ly, that of the highly visual tiger beetle larvae (18). In the strepsipteran eye no screening pigment appears to be present (19). The effective image resolution within each eyelet of X. peckii therefore depends not only on the number of photoreceptors but also on the extent of optical pooling, which remains to be seen in the estimated. The image resolution would also be influenced by the degree of overlap between the visual fields of neighboring eyelets. The acceptance angle of an individual eyelet can be estimated if the focal length of the lens is known. On the basis of measurements of the image magnification (23), a focal length of 44 ± 5 μm (n = 21) and an acceptance angle of 33° ± 6° (n = 10) was calculated. Thus, the values for the acceptance angle are, if at all, only slightly greater than those of the inter-eyelet angle of 27° ± 6°. Our model (Fig. 4) assumes no overlap, but a small amount of overlap is conceivable and could be consistent with the unusual absence of clearly definable caudelles of the medulla.

For more than a century the arthropod eye has been extensively studied in structure and function, and many common features are conserved throughout this group. Although the detailed modes of function of arthropod eyes vary considerably (8), it is remarkable how profoundly the structural features of the eye of Strepisiptera have changed. The course of its evolution is unclear, but it is certainly noteworthy; after all, its organization may be comparable to DΔψ values of conventional compound eyes.

11. The sensitivity [from (8)] was calculated as S = [(n/4)[f(A)]/f(1) − exp(−A)], where A is aperture (65 μm, measured), f is focal length (44 μm, calculated), d is receptor diameter (1.5 μm, estimated), x is receptor length (11 μm, measured), and k is the absorption coefficient. We used for k [like (8); see all invertebrates in table 5] the estimate of 0.0067 μm−1 based on measurements by M. S. Bruno, S. N. Barnes, T. H. Goldsmith, J. Comp. Physiol. A 120, 123 (1977).
13. Male X. peckii were collected as late pupae within their wasp hosts near Ithaca, NY.
14. The heads of late pupae and adults were stained with osmium or reduced silver. We estimated the number of rhabdoms (or functional receptor units) from light microscopy examination of sections of the retina.
15. Volumes were estimated by serial reconstruction. Outlines of neuromasts were scanned in, measured in Adobe Photoshop, multiplied by section thickness, and the relative proportion of the summed volumes was calculated.
16. If each photoreceptor of X. peckii required its own lens, then the diameter of each lens would only be 6 μm (65 μm/11 receptors), resulting in a DΔψ value of 0.24, and therefore diffraction would be limiting. (8). A single-lens eye with a wide field of view, however, requires a very short focal length for a small insect and thus a very thick lens, which usually has pronounced spherical and chromatic aberrations. Thus, pooling the information of a number of simple-lens eyes with a restricted field of view (“chuck sampling”) may be a good compromise.
23. The setup for image magnification measurements was as described in (5). The focal length f was calculated using the lens formula 1/f = 1/U + 1/V, where O is object size, U is object distance, and V is image size. The acceptance angle is determined by f and the extension of the retina behind the lens.
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Characterization of the Calmodulin-Dependent Glutamate Receptor System Patricia A. Vellutino,*† Oussama El Far,‡ Elisa Boffill-Cardona,§ Christian Nolteno,∥ Michael Freissmuth,‡ Andreas Karschin,∥ José M. Airas,∥ Heinrich Betz,‡ † Stefan Boehm‡

Glutamatergic neurotransmission is controlled by presynaptic metabotropic glutamate receptors (mGluRs). A subdomain in the intracellular carboxyl-terminal tail of group III mGluRs binds calmodulin and heterotrimeric guanosine triphosphate–binding protein (G protein) βγ subunits in a mutually exclusive manner. Mutations interfering with calmodulin binding and calmodulin antagonists inhibit G protein–mediated modulation of ionic currents by mGluR7. Calmodulin antagonists also prevent inhibition of excitatory neurotransmission via presynaptic mGluRs. These results reveal a novel mechanism of presynaptic modulation in which Ca2+-calmodulin is required to release G protein βγ subunits from the C-tail of group III mGluRs in order to mediate glutamatergic autoinhibition.

