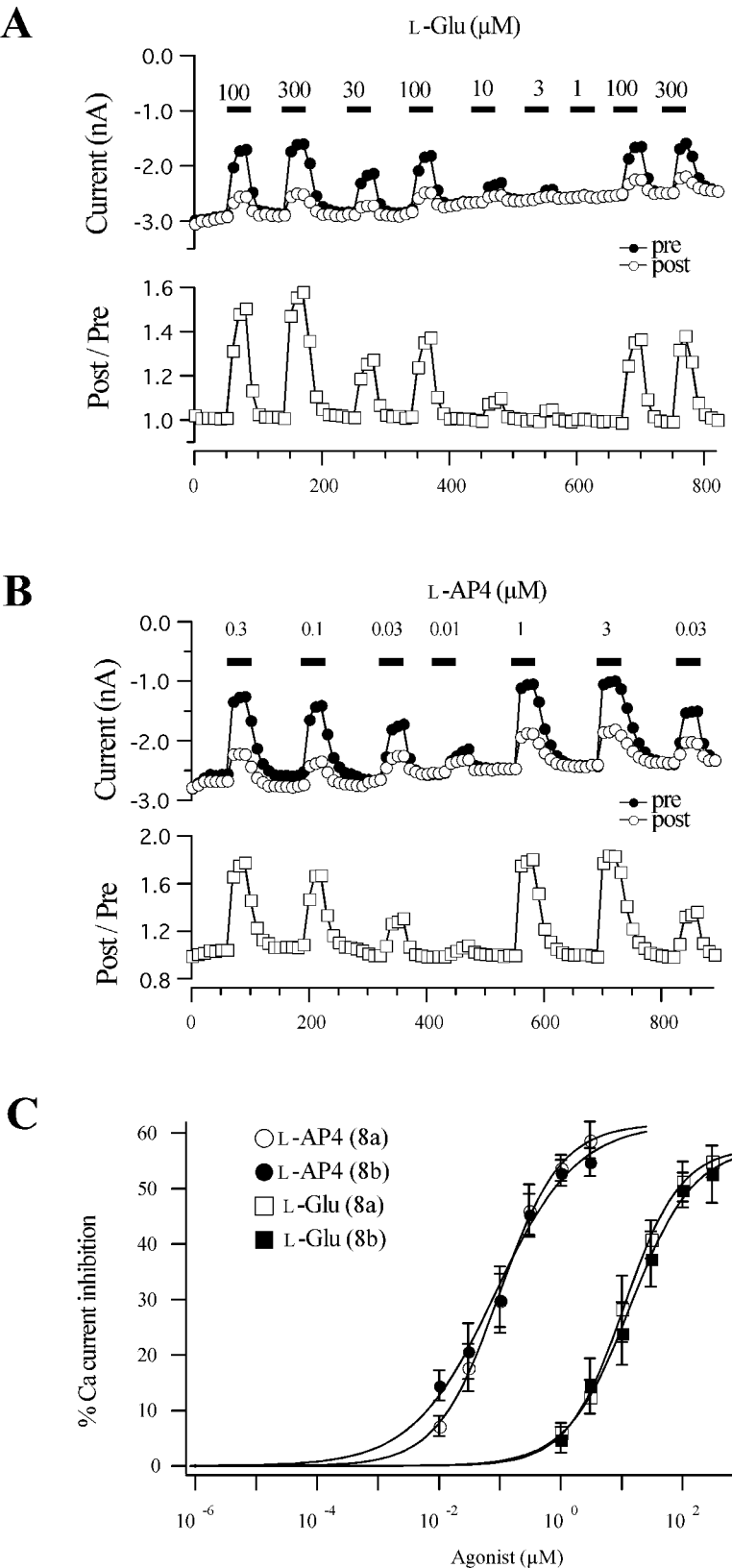

rmGluR8b injected
1 nA
20 ms
1 µM L-AP4
Control

Fig. 4


A
L-Glu (μM)
100
300
30
100
10
3
1
100
300
Current (nA)
pre
post
Post / Pre
B
L-AP4 (μM)
0.3
0.1
0.03
0.01
1
3
0.03
Current (nA)
pre
post
Post / Pre
C
L-AP4 (8a)
L-AP4 (8b)
L-Glu (8a)
L-Glu (8b)
% Ca current inhibition
Agonist (μM)

