Cloning and Functional Expression of a *Drosophila* Metabotropic Glutamate Receptor Expressed in the Embryonic CNS

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The excitatory neurotransmitter glutamate plays important roles in the mammalian brain, ranging from synaptic plasticity to memory. To mediate these functions, glutamate activates two types of receptors: ligand-gated channels and metabotropic receptors coupled to G-proteins. Both families of glutamate receptors share no sequence homology and possess original structural features compared with other ligand-gated channels and G-protein-coupled receptors, respectively. Glutamate-gated receptor-channel subunits have already been characterized in invertebrates. Here we report the cloning and functional characterization of an invertebrate metabotropic glutamate receptor (DmGluRA) isolated from *Drosophila melanogaster*. This receptor displays 45 and 43% amino acid sequence identity with its mammalian homologs mGluR3 and mGluR2, respectively. Moreover, its pharmacology and transduction mechanisms are surprisingly similar to those of mGluR2 and mGluR3. DmGluRA is expressed in the CNS of the late embryo. These results indicate that the original structural features of both glutamate receptor types are conserved from insects to mammals and suggest that the functions of these receptors have been highly conserved during evolution.

Key words: CNS; Drosophila melanogaster*; glutamate; G-protein coupling; metabotropic glutamate receptor; embryogenesis; adenylyl cyclase inhibition*

Glutamate is the major excitatory neurotransmitter in the vertebrate CNS. To mediate fast synaptic transmission, it activates AMPA- and NMDA-selective ionotropic receptors (Hollmann and Heinemann, 1994), the latter playing important roles in synaptic plasticity, neurotoxicity, development, and memory formation. In addition, glutamate activates G-protein-coupled metabotropic receptors (mGluRs), which can modulate or mediate neurotransmission and play a role in long-term changes in synaptic efficacy (Pin and Duvoisin, 1995). This makes them essential for functions as diverse and important as developmental plasticity (Dudek and Bear, 1989; Catania et al., 1994), vision (Masu et al., 1995), olfactory memory (Hayashi et al., 1993; Kaba et al., 1994), spatial memory, and motor control (Aiba et al., 1994; Conquet et al., 1994). Eight mGluRs (mGluR1-8) have been cloned in vertebrates (Nakanishi, 1994; Pin and Duvoisin, 1995). They can be classified into three groups based on their amino acid sequence identity, pharmacology, and transduction mechanism. mGluR1 and mGluR5 are coupled to phospholipase C (PLC) and constitute the first group. mGluR2/3 and mGluR4, -6, -7, and -8

inhibit adenylyl cyclase (AC) and constitute the second and third groups, respectively.

Both ionotropic and metabotropic glutamate receptors share no sequence and structural homology with the other ligand-gated channels and G-protein-coupled receptors (GPCRs), respectively (Hollmann and Heinemann, 1994). Indeed, metabotropic glutamate receptors constitute, with the calcium-sensing receptor (Brown et al., 1993), a new family of GPCRs. Their ligand binding site is located in a large N-terminal extracellular domain homologous to bacterial periplasmic binding proteins (O'Hara et al., 1993), whereas for most other GPCRs it is located in a cavity formed by the seven transmembrane domains. Moreover, the specificity of interaction with G-proteins is determined by the second intracellular loop in mGluRs (Pin et al., 1994; Gomeza et al., 1996a) and by the third intracellular loop in most other GPCRs (Liu et al., 1995).

In arthropods, glutamate is well known as the transmitter at the neuromuscular junction (Jan and Jan, 1976; Delgado et al., 1989). It is also recognized as a transmitter in the invertebrate CNS (Bicker et al., 1988), where it has both excitatory and inhibitory actions (Bolshakov et al., 1991; Kehoe, 1994; Parker, 1994). Indeed, subunits of glutamate-gated cationic channel receptors have been cloned in nematodes (Hart et al., 1995; Maricq et al., 1995), in *Lymnea* (Hutton et al., 1991; Stühmer et al., 1996), and in *Drosophila* (Schuster et al., 1991; Ultsch et al., 1992, 1993). These cationic receptor subunits are homologous to the mammalian ionotropic glutamate receptor subunits. In contrast, an anionic glutamate-gated receptor cloned from *Caenorhabditis elegans* (Cully et al., 1994) is homologous to the mammalian GABA/glycine receptor subunits. The *Drosophila* subunit DGluRI, the molluscan kainate-type receptor, and the nematode AMPA-type receptor GLR-1 are expressed in the CNS, whereas the *Drosophila* receptor subunit DGluRII is expressed in muscle cells. Mutations in GLR-1 lead to abnormal mechanosensory signaling (Hart

et al., 1995; Maricq et al., 1995). Moreover, a sequence homologous to mammalian mGluRs has been found by systematic sequencing of *C. elegans* genome (Wilson et al., 1994). Taken together, these data suggest important functions for glutamate in invertebrates.

Here we report the isolation of a cDNA encoding a *Drosophila* metabotropic glutamate receptor, called DmGluRA, homologous to the mammalian mGluR family and especially to group II mGluRs. In mammalian-transfected cells, DmGluRA behaves as a functional glutamate receptor negatively coupled to AC. Its pharmacology is very close to that of mGluR2. It is expressed in the adult and in the CNS of the *Drosophila* late embryo.

MATERIALS AND METHODS

Materials. AMPA, L-quisqualate, (2S,3S,4S)-α-(carboxycyclopropyl)-glycine (L-CCG-I), (2S, 3S, 4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG-I), (S)-4-carboxy-3-hydroxyphenylglycine (4C3HPG), (S)-3-hydroxyphenylglycine (3HPG), (RS)-3,5-dihydroxyphenylglycine (DHPG), L-2-amino-4-phosphonobutanoate (L-AP4), (S)-2-amino-2-methyl-4-phosphonobutyrate (MAP4), L-serine-o-phosphate (L-SOP), (RS)-α-methyl-4-phosphonophenylglycine (MPPG), (RS)-α-methyl-4-carboxyphenylglycine (MCPG), (RS)-α-methyl-4-tetrazolylphenylglycine (MTPG), and (RS)-α-methyl-4-sulfonophenylglycine (MSPG) were obtained from Tocris Cookson (Essex, UK). The two isomers 1S,3S-1-amino-1,3-cyclopentanedicarboxylate (1S,3S-ACPD) and 1S,3R-1-amino-1,3-cyclopentanedicarboxylate (1S,3R-ACPD) were a generous gift of Dr. K. Curry (Vancouver, Canada). NMDA, glutamate, and kainic acid were from Sigma-Aldrich (L'isle d'Abeau, France).

Cloning of DmGluRA. A set of primers was used to amplify the cDNA fragments coding for the transmembrane region. The sense primer aMDM55 (5′-TA(C/T) AG(C/T) GT(G/A/T/C) CT(A/G) CTI AC(G/A/T/C) AA(A/G) AC-3′) and the antisense primer aMDM43 (5′-CC(A/G) AT(G/A/T/C) AT(A/G) CAI GTI GT(A/G) TAC AT-3′) correspond to the consensus sequences found in transmembrane domains III and VI. A cDNA library (gift from L. Jan, University of California San Francisco, San Francisco, CA) prepared from heads of wild-type Oregon R *Drosophila* was used as a template. A band of 480 bp was obtained after 40 cycles of amplification (denaturation at 94°C for 1 min, annealing at 52°C for 45 sec, and elongation at 72°C for 1 min). After subcloning into the pBluescript vector, the PCR fragments were sequenced using the dideoxy technique. One clone homologous to mGluRs was obtained and random prime-labeled to screen the cDNA library at high stringency (42°C, 50% formamide, 5× SSC, 5× PAF, 0.5% SDS, and 20 μg/ml salmon sperm DNA).