Glutamate Receptor Signaling

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**References and Notes
2. Calmodulin dependence of presynaptic metabotropic glutamate receptor signaling. A subdomain in the intracellular carboxyl-terminal tail of group III mGluRs binds calmodulin and heterotrimeric guanosine triphosphate–binding protein (G protein) βγ subunits in a mutually exclusive manner. Mutations interfering with calmodulin binding and calmodulin antagonists inhibit G protein–mediated modulation of ionic currents by mGluR7. Calmodulin antagonists also prevent inhibition of excitatory neurotransmission via presynaptic mGluRs. These results reveal a novel mechanism of presynaptic modulation in which Ca2+-calmodulin is required to release G protein βγ subunits from the C-tail of group III mGluRs in order to mediate glutamatergic autoinhibition.

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Mouse brain cDNA was used to amplify (7) the sequence encoding the C-tail of the group III receptor 7. The variant C-tails, m-7A and m-7B (Fig. 1A), were expressed as recombinant proteins fused to glutathione S-transferase (GST) and used in an affinity purification procedure (8) from rat brain extract (Fig. 1B). Comparison of Coomassie blue-stained gel lanes with parallel samples obtained with immobilized GST indicated that an 18-kD protein bound specifically to both GST-7A (Fig. 1B) and GST-7B that was not stained with silver (Fig. 1C). Poor reactivity toward silver is associated with acidic proteins; immunostaining identified the 18-kD band as calmodulin (molecular mass 18 kD; pl ~ 4), a Ca²⁺ sensor protein (9) known to bind to mGluR 5 (10). Purification in the presence of Ca²⁺ or of EGTA revealed that calmodulin binding to both GST-7A and GST-7B was Ca²⁺ dependent (Fig. 1D). The binding of calmodulin occurred at <100 nM Ca²⁺ and with a dissociation constant K_d of 57 nM at saturating Ca²⁺ concentrations (11).

The interaction between Ca²⁺-calmodulin and the C-tail of mGluR 7 was confirmed in binary binding assays (8) with immobilized fusion proteins and purified calmodulin. A fusion protein encompassing the 27 COOH-terminal amino acids of mGluR 7A (C27; see Fig. 1A) failed to bind calmodulin, but fusion constructs N38 and N25 containing the common NH₂-terminal fragments (Fig. 1A) both bound calmodulin (12). The 25 amino acids of N25 are highly conserved in all members of group III but not other mGluRs and display homology to a consensus calmodulin binding sequence (13). Calmodulin binding to GST-7A was fully prevented by the irreversible calmodulin antagonist ophiobolin A (12, 14). Also, mGluR 7 solubilized from rat hippocampal synaptosomes bound to Ca²⁺-calmodulin immobilized on agarose beads, thus confirming binding of Ca²⁺-calmodulin by a native group III mGluR (15).

The tail regions of heptahelical receptors contribute to G protein coupling. We therefore examined whether binding of calmodulin to mGluR 7 might interfere with its interaction with trimeric G protein subunits. When detergent extracts of porcine brain membranes were used in pull-down experiments with immobilized GST-7A (16), a band migrating at ~35 kD was retained and recognized by an antiserum to G protein β subunits

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**Fig. 1.** A Ca²⁺-dependent calmodulin binding site exists on the C-tails of mGluR 7 splice variants. (A) Alignment of the mGluR 7A and 7B tail sequences. Partial mGluR 7A sequences used in pull-down experiments are indicated by bars. Italic characters represent regions of alternative splicing; bold characters indicate the potential calmodulin binding domain. (B) The C-tail of mGluR 7A fused to GST (GST-7A; top) and GST alone (bottom) were immobilized on glutathione-Sepharose and incubated with a cytosolic extract of rat brain (lane 1). After collection of the flow-through (lane FT) and sequential washes (lanes W1 to W3), bound proteins were sequentially eluted with 15 mM glutathione (lanes E1 and E2) and SDS-PAGE sample buffer (lane E3). SDS-PAGE revealed that GST-7A, but not GST, bound an 18-kD protein. (C) Brain cytosolic proteins bound to GST-7A or GST-7B were eluted with SDS-PAGE sample buffer and analyzed by Coomassie blue and silver staining. Note that the 18-kD band revealed by Coomassie staining was not stained by silver. Protein immunoblotting identified the 18-kD copurifying protein as calmodulin (CaM). (D) Incubating rat brain cytosolic proteins with GST, GST-7A, or GST-7B in the presence of CaCl₂ (1 mM) or EGTA (5 mM) revealed that the binding of calmodulin to the mGluR 7 tail regions is Ca²⁺ dependent. The experiments shown in (B) to (D) were performed 2 to 10 times.