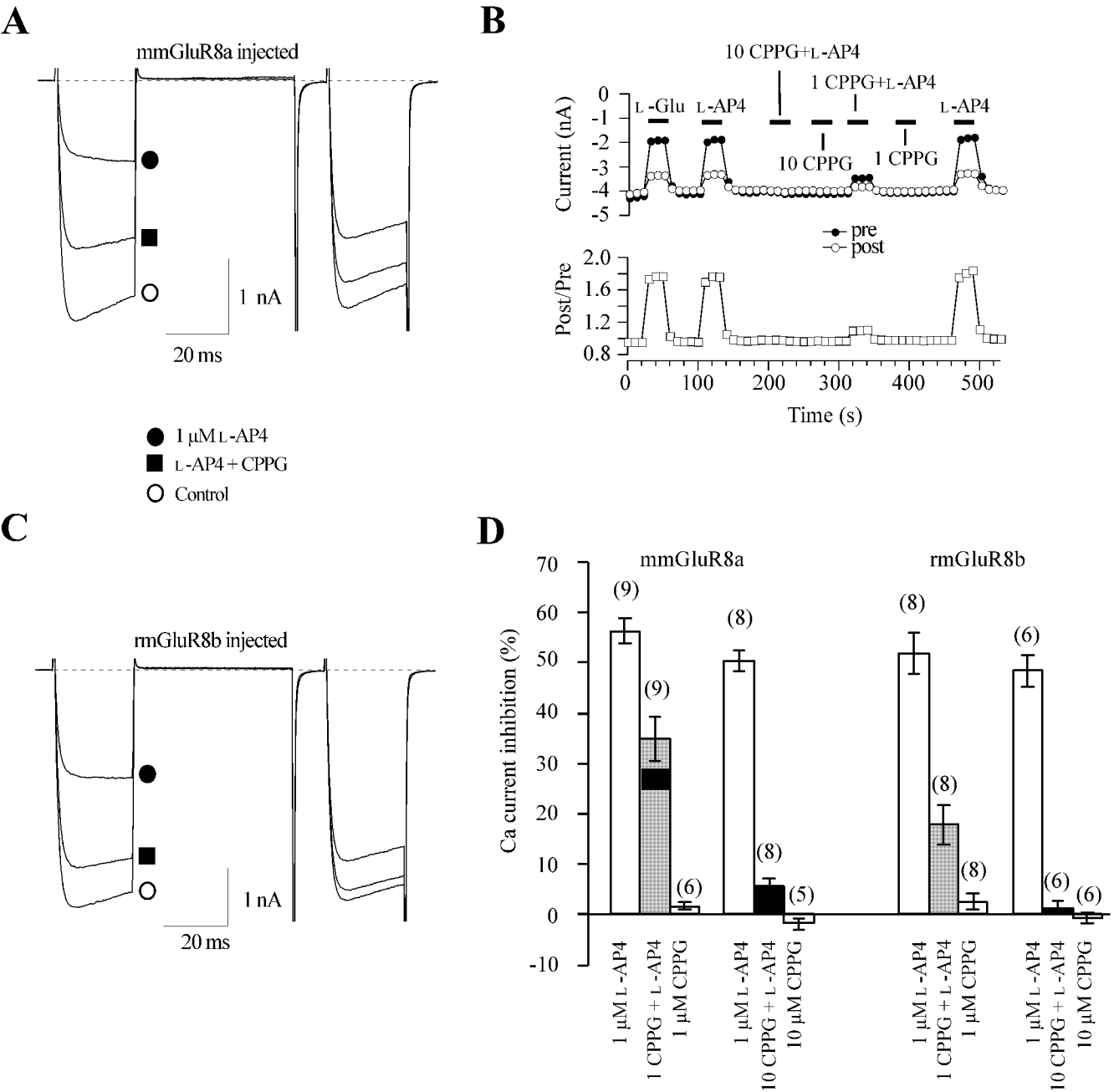
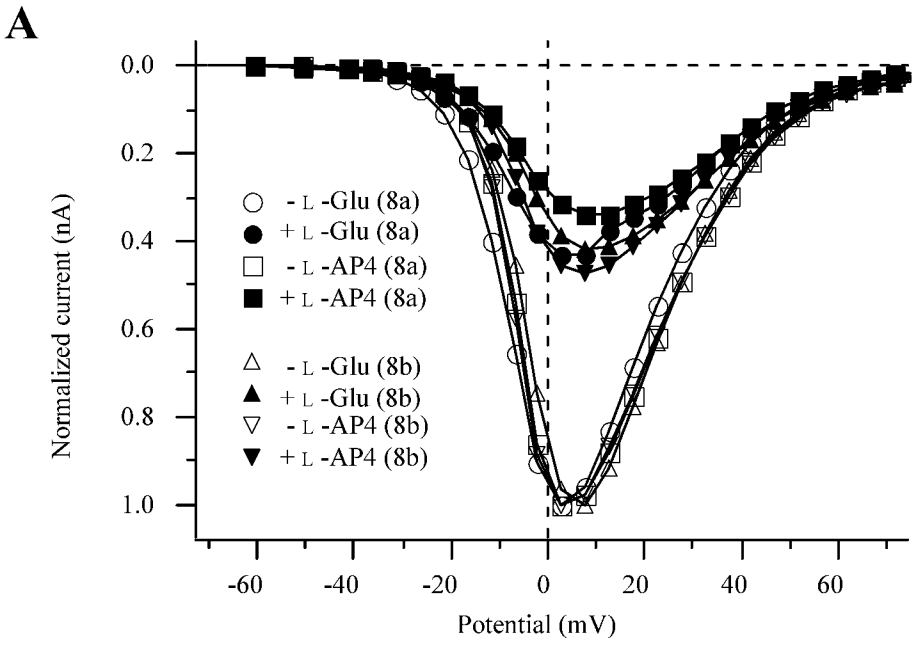