Sequence of the 3358 bp DmGluRA cDNA was performed on both strands by the dideoxy technique using successive synthetic oligonucleotides.

Construction of DmGluRA expression vector. The *Bam*HI–*Asn* I fragment of DmGluRA was inserted into the expression vector pRK7 digested with *Bam*HI, with *Sma*I giving the pRKDGAs clone.

DmGluRA Kosak sequence was modified by PCR to fit the mammalian consensus sequence (Cavener, 1987). Sense primer was (5′-CGG GGA T*CC ACC* ATG AAA CAG AAA AAT AAT AAC-3′) with a *Bam*HI restriction site, and antisense primer was (5′-CGT AAA AAG AAC AAC GCC G-3′). The PCR product and the pRKDGAs clone were digested with *Bam*HI and *Ppu*M I, gel-purified, and ligated. The resulting recombinant was called KosDMGRAs.

Culture and transfection of HEK 293 cells. Cells were cultured in DMEM (Life Technologies, Gaithersburg MD) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin, 100 U/ml final). Electroporation was performed in a total volume of 300 μl with 12.5 μg of carrier DNA (plasmid DNA without insert), 5 μg of KosDMGRAs with 6 μg of Gqi9, Gqi5, or Gqwt (in pCDNAI, Invitrogen, San Diego, CA), or with 600 ng luteinizing hormone (LH) receptor (in pRK8) for 1×10^7 cells in electroporation buffer (K_2HPO_4, 50 mM; CH3COOK, 20 mM; KOH, 20 mM). After electroporation (270 V, 950 μF, Biorad gene pulser electroporator), cells were resuspended in DMEM supplemented with 10% FCS and antibiotics and split into 12-well clusters (5×10^6 cells per cluster) precoated with poly-L-ornithine [15 μg/ml; molecular weight (MW) 40,000; Sigma-Aldrich].

Determination of cAMP accumulation. The cellular cAMP production was measured using the prelabeling technique. Four hours after being electroporated, cells were washed and incubated for 14 hr in DMEM glutamax I (Life Technologies, Cergy Pontoise, France) containing 1 μCi/ml [³H] adenine (27 Ci/mmol) and pertussis toxin (PTX) (100 ng/ml) when indicated. Cells were then washed three times and incubated for 1.5 hr at 37°C in 1 ml of HEPES buffer saline (in mM): 146 NaCl, 4.2 KCl, 0.5 MgCl₂, 20 HEPES, and glucose 0.1%, pH 7.4. Cells were then washed again twice with the same medium, incubated for 8 min with 0.75 mM 3-isobutyl-1-methyl-xanthine (Sigma), and stimulated for 10 min with 100 ng/ml LH (Sigma) or 1 mM Glu or both. The reaction was stopped by aspiration of the media and addition of 1 ml of ice-cold 5% trichloroacetic acid. Cells were scraped, and 100 μl of 10 mM ATP and 10 mM cAMP were added to the mixture. Cellular proteins were removed by centrifugation at $5000 \times g$, and [³H]-ATP and [³H]-cAMP were separated by sequential chromatography on Dowex and alumina columns. cAMP formation is expressed as percentage conversion of [³H]-ATP to [³H]-cAMP: ([³H]-cAMP × 100)/([³H]-cAMP + [³H]-ATP).

Determination of inositol phosphate (IP) accumulation. Determination of IP accumulation in transfected cells was performed as described previously (Joly et al., 1995). Four hours after electroporation, cells were washed and incubated for 14 hr in DMEM-glutamax I containing 1 μCi/ml *myo*-[³H]inositol (22.9 Ci/mmol). Cells were then washed three times and incubated in HEPES saline buffer for 1 hr with 3 μg/ml glutamate pyruvate transaminase (Boehringer Mannheim, Meylan, France) and 2 mM pyruvate. Cells were then washed twice and incubated for 5 min in medium containing 10 mM LiCl. Agonists were then added for a period of 30 min. Antagonists were added just before agonists during the LiCl preincubation. The reaction was stopped by replacing the incubation medium with 1 ml 5% perchloric acid. The total IP was then extracted and purified on Dowex columns as described previously (Joly et al., 1995). Total radioactivity remaining in the membrane fraction was counted after solubilization in 10% Triton 0.1N NaOH and used as standard. Results are expressed as the amount of IP produced over the radioactivity present in the membranes.

RT-PCR of DmGluRA. Total RNA was extracted from *Drosophila* by the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). PolyA RNA was obtained with the RNA Flash Kit (Bioprobe Systems, Montreuil, France). One microgram of polyA RNA was reverse-transcribed with 50 μ M-MLV reverse transcriptase (Life Technologies) in a 20 μl reaction containing 100 pmol of nonamer random primers (Bioprobe Systems, Montreuil, France), 1 mM dNTPs, and 10 mM DTT in the reaction buffer supplied by the manufacturer. The PCR reaction was performed using 2 μl of the RT reaction with the sense primer dec 8s (5′-ATG CGA CGA TAG TTA CCG G-3′) and the antisense primer dec 18r (5′-ACC TAC TTC GGC CTG TTC A-3′). Reaction was as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 45 sec, and elongation at 72°C for 1 min. The PCR products were subcloned into pGEM-T vector (Promega, Charbonnières, France) and sequenced. A Southern blot of these PCR products was realized at high stringency (42°C, 50% formamide, 5× SSC, 5× PAF, 0.5% SDS, and 20 μg/ml salmon sperm DNA) using a radiolabeled DmGluRA probe.

Whole-mount embryo in situ *hybridization.* DmGluRA transcripts were localized by whole-mount *in situ* hybridization with a digoxygenin-labeled probe (Lehmann and Tautz, 1994). Two hundred nanograms of the *Eco*RI fragment of DmGluRA were amplified with 0.6 mM of a mix of 6 sense or 6 antisense oligos dispersed on the whole sequence, with DIG DNA labeling mix (Boehringer Mannheim) diluted 5×, and with 1 U of Vent DNA polymerase. The amplification was performed using 30 cycles: 94°C for 1 min, 52°C for 45 sec, and 72°C for 1 min. The probe was then boiled for 30 min to reduce the DNA size. Dechorionated and devitelinized embryos were incubated overnight at 46°C in 50% formamide, 5× SSC, 0.1% Tween 20, 100 μg/ml salmon sperm DNA, and 50 μg/ml heparin solution, with the probe diluted 60 times. Embryos were then washed progressively in 1× PBS, 0.1% Tween 20 at 46°C, incubated with anti-digoxygenine antibody conjugated with alkaline phosphatase for 1 hr at room temperature, washed again, and incubated with alkaline phosphatase substrates.