**Fig. 2.** Binding of Giβγ to the C-tail of mGluR 7A. (A) A CHAPS extract of porcine brain membranes (input = lane in) was incubated with the mGluR 7A tail region (GST-7A) or GST. Unbound proteins were recovered in the supernatant (lanes sn); after three washes (lanes 1 to 3), bound proteins were eluted with 30 mM glutathione (lanes el). Equal fraction volumes were subjected to SDS-PAGE and protein immunoblotting with a mixture of antibodies to Go and Gβ. (B) Interaction between purified oligomeric G proteins (30 pmol) and GST-7A or GST in the presence (+CaM) and absence of Ca²⁺ and calmodulin. For conditions and lane labels, see (A). (C) Binding of purified recombinant Go_{i1} (60 pmol) to GST or GST-7A in the presence (+CaM) and absence (−CaM) of Gβγ dimers (120 pmol). (D) Binding of purified Gβγ (30 pmol) to GST-7A and GST-7A−ΔCaM in the absence (−CaM) and presence (+CaM) of Ca²⁺ and calmodulin. In (C) and (D), only the input (in), third wash (3), and elution (el) lanes are shown. All data were corroborated in three to five separate experiments.
(Gβγ) (Fig. 2A). In contrast, only trace amounts of G protein α subunits (Gα) were detected. A specific interaction with G protein βγ dimers (Gβγ) was confirmed using purified proteins (16). Because group III mGluRs signal via pertussis toxin-sensitive G proteins (2, 17), we tested binding with a mixture of heterotrimeric Gi and Go, GST-7A, but not GST, bound the β subunit (Fig. 2B). Gβγ chromatographically resolved from α subunits also associated with GST-7A (Fig. 2D). In contrast, GST-7A failed to specifically bind Gα alone, but retained it in the presence of Gβγ (Fig. 2C). Furthermore, GST-7A did not accelerate the rate of guanosine 5'-O-(3'-triotriphosphate) (GTPγS) binding to Gα in both the absence and the presence of βγ dimers (18), which is consistent with the m-7A tail not contacting Gα in the bound trimeric G protein. Ca2+-calmodulin inhibited the interaction between GST-7A and Gβγ, regardless of whether incubations contained heterotrimeric G proteins (Fig. 2B) or βγ dimers (Fig. 2D). Thus, binding of Gβγ and calmodulin is specific and mutually exclusive.

To assay transmitter release and its regulation by presynaptic receptors, we monitored autaptic currents of hippocampal neurons on micro-islands of glial cells (19, 20). The group III–selective mGluR agonist L-AP4 (6, 17) caused inhibition of glutamatergic signaling (Fig. 3, A, B, and D), but not GABAAergic (21), autaptic currents (GABA, γ-aminobutyric acid). This effect was not observed in cultures treated with pertussis toxin, indicating that L-AP4 activates G/Go proteins (2, 17) (Fig. 3A). L-AP4 did not affect glutamate-evoked currents, consistent with its presynaptic action (Fig. 3C). Reverse transcription polymerase chain reaction (RT-PCR) analysis of RNA from cultured hippocampal neurons (12) corroborated the expression of group III mGluRs 4, 7A, 7B, and 8 in rat hippocampus (22). A concentration-response curve for the inhibition of glutamatergic transmission by L-AP4 revealed a median effective concentration of 60 ± 18 μM (Fig. 3A). This inhibition was attenuated by the group III–preferring mGluR agonists (RS)-α-methylserine-O-phosphate (MSOP) (23) and (RS)-α-methyl-4-phosphonophenylglycine (MPPG) (3), but not by (RS)-α-methylserine-O-phosphate monophosphophoryl ester (MSOPPE) (23), which preferentially blocks group II mGluRs (Fig. 3B); none of these antagonists had an effect when applied alone. Hence, L-AP4 inhibits glutamate release via group III mGluRs, with a predominant role of the low-affinity receptors mGluR 7A or 7B (17).