A
mmGluR8a injected
1 nA
20 ms
1 μM L-AP4
L-AP4 + CPPG
Control
B
10 CPPG+L-AP4
1 CPPG+L-AP4
L-Glu
L-AP4
L-AP4
10 CPPG
1 CPPG
Current (nA)
pre
post
Post/Pre
Time (s)
C
rmGluR8b injected
1 nA
20 ms
D
mmGluR8a
rmGluR8b
Ca current inhibition (%)
1 μM L-AP4
1 CPPG + L-AP4
1 μM CPPG
10 CPPG + L-AP4
10 μM CPPG

Fig. 6


A
0.0
0.2
0.4
0.6
0.8
1.0
Normalized current (nA)
- L -Glu (8a)
+ L -Glu (8a)
- L -AP4 (8a)
+ L -AP4 (8a)
- L -Glu (8b)
+ L -Glu (8b)
- L -AP4 (8b)
+ L -AP4 (8b)
-60
-40
-20
0
20
40
60
Potential (mV)

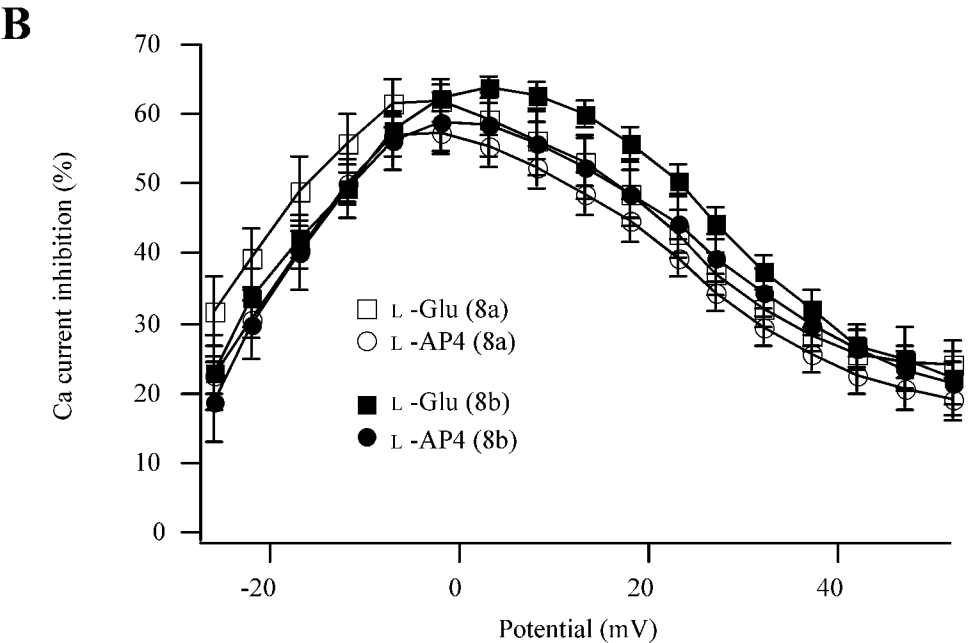

B
70
60
50
40
30
20
10
0
Ca current inhibition (%)
L -Glu (8a)
L -AP4 (8a)
L -Glu (8b)
L -AP4 (8b)
-20
0
20
40
Potential (mV)