In situ *hybridization to polytene chromosome. Drosophila* salivary gland polytene chromosome squashes were hybridized with biotinylated DNA probes obtained by nick-translation. Hybridizations were performed as described (Pardue, 1994) at 37°C with 50% formamide and 4× SSC.

RESULTS

Cloning and analysis of DmGluRA

Cloning of *Drosophila* mGluRs homologs was performed through a PCR approach using degenerated oligonucleotides corresponding to the transmembrane domains III and VI highly conserved in the mGluR family. From all PCR products obtained using *Drosophila* head cDNAs as template, one sequence was found to be homologous to rat mGluRs. This fragment was used to screen a λ-Zap *Drosophila* head cDNA library to get the corresponding cDNA, which was called DmGluRA. It contains a 2.9-kb-long open reading frame with 5′ and 3′ untranslated regions but no polyadenylation signal.

The deduced amino acid sequence of DmGluRA contains 977 amino acid residues corresponding to a protein of a predicted MW of 108 kDa. This protein sequence reveals that all of the structural features characteristic of the mammalian mGluRs are conserved in DmGluRA: a putative N-terminal signal peptide, a large extracellular domain with a cysteine-rich region, seven putative transmembrane segments that give rise to three short intracellular loops (i1, i2, i3), and a C-terminal tail (Fig. 1). All of the cysteines conserved in the mammalian mGluRs are also conserved in DmGluRA.

DmGluRA is most homologous to group II mGluRs (45% amino acid identity with mGluR3 and 43% with mGluR2). A dendrogram constructed with the percentages of identity between DmGluRA and rat mGluRs and the bovine parathyroid calcium-sensing receptor (CaR) shows that DmGluRA belongs to the subgroup formed by groups II and III mammalian mGluRs (Fig. 2). DmGluRA branches with group II rather than group III, although this relies only on a slight difference in the percentage of identity (5%).

Transduction mechanism of DmGluRA

To determine its transduction pathway, DmGluRA was transiently expressed into human embryonic kidney (HEK 293) cells. Glutamate elicited neither cAMP nor IP formation in cells expressing DmGluRA (Fig. 3*A,B*). In cells coexpressing DmGluRA and the LH receptor, glutamate induced a strong inhibition of the LH-stimulated cAMP production (Fig. 3*A*). No such inhibition was observed after pretreatment of the cells with *Bordetella* PTX (Fig. 3*A*) or in cells expressing the LH receptor alone (data not shown). PTX is known to prevent the activation of G_i and G_o G-proteins, the G_i proteins often being involved in the negative coupling of receptors to AC. This indicates that DmGluRA is negatively coupled to AC like the group II and III mammalian mGluRs.

To further define the G-protein-coupling specificity of DmGluRA, its coupling to chimeric G-proteins was analyzed. The α-subunit of G_q ($G_{\alpha q}$) is known to couple GPCRs to PLC, whereas the $G_{\alpha i}$ subunits negatively couple GPCRs to AC, the coupling to K^+ and Ca^{2+} channels being mediated by either the G_i or G_o type of G-proteins (Birnbaumer and Birnbaumer, 1995). The few last C-terminal residues of these α-subunits are known to be involved in the specificity of interaction between the G-protein and the receptor (Conklin and Bourne, 1993). Accordingly, the chimeric $G_{\alpha q}$-proteins in which the five or nine C-terminal residues have been replaced by those of $G_{\alpha i}$ ($G_{\alpha qi5}$ or $G_{\alpha qi9}$) or $G_{\alpha o}$ ($G_{\alpha qo5}$) allow many $G_i/_o$-coupled receptors to activate PLC (Conklin et al., 1993). In cells coexpressing DmGluRA and these chimeric G_α proteins, glutamate elicited a threefold stimulation of IP production (Fig. 3*B*). No such stimulation was observed when DmGluRA was coexpressed with the wild-type $G_{\alpha q}$ protein, indi-

Table 1. Pharmacological characterization of DmGluRA

Agonists	EC$_{50}$ SEM	Antagonists	IC$_{50}$ SEM
Glu	1.2 ± 0.05	(±)MCPG	>1000
1S,3S-ACPD	41 ± 6	MCCG-I	201 ± 45
1S,3R-ACPD	65 ± 2	(RS)-MPPG	513 ± 125
L-CCG-I	11 ± 1	(RS)-MSPG	>1000
(S)-4C3HPG	20 ± 3	(RS)-MTPG	348 ± 32
L-Quis	28 ± 9	(S)-MAP4	>1000
L-AP4	>1000		

Values are EC$_{50}$ and IC$_{50}$ (in μM), determined as described in Materials and Methods, and are means ± SEM of three independent experiments performed in triplicates. For agonists, >1000 means the molecule at 1 mM induced a response <50% of the maximal effect of glutamate. For antagonists, >1000 means the molecule at 1 mM induced <50% inhibition of the 3μM glutamate effect.

cating that the C-terminal sequence of $G_{\alpha i}$ or $G_{\alpha o}$ is necessary for the coupling of the G-protein to DmGluRA.

Pharmacology of DmGluRA

The measurement of IP production after coexpression of DmGluRA and $G_{\alpha qi9}$ was more sensitive and accurate than the measurement of the inhibition of LH-stimulated cAMP production. Moreover, the pharmacological profiles of the G_i-coupled mGluR2 and mGluR4 determined by this IP assay were found to be similar to those reported previously by measuring AC inhibition in cell lines stably expressing these receptors (Gomeza et al., 1996b). This assay therefore was used to establish the pharmacology of DmGluRA. None of the ionotropic receptor agonists—AMPA, kainate, and NMDA—had any activity at 300 μM. The specific and potent group I mGluRs agonists 3HPG and DHPG (Ito et al., 1992) were inactive at a concentration up to 100 μM; however, group II and III agonists 1S,3S-ACPD and L-CCG-I (Hayashi et al., 1992; Cartmell et al., 1993) were active. Among group III-specific agonists (Pin and Duvoisin, 1995), L-AP4 gave a small response and L-SOP was inactive at 1 mM. 4C3HPG, which is a group I antagonist and a group II agonist (Hayashi et al., 1994), was found to be an agonist of DmGluRA. The order of potency of the different agonists of DmGluRA was Glu > L-CCG-I > 4C3HPG > quisqualate > 1S,3S-ACPD ≈ 1S,3R-ACPD > L-AP4 (Fig. 3*C*, Table 1). Among these, Glu had the best efficacy followed by 4C3HPG, 1S,3S-ACPD, 1S,3R-ACPD, and L-CCG-I.

Among the antagonists tested, the group II-specific antagonist MCCG-I, as well as MPPG and MTPG (group II and group III antagonists) (Roberts, 1995), totally inhibited the glutamate response in a dose-dependent manner (Fig. 3*D*). Their order of potency was MCCG-I > MPPG ≈ MTPG. MAP4, a group III antagonist (Roberts, 1995), and MCPG, more potent on group I mGluRs, reduced the glutamate response to 50 and 32% of the maximum at 1 mM, respectively.