Detection of receptors to the calmodulin antagonists ophiobolin A (14) or calmidazolium (24) abolished presynaptic inhibition by L-AP4 (Fig. 3, D and E). This effect was specific to mGluRs, because inhibition by somatostatin (Fig. 3F) or cyclopentyladenosine (CPA; Fig. 3, D and F)—which act via presynaptic SRIF (20) or A1 (25) receptors, respectively—was not altered. Control experiments showed that L-AP4 (300 μM) had no significant effect on the average frequency and mean amplitude of miniature excitatory postsynaptic currents (MEPCs), which occur independently of Ca2+ entry, but significantly reduced the frequency of MEPCs raised by depolarization with 10 mM KCl (21).

To directly demonstrate that calmodulin binding is critical for mGluR7 signaling, we generated a mutant 7A-ΔCaM, in which amino acids 864 to 876 (KAVVTAATMSSRL) (26) were deleted from the tail’s calmodulin binding site. GST-7A-ΔCaM failed to interact with calmodulin under the conditions described previously (8, 12) but bound Gβγ as efficiently as the wild-type receptor (Fig. 2D).

Calmodulin proved unable to compete with binding of Gβγ to this fusion construct (Fig. 2D). Only 6.8 ± 6.7% of control Gβγ binding was found by densitometry with GST-7A in the presence of Ca2+-calmodulin, whereas GST-7A-ΔCaM bound 90.6 ± 19% under the same conditions (n = 3). We therefore tested a full-length mGluR7A-ΔCaM construct for functional coupling by coexpression with concatenated pairs of G protein–activated inwardly rectifying Kir channels (27). HEK 293 cells cotransfected with wild-type mGluR7A and Kir3.1/3.2 generated an inward current of -693 ± 190 pA (n = 13) in response to hyperpolarizing voltage

**Fig. 3.** Presynaptic inhibition of glutamate release at hippocampal autapses by group III metabotropic glutamate receptors is attenuated selectively by calmodulin antagonists. (A) Concentration-response relation for the reduction of glutamatergic autaptic currents by L-AP4 [as shown in (D)] in either untreated neurons (○) or in neurons treated with pertussis toxin (100 ng/ml) for 24 hours (●), n = 4 to 8. (B) Inhibitory action of 300 μM L-AP4 alone in the presence of 100 μM MSOP, MPPG, MSOPPE, or CPA (C), and 1 μM somatostatin (S) before (white bars) and after (black bars) 3-min applications of either 25 μM ophiobolin A (as shown in (D)) or 1 μM calmidazolium (as shown in (E); calmidazolium was also present during the second application of agonists); n = 4 to 10.
performed in 25 mM external K in the absence of ophiobolin A (right panel). Dashed line indicates zero current level; all recordings were conducted increase in inward current was seen even in the absence of ophiobolin A (right panel). Dashed line indicates zero current level; all recordings were performed in 25 mM external K. (B) Whole-cell current responses to 1-s voltage ramps between −150 and +60 mV recorded from cells coexpressed with mGlur 7A and Kir3.1/3.2 in the presence of 100 μM L-AP4 before and after incubation with ophiobolin A. The thick black trace represents the subtraction current (SC). (C) Kir3.1/3.2 current amplitudes at −100 mV in the presence of 100 μM L-AP4 before (−546 ± 210 pA) and after a 2-min incubation with 25 μM ophiobolin A (−106 ± 27 pA; n = 11 each) for mGlur 7A (white bar), and without ophiobolin A for mGlur 7A–ΔCaM (black bar).

Steps that were prominently enhanced by 100 μM L-AP4 (−1239 ± 397 pA for Kir3.1/3.2, n = 13) (Fig. 4A). In cells responding to L-AP4, the current-voltage relation of basal and agonist-induced currents showed steep voltage dependence with strong rectification typical of h<sub>in</sub> currents (Fig. 4B). Both the onset of current activation by L-AP4 and recovery upon removal of the ligand occurred rapidly with a rate of 2 to 3 s<sup>−1</sup>, suggesting that the Kir open probability is regulated by mGlur 7A via G<sub>B</sub> (31). Channel activity dependence of autoinhibition previously observed at glutamatergic synapses (33).

References and Notes

To generate sequences encoding the COOH-terminal region of mGlur 7 (N. Okamoto et al., J. Biol. Chem. 269, 1231 (1994)), we used oligonucleotides complementary to the codons of amino acids 851 to 857 [H. Betz, M. Barnes-Davies, K. Onodera, Science 274, 594 (1996)].