Fig. 7

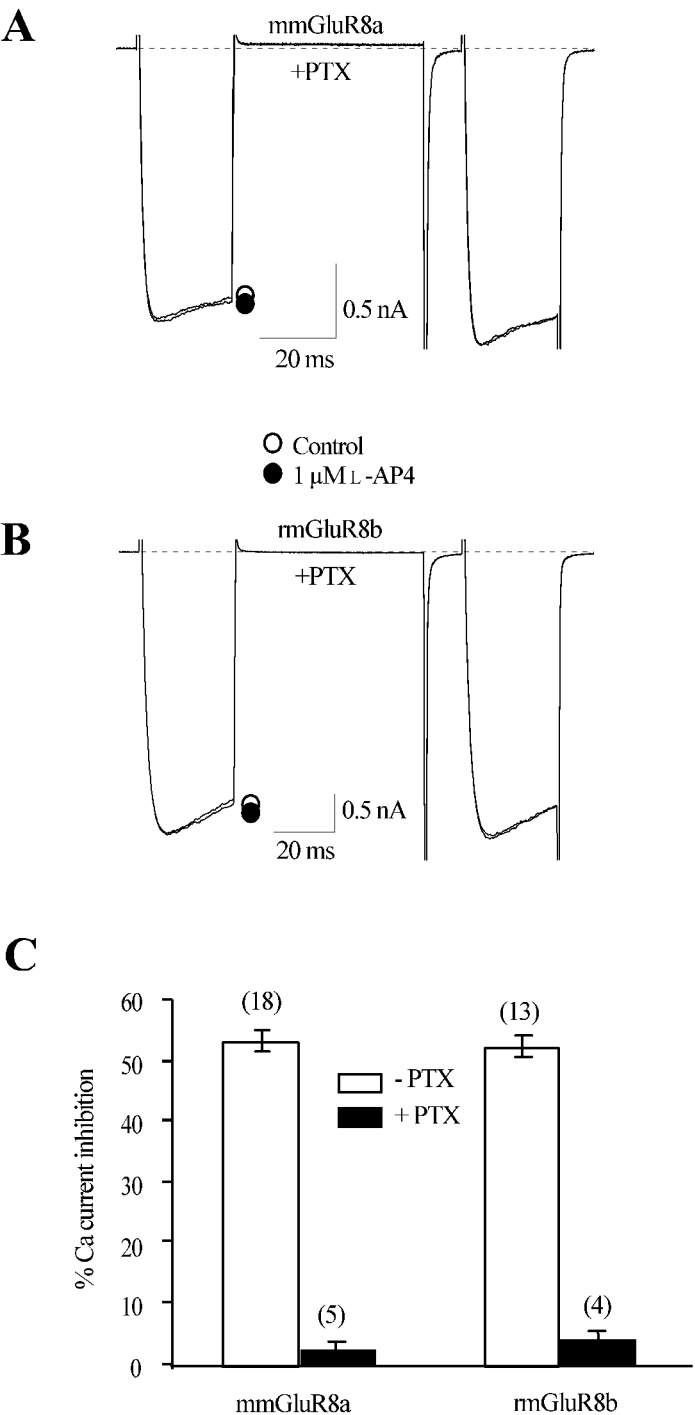
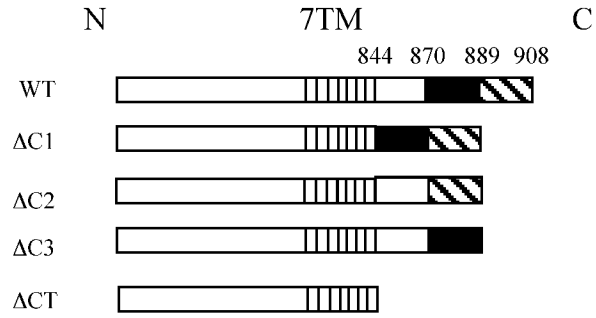
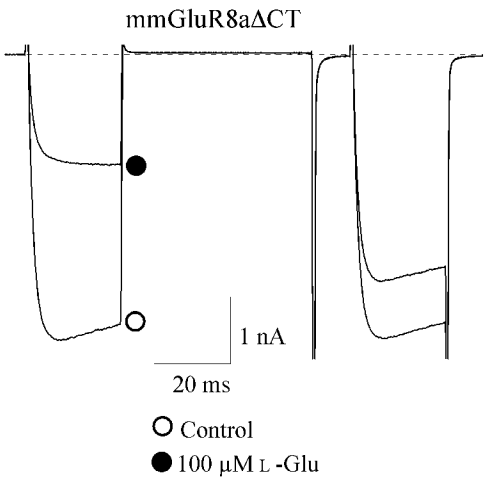

A
mmGluR8a
+PTX
0.5 nA
20 ms
Control
1 μM L-AP4
B
rmGluR8b
+PTX
0.5 nA
20 ms
C
% Ca current inhibition
60
50
40
30
20
10
0
(18)
(5)
(13)
(4)
- PTX
+ PTX
mmGluR8a
rmGluR8b

Fig. 8

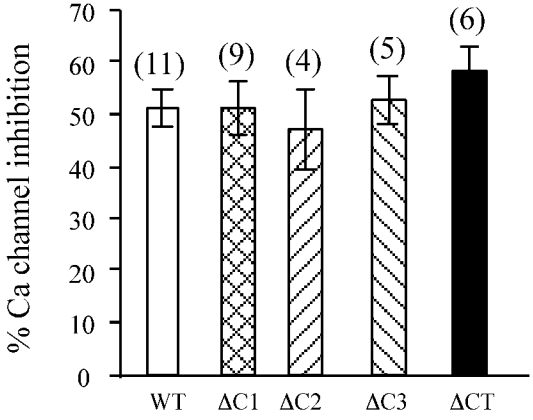
A


N
7TM
C
844 870 889 908
WT
ΔC1
ΔC2
ΔC3
ΔCT

B


mmGluR8aΔCT
1 nA
20 ms
Control
100 μM L-Glu

C


% Ca channel inhibition
70
60
50
40
30
20
10
0
(11)
(9)
(4)
(5)
(6)
WT
ΔC1
ΔC2
ΔC3
ΔCT