Localization of DmGluRA mRNAs

Embryonic expression of DmGluRA was studied by *in situ* hybridization with a digoxigenin-labeled antisense probe. No signal was detected until stage 10, where a transient labeling was observed in the cephalic region (Fig. 4*A*). No such labeling was observed when the sense probe was used (data not shown). At this stage, stomodeal invagination takes place, giving rise to the anterior and posterior labia. In the posterior labium, two symmetrical clusters of two or three epithelial cells were labeled. In the anterior labium, two clusters of 5–10 cells were also labeled. The anterior and posterior clusters were close to the median plane in the early stage 10, but the anterior ones moved to the lateral ends of the
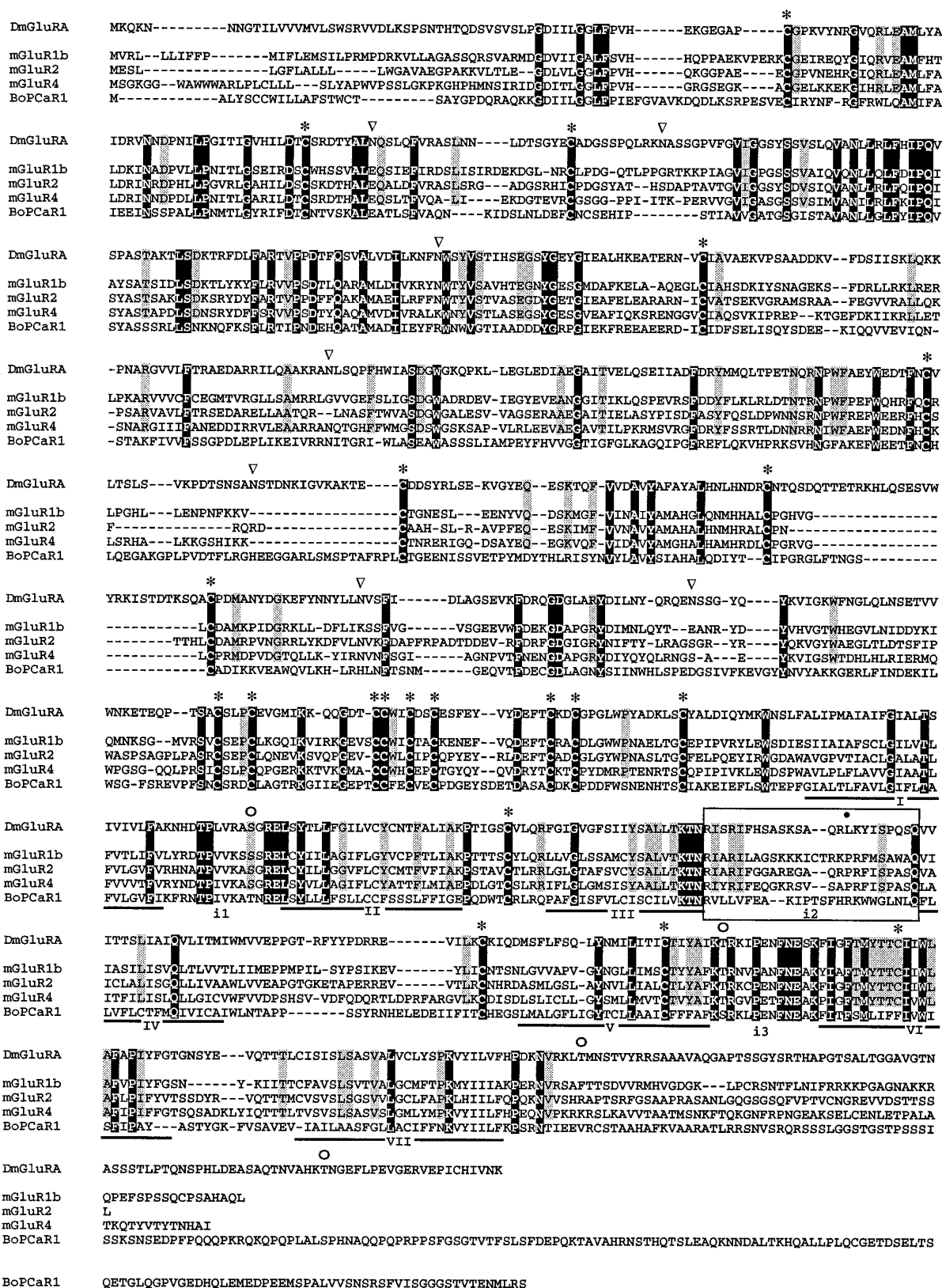
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DmGluRA    MKQKN---------NNGTILVVVMVLSWSRVVDLKSPSNTHTQDSVSVSLPGDIILGGLFPVH------EKGEGAP-----CGPKVYNRGVQRLEAMLYA

mGluR1b    MVRL--LLIFFP-------MIFLEMSILPRMPDRKVLLAGASSQRSVARMDGDVIIGALFSVH------HQPPAEKVPERKCGEIREQYGIQRVEAMFHT
mGluR2     MESL---------------LGFLALLL-----LWGAVAEGPAKKVLTLE--GDLVLGGLFPVH------QKGGPAE----ECGPVNEHRGIQRLEAMLFA
mGluR4     MSGKGG--WAWWWARLPLCLLL---SLYAPWVPSSLGKPKGHPHMNSIRIDGDITLGGLFPVH------GRGSEGK----ACGELKKEKGIHRLEAMLFA
BoPCaR1    M------------ALYSCCWILLAFSTWCT---------SAYGPDQRAQKKGDIILGGLFPIEFGVAVKDQDLKSRPESVECIRYNF-RGFRWLQAMIFA

                            *                ▽                             *              ▽
DmGluRA    IDRVNNDPNILEGITIGVHILDTCSRDTYALNQSLQFVRASLNN----LDTSGYECADGSSPQLRKNASSGPVFGVIGGSYSSVSLQVANLLRLFHIPQV

mGluR1b    LDKINADPVLLPNITLGSEIRDSCWHSSVALEQSIEFIRDSLISIRDEKDGL-NRCLPDG-QTLPPGRTKKPIAGVICPGSSSVAIQVANLLQLFDIPQI
mGluR2     LDRINRDPHLLPGVRLGAHILDSCSKDTHALEQALDFVRASLSRG---ADGSRHICPDGSYAT--HSDAPTAVTGVICGSYSDVSIQVANLLRLFQIPQI
mGluR4     LDRINNDPDLLPNITLGARILDTCSRDTHALEQSLTFVQA-LI----EKDGTEVRCGSGG-PPI-ITK-PERVVGVICGSGSVSIMVANILRLFKIPQI
BoPCaR1    IEEINSSPALLPNMTLGYRIFDTCNTVSKALEATLSFVAQN----KIDSLNLDEFCNCSEHIP--------STIAVVGATGSGISTAVANLLGLFYIPQV

                                                              ▽
DmGluRA    SPASTAKTLSDKTRFDLFARTVPPDTFQSVALVDILKNFNWSYVSTIHSEGSYGEYGIEALHKEATERN-VCIAVAEKVPSAADDKV--FDSIISKLQKK

mGluR1b    AYSATSIDLSDKTLYKYFLRVVPSDTLQARAMLDIVKRYNWTYVSAVHTEGNYGESGMDAFKELA-AQEGLCIAHSDKIYSNAGEKS--FDRLLRKLRER
mGluR2     SYASTSAKLSDKSRYDYFARTVPPDFFQAKAMAEILRFFNWTYVSTVASEGDYGETGIEAFELEARARN-ICVATSEKVGRAMSRAA--FEGVVRALLQK
mGluR4     SYASTAPDLSDNSRYDFFSRVVPSDTYQAQAMVDIVRALKWNYVSTLASEGSYGESGVEAFIQKSRENGGVCIAQSVKIPREP--KTGEFDKIIKRLLET
BoPCaR1    SYASSSRLLSNKNQFKSFLRTIPNDEHQATAMADIIEYFRWNWVGTIAADDDYGRPGIEKFREEAEERD-ICIDFSELISQYSDEE--KIQQVVEVIQN-

                                      ▽                                        *
DmGluRA    -PNARGVVLFTRAEDARRILQAAKRANLSQPFHWIASDGWGKQPKL-LEGLEDIAEGAITVELQSEIIADEDRYMMQLTPETNQRNPWFAEYWEDTFNCV

mGluR1b    LPKARVVVCFCEGMTVRGLLSAMRRLGVVGEFSLIGSDGWADRDEV-IEGYEVEANGGITIKLQSPEVRSFDDYFLKLRLDTNTRNPWFPEFWQHRFQCR
mGluR2     -PSARVAVLFTRSEDARELLAATQR--LNASFTWVASDGWGALESV-VAGSERAAEGAITIELASYPISDFASYFQSLDPWNNSRNPWFREFWEERFHCS
mGluR4     -SNARGIIIFANEDDIRRVLEAARRANQTGHFFWMGSDSWGSKSAP-VLRLEEVAEGAVTILPKRMSVRGFDRYFSSRTLDNNRRNIWFAEFWEDNFHCK
BoPCaR1    -STAKFIVVFSSGPDLEPLIKEIVRRNITGRI-WLASEAWASSSLIAMPEYFHVVGGTIGFGLKAGQIPGFREFLQKVHPRKSVHNGFAKEFWEETFNCH

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DmGluRA    LTSLS---VKPDTSNSANSTDNKIGVKAKTE----CDDSYRLSE-KVGYEQ--ESKTQF-VVDAVYAFAYALHNLHNDRCNTQSDQTTETRKHLQSESVW

mGluR1b    LPGHL---LENPNFKKV----------------CTGNESL---EENYVQ--DSKMGFVINAIYAMAHGLQNMHHAL-CPGHVG---------------
mGluR2     F--------------RQRD---------------CAAH-SL-R-AVPFEQ--ESKIMF-VVNAVYAMAHALHNMHRAL-CPN----------------
mGluR4     LSRHA---LKKGSHIKK----------------CTNRERIGQ-DSAYEQ--EGKVQF-VIDAVYAMGHALHAMHRDL-CPGRVG---------------
BoPCaR1    LQEGAKGPLPVDTFLRGHEEGGARLSMSPTAFRPLCTGEENISSVETPYMDYTHLRISYNVYLAVYSIAHALQDIYT--CIPGRGLFTNGS---------

                        *              ▽                                        ▽
DmGluRA    YRKISTDTKSQACPDMANYDGKEFYNNYLLNVSFI------DLAGSEVKFDRQGGLARYDILNY-QRQENSSG-YQ----YKVIGKWFNGLQLNSETVV

mGluR1b    -----------LCDAMKPIDGRKLL-DFLIKSSFVG------VSGEEVWFDEKGDAPGRYDIMNLQYT--EANR-YD----YVHVGTWHEGVLNIDDYKI
mGluR2     --------TTHLCDAMRPVNGRRLYKDFVLNVKFDAPFRPADTDEN-RFDRFGIGIRYNIFTY-LRAGSGR--YR----YQKVGYWAEGLTLDTSFIP
mGluR4     -----------LCPRMDPVDGTQLLK-YIRNVNFNFSGI----AGNPVTFNENGDAPGRYDIYQYQLRNGS-A---E----YKVIGSWTDHLHLRIERMQ
BoPCaR1    -----------CADIKKVEAWQVLKH-LRHLNFTSNM------GEQVTFDEC-GDLAGNMSIINWHLSPEDGSIVFKEVGYYMNVYAKKGERLFINDEKIL

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DmGluRA    WNKETEQP--TSACSLPCEVGMIKK-QQGDT--CCWICDSCESFEY--VYDEFTCKDCGPGLWPYADKLSCYALDIQYMKWNSLFALIPMAIAIFGIALTS

mGluR1b    QMNKSG--MVRSVGCSEPCLKGQIKVIRKGEVSCCWICTACKENEF--VQDEFTCRACDLGWWPNAELTGCEPIPVRYLBWSDIESIIAIAFSCLGILVTL
mGluR2     WASPSAGPLPASRCSEPCLQNEVKSVQPGEV--CCWLCIPCQPYEY--RLDEFTCADCGLGYWPNASLTGCFELPQEYIRWGDAWAVGPVTIACLGALALL
mGluR4     WPGSG--QQLPRSICSLPCQPGERKKTVKGMA--CCWHCEPCTGYQY--QVDRYTCKTCPYDMRPTENRTSCQPIPIVKLEWDSPWAVLPLFLAVVGIAAL
BoPCaR1    WSG-FSREVPFSNCSRDCLAGTRKGIIEGEPTCCFECVECPDGEYSDETDASACDKCPDDFWSNENHTSCIAKEIEFLSWTEPFGIALTLFAVLGIFLTA
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DmGluRA    IVIVLFAKNHDTPLVRASGRELSYTLLFGILVCYCNTFALIAKPTIGSCVLQRFGIGVGFSIIYSALLTKTNRISRIFHSASKSA--QRLKYISPQSQVV

mGluR1b    FVTLIFVLYRDTPVVKSSSREICYIILAGIFLGYVCPFTLIAKPTTTSCYLQRLLVGLSSAMCYSALVTKTNRIARILAGSKKKICTRKPRFMSAWAQVI
mGluR2     FVLGVFVRHNATPVVKASGRELCYILLGGVFLCYCMTFVFIAKPSTAVCTLRRLGLGTAFSVCYSALLTKTNRIARIFGGAREGA--QRPRFISPASQVA
mGluR4     FVVVTVRYNDTPIVKASGRELSYVLLAGIFLCYATTFLMIAEPGKGRSVHQDWTCKTCPYDMRPTENRTSCQPIPIVKLEWDSPWAVLPLFLAVVGIAAL
BoPCaR1    FVLGVFIKFRNTPIVKATNREISMLLLFSLLCCFSSSLFFIGEPQDWTCRLRQPAFGISFVLCISCILVKTNRVLLVFEA--KIPTSFHRKWWGLNLQFL
           ─────   ─────────    ──────────   ────────────   ─────────────────    ────────────────
             i1               II                        III                           i2

                                                              *            *                      *
DmGluRA    ITTSLIAIQVLITMIWMVVEPPGT-RFYYPDRRE-------VILKCKIQDMSFLFSQ-LYNMIITICTIYAIKTRKIPENFNESKFIGFTMYTTCIIWL

mGluR1b    IASILISVQLTLVVTLIIMEPPMPIL-SYPSIKEV-------YLICNTSNLGVVAPV-GYNGLLIMSCTYYAFKTRNVPANFNEAKYIAFTMYTTCIIWL
mGluR2     ICLALISGQLLIVAAWLVVEAPGTGKETAPERREV-----VTLRCNHRDASMLGSL-AYNVLLIALCTLYAFKTRKCPENFNEAKFIGFTMYTTCIIWL
mGluR4     ITFILISLQLLGICVWFVVDPSHSV-VDFQDQRTLDPRFARGVLKCDISDLSLICLL-GYSMLLMVTCTVZAIKTRGVPETFNEAKPIGFTMYTTCIVWL
BoPCaR1    LVFLCTFMQIVICAIWLNTAPP-------SSYRNHELEDEIIFITCHEGSLMALGFLIGYTCLLAAICFFFAFKSRKLPENFNEAKFITPSMLIFFIWI
           ──────────                                            ─────────      ─────────────────     ────
             IV                                                    V            i3               VI

DmGluRA    AFAPIYFGTGNSYE---VQTTTLCISISLSASVAIVCLYSPKVYILVFHPDKNVRKLTMNSTVYRRSAAAVAQGAPTSSGYSRTHAPGTSALTGGAVGTN

mGluR1b    AFVPIYFGSN------Y-KIITTCFAVSLSVTVAIGCMFTPKMYIIIAKPERNVRSAFTTSDVVRMHVGDGK---LPCRSNTFLNIFRRKKPGAGNAKKR
mGluR2     APLPIFYVTSSDYR---VQTTTMCVSVSLSGGSVVIGCLFAPKLHIILFQPQKNVVSHRAPTSRFGSAAPRASANLGQGSGSQFVPTVCNGREVVDSTTSS
mGluR4     APIPIFFGTSQSADKLYIQTTTLTVSVSLSASVSIGMLYMPKVYIILFHPEQNVPKRKRSLKAVVTAATMSNKFTQKGNFRPNGEAKSELCENLETPALA
BoPCaR1    SGIPAY---ASTYGK-FVSAVEV-IAILAASFGLLACIFFNKVYIILFKPSRNTIEEVRCSTAAHAFKVAARATLRRSNVSRQRSSSLGGSTGSTPSSSI
           ───────────────────
                 VII
                       O
DmGluRA    ASSSTLPTQNSPHLDEASAQTNVAHKTNGEFLPEVGERVEPICHIVNK

mGluR1b    QPEFSPSSQCPSAHAQL
mGluR2     L
mGluR4     TKQTYVTYTNHAI
BoPCaR1    SSKSNSEDPFFPQQQPKRQKQPQPLALSPHNAQQPQPRPPSFGSGTVTFSLSFDEPQKTAVAHRNSTHQTSLEAQKNNDALTKHQALLPLQCGETDSELTS


BoPCaR1    QETGLQGPVGEDHQLEMEDPEEMSPALVVSNSRSFVISGGGSTVTENMLRS
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Figure 1. Multiple alignment of DmGluRA with one rat mGluR from each group and with the bovine calcium-sensing receptor. Residues conserved in the entire mGluR family (including those not represented in the figure) and in the calcium-sensing receptor are *highlighted* in *black*. Residues conserved only in the entire mGluR family are *highlighted* in *gray*. Conserved cysteines are indicated by an *asterisk*, and potential N-glycosylation sites in DmGluRA by ▽. Leucine 730 of DmGluRA is indicated by ●, and potential phosphorylation sites of this receptor (by protein kinase A and casein kinase II) are indicated by O. The sequence of the second intracellular loop is *bordered* in *black*. The position of transmembrane domains I–VII is *underlined* in *black*. The sequence of the cDNA, from which the DmGluRA protein was deduced, has been given the EMBL accession number X99675.
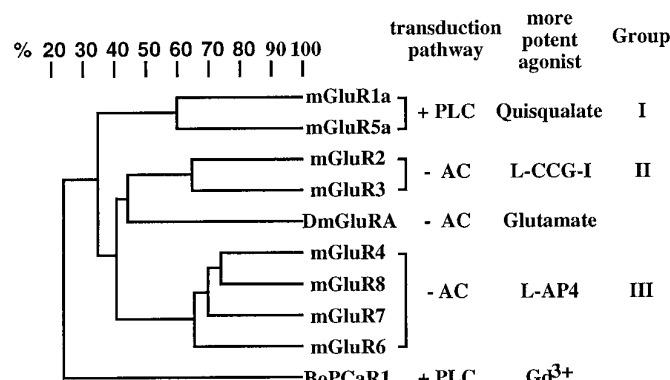
Figure 2. UPGMA dendrogram using pairwise amino acid identities (indicated in percentages) between all members of the mGluR family and bovine parathyroid CaR as an outgroup. Cophenetic correlation coefficient was 0.99, indicating an overall very good fit.
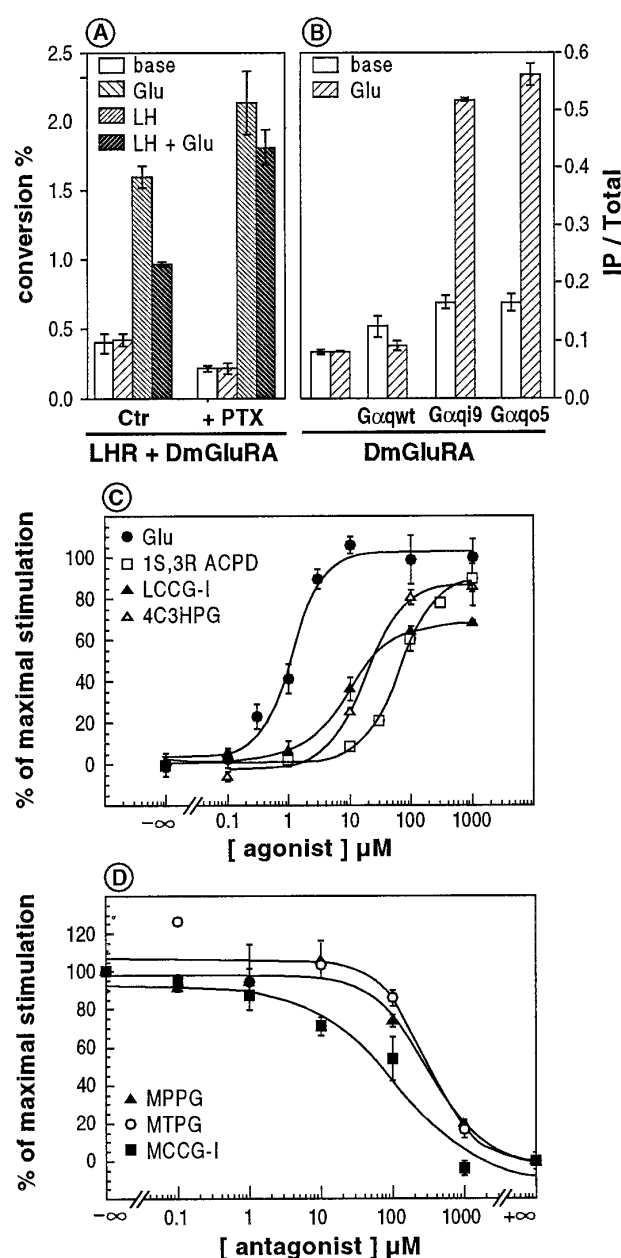

Figure 3. Transduction mechanism and pharmacology of DmGluRA. *A*, Cotransfection of DmGluRA and LH receptor (*LHR*). Percentage of conversion of ATP in cAMP is estimated in the presence of either 1 mM glutamate or 100 ng/ml LH or in the presence of both agonists. The same experiment is repeated on cells pretreated with 100 ng/ml PTX. *B*, Measure of IP production after application of 1 mM glutamate on cells expressing DmGluRA alone or DmGluRA with wild-type $G_{\alpha q}$ ($G\alpha qwt$) or $G_{\alpha qi9}$ or $G_{\alpha qo5}$. *C*, Dose–response curves of agonist-induced IP stimulation (expressed in percent of maximal glutamate stimulation) when DmGluRA was cotransfected with $G_{\alpha qi9}$. *D*, Activation of IP production by 3 μM glutamate in the presence of variable concentrations of antagonists. In *C* and *D*, curve fittings were realized using Kaleidagraph software [fitting equation: $y = (m1 - m2)/(1 + (x/m4)\ m3) + m2$; m1 = response when $x = 0$; m2 = response when $x = \infty$; m3 = slope; m4 = EC$_{50}$ or IC$_{50}$]. Values are means ± SEM of triplicate determinations from typical experiments.

labium in the late stage 10. Moreover, the cells of these anterior clusters were not only epithelial but were also subepithelial. This labeling completely disappeared at stage 11. Later in development, at stage 14, we observed a weak labeling restricted to the CNS (Fig. 4*B*). This labeling became stronger until stage 17 (Fig. 4*C*). At this stage, DmGluRA RNAs were expressed in many of the ventrolateral cells of the abdominal CNS as well as in 6–10 median cells of every abdominal segment (Fig. 4*D*). The expression in the thoracic segments and in sub- and supraesophageal ganglions was high and difficult to resolve at the cellular level.

The expression of DmGluRA in the adult was detected by RT-PCR (Fig. 5), in both the head (including the brain) and the rest of the body of the animal (including the thoracic and abdominal ganglia).

Mapping of the gene encoding DmGluRA

The location of the gene encoding DmGluRA was identified by *in situ* hybridization of biotin-labeled DmGluRA probes to *Drosophila* salivary gland chromosomes. Hybridization was observed at position 102 CD of the fourth chromosome (data not shown).

DISCUSSION

We report here the cloning and functional characterization of an invertebrate mGluR: DmGluRA. Its deduced primary sequence reveals that all of the characteristics of mammalian mGluRs are conserved in DmGluRA. Moreover, the 19 cysteine residues conserved in the mammalian mGluR-CaR receptor family are also conserved in DmGluRA. These residues are therefore likely to play important roles in the functioning of these receptor proteins, possibly being involved in intramolecular transduction (O'Hara et al., 1993). DmGluRA is more related to group II mammalian mGluRs, as shown by the unweighted pair-group method of arithmetic averages (UPGMA) analysis. It shares ~45 and 43% amino acid identity with mGluR3 and mGluR2, respectively. Similar percentages of identity were reported between two *Drosophila* ionotropic GluRs and their mammalian homologs (44% for DGluR I with GluR3, 46% for DNMDAR-I with NMDARI) (Ultsch et al., 1992, 1993). These observations suggest that the group II mGluR existed in the common ancestor of arthropods and vertebrates. Consequently, we can suppose that mGluRs corresponding to the other subgroups are also present in *Drosophila*.

When expressed in HEK 293 cells, DmGluRA activates neither AC nor PLC but is negatively coupled to AC in a PTX-sensitive manner, like its mammalian homologs. Because $G_{\alpha o}$ is absent in HEK 293 cells (Law et al., 1993), our results indicate that DmGluRAs do couple to human $G_{\alpha i}$-type G-proteins. Other *Drosophila* receptors, tyramine/octopamine receptor (Arakawa et
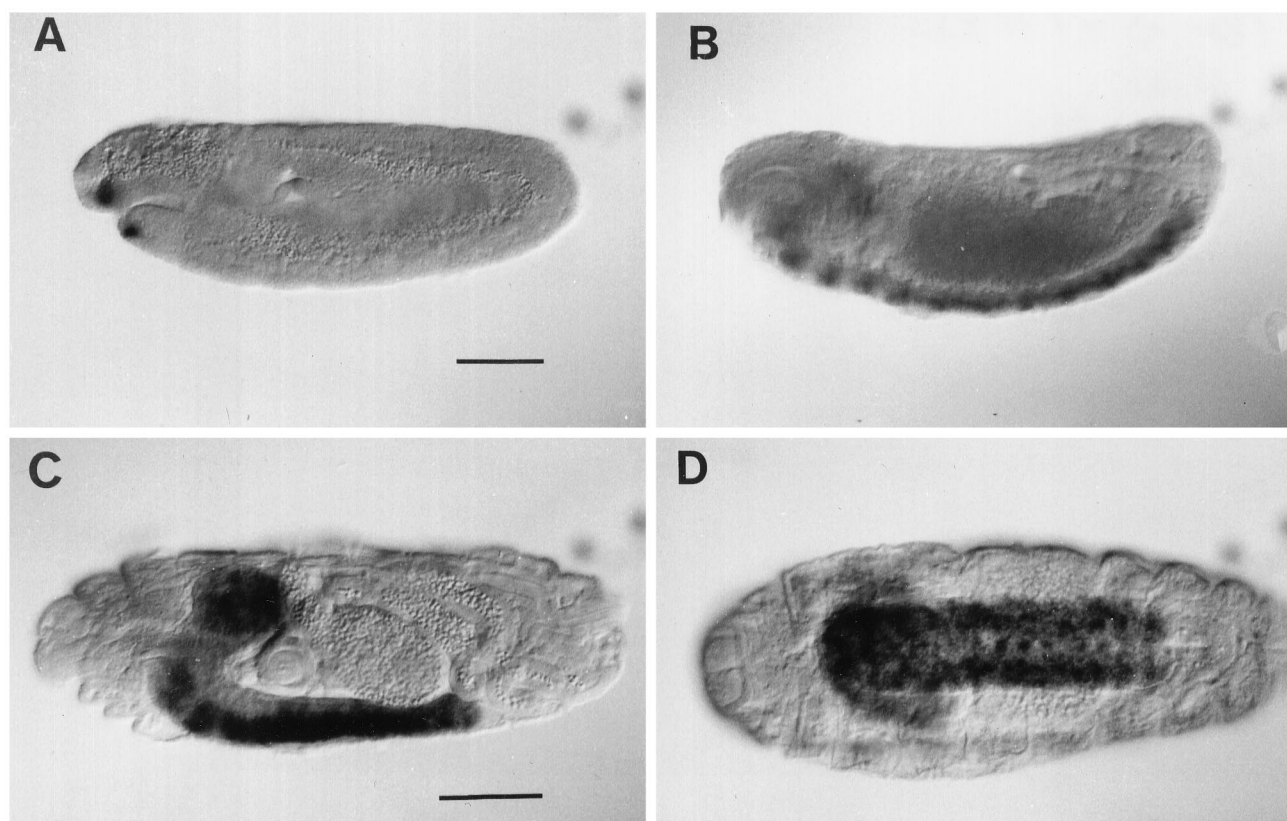
Figure 4. *In situ* hybridization on whole-mount embryos with a digoxigenin-labeled antisense DmGluRA probe. In *A–D*, anterior is on the *left*, and when the view is lateral, dorsal is on the *top*. *A*, Stage 10 embryo, lateral view. *B*, Stage 14 embryo, lateral view, same scale as *A*. *C*, stage 16 embryo, lateral view. *D*, Stage 16 embryo, ventral view, same scale as *C*. Scale bar, 100 μm.

al., 1990; Saudou et al., 1990) and $5HT_{1ADro}$ and $5HT_{1BDro}$ receptors (Saudou et al., 1992; Colas et al., 1995), also couple to mammalian $G_{i/o}$ proteins.

DmGluRA coupling to G-proteins was examined further using $G_{\alpha qi/o}$ chimeric G-proteins. Like other $G_{i/o}$-coupled GPCRs (Conklin et al., 1993), DmGluRA is able to activate these chimeric $G_{\alpha qi/o}$ subunits but does not activate the wild-type $G_{\alpha q}$. Similar results were obtained with the rat mGluR2 and mGluR4 (Gomeza et al., 1996b). Therefore, the C-terminal region of the

Figure 5. PCR analysis of the expression pattern of DmGluRA in the adult. PCR products of 485 bp length, hybridizing with a radiolabeled DmGluRA probe, were obtained from heads (*lane 1*) and the remaining body fraction (*lane 2*) as well as from the positive control performed on the DmGluRA cDNA (*lane 5*). *Lanes 3* and *4* are negative controls of RT (PCR reaction realized on the product of RT made without RNAs) and PCR (PCR reaction realized without any substrate), respectively.

α-subunit is also critical for the specific coupling of G-proteins to mGluRs. Moreover, these results suggest that DmGluRA can couple to *Drosophila* $G_{\alpha i}$ and $G_{\alpha o}$ proteins (Provost et al., 1988; Thambi et al., 1989). Interestingly, like DmGluRA, $G_{\alpha o}$ protein is expressed in the embryonic CNS. Therefore, in its native environment, DmGluRA may not only inhibit AC activity but may also regulate the activity of K^+ and Ca^{2+} channels (Harris-Warrick et al., 1988; Birnbaumer and Birnbaumer, 1995).

Taken together, these results indicate that DmGluRA shares a similar G-protein-coupling specificity with its mammalian homologs. We recently reported that i2 of mGluRs plays a critical role in defining the G-protein-coupling specificity of these receptors (Gomeza et al., 1996a). This is in contrast to most other GPCRs in which i3 plays such a role (Liu et al., 1995). In agreement with this proposal, the residues conserved in the second intracellular loop of G_i-coupled rat mGluRs, but not in PLC-coupled ones, are also conserved in DmGluRA (Fig. 1). Interestingly, a proline residue found in the C-terminal part of i2 in all mammalian mGluRs is not conserved in DmGluRA (Fig. 1, position 730 of DmGluRA). Although a proline residue is likely to be important for the secondary structure of this loop, our observation that DmGluRA has the same G-protein-coupling specificity as group II and III rat mGluRs suggests that this residue is not critical for a functional and specific coupling of these receptors to G-proteins.

The pharmacology of DmGluRA is similar to that of group II mammalian mGluRs (Tanabe et al., 1992, 1993; Pin and Duvoisin, 1995). This is remarkable because the amino acid identity of DmGluRA with mGluR2/3 in the supposed glutamate binding
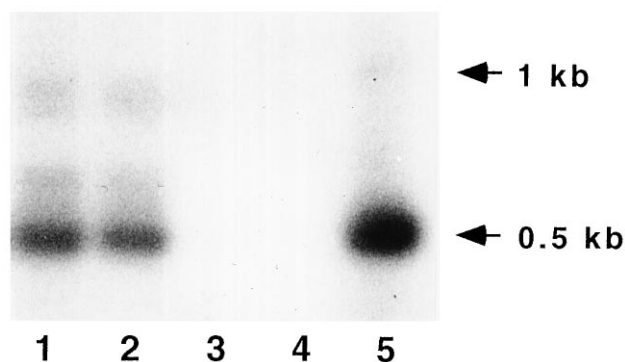
domain, i.e., the region homologous to bacterial periplasmic proteins, is only 45%. This means that the critical amino acids that determine the pharmacophore of mGluR2/3 are conserved in DmGluRA. Such a pharmacological profile conservation for G-protein-coupled receptors is seldom observed through evolution. *Drosophila* peptide receptors (Li et al., 1991, 1992; Monnier et al., 1992), cloned *Drosophila* serotonin receptors 5-HT1ADro, 5-HT$_{1BDro}$, and 5-HT$_{7Dro}$ (Witz et al., 1990; Saudou et al., 1992; Colas et al., 1995), the cloned *Drosophila* dopamine receptor (Gotzes et al., 1994), and the invertebrate octopamine and tyramine receptors (Arakawa et al., 1990; Saudou et al., 1990; Roeder, 1995), considered to be noradrenergic receptor homologs, display important pharmacological differences with their mammalian homologs; however, the recently cloned 5-HT$_2$ *Drosophila* receptor is clearly pharmacologically related to the mammalian homolog 5-HT2B (Colas et al., 1995).

A first transient expression of DmGluRA revealed by *in situ* hybridization takes place at stage 10. From the comparison with the fate map established at this stage (Jürgens et al., 1986), we suppose that the posterior labial labeling is situated within the hypopharyngeal presumptive territory, whereas the anterior labial labeling is situated within the clypeolabrum presumptive territory. More work is necessary, however, to determine which cell types express this mRNA and whether this is of any physiological relevance. A second period of expression of DmGluRA starts at stage 14 in the CNS and culminates at stage 17 when the larval CNS is formed. This expression is nearly synchronous with the expression of glutamate in motoneurones, which is first observed at 12.5–13.0 hr after egg laying (stage 15) (Broadie and Bate, 1993). Moreover, DmGluRA transcription appears at a period of neuronal differentiation and synaptogenesis. Indeed, the contact between motoneurones and their muscle target occurs at 12.5–13.0 hr AEL, and synaptic proteins begin to be expressed at stage 14 (DiAntonio et al., 1993). It is therefore likely that DmGluRA plays a role in embryonic glutamatergic synapses and is possibly involved in CNS development. Interestingly, mGluR5 and the mammalian DmGluRA homolog mGluR3 are expressed at a high level during the first postnatal weeks when synaptogenesis occurs (Catania et al., 1994; Romano et al., 1996). A role for these receptors in CNS development has therefore been proposed.

Taken together, these results indicate that in addition to ionotropic receptors, metabotropic glutamate receptors are also present in *Drosophila*, suggesting major roles for glutamate in the CNS of insects. The functions of these receptors, in either the development or physiology of the adult nervous system, will be approached further using genetic analysis.

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