To produce GST fusion proteins, we subcloned cDNAs into the Eco RI and Sal I sites of pGEX-5X1 before transformation into E. coli BL21 (Stratagene). The cytosol of bacteria expressing the GST-fusion proteins was incubated with glutathione Sepharose 4B for 2 hours at 4°C followed by sequential washes (washes 1 to 3) in binding buffer [BB; 20 mM Heps, 100 mM KCl (pH 7.4), and protease inhibitor cocktail Complete (Boehringer-Mannheim)] containing 0.1% (w/v) Triton X-100, and BB containing 2 mM MgCl<sub>2</sub> and 0.5 mM adenosine triphosphate (ATP). Immobilized fusion proteins were then used in binding assays. Cytosolic fractions were obtained from freshly dissected rat brain, and SDS–polyacrylamide gel electrophoresis (PAGE) and protein immunoblotting were performed according to published procedures (L. L. Pellegrini, V. O’Connor, L. Zottles, H. Betz, EMBO J. 14, 4705 (1995)). The fractions (∼5 mg protein/ml) were dialyzed into BB, cleared by centrifugation, and preadsorbed by incubation for 2 hours at 4°C with GST bound to glutathione-Sepharose 4B (Pharmacia). Aliquots of these fractions were supplemented with 1 mM CaCl<sub>2</sub> or 5 mM EGTA (when indicated) and incubated for 2 hours at 4°C with the respective GST fusion protein prebound to glutathione-Sepharose (1 ml). After removal of unbound proteins and washing of the beads in BB containing the appropriate CaCl<sub>2</sub> (1 mM) or EGTA (5 mM) supplement, bound proteins were eluted with 15 mM reduced glutathione or SDS-PAGE sample buffer. In binary binding reactions, 20 μg of immobilized fusion protein (GST-7A) was incubated with 100 μg of calmodulin (Calbiochem) for 2 hours at 4°C in BB supplemented with either CaCl<sub>2</sub> or EGTA. After repeated washing, the beads were eluted with SDS–PAGE sample buffer.


Affinity measurements were done by fluorescence spectroscopy using dansylated calmodulin.

V. O’Connor, O. El Far, J. Airas, unpublished data.


V. O’Connor, unpublished data.

Porcine brain membranes (prepared with EDTA and EGTA, 1 mM each) were extracted in the presence of 10 mM CHAPS (29). Oligomeric G proteins [G<sub>i</sub> and G<sub>β</sub>~>80% G<sub>β</sub>], purified from bovine or porcine brain membranes; G<sub>i</sub>βγ was chromatographically resolved from Go [R. Jockers et al., J. Biol. Chem. 269, 32077 (1994)]; Detergent extracts (containing 100 μg of protein), 30 pmol of purified oligomeric G proteins or G<sub>i</sub>γγ, were added to the indicated amounts of purified recombinant G<sub>i</sub>βγ [S. M. Munday and M. E. Linder, Methods Enzymol. 237, 325 (1994)], incubated with 10 μg of GST, GST-7A, or GST-7A–ΔCaM for 1 hour at 20°C in GBB (20 mM GTPγS, 100 mM Tris–HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 8 mM CHAPS, and 10 μM guanosine diphosphate) in a total volume of 0.14 ml. After addition of 200 pmol of calmodulin and 2 mM Ca<sup>2+</sup>, preequilibrated glutathione...
one-Sepharose 4B beads (20 μl of a 50% slurry) were added, and incubation continued for 2 hours at 4°C. Ca²⁺ did not affect the binding of Gbg to GST-7A. After repeated washing, bound proteins were eluted with 30 mM glutathione or SDS sample buffer. Immunoblots were done with antiserum 7 (Gbg-common) or antiserum 11 (Gα-common) (29).

18. M. Freissmuth, C. Nanoff, E. Bofill-Cardona, data not shown.
19. Hippocampi from neonatal Sprague-Dawley rats were dissociated and plated onto micro-islands of glia cells according to published methods (20) [J. M. Bekkers and C. F. Stevens, Proc. Natl. Acad. Sci. U.S.A. 88, 7834 (1991)]. After 10 to 15 days in vitro, autaptic currents were recorded at 20° to 24°C from neuronal somata (20). Neurons were clamped at -70 mV and depolarized for 1 to 2 ms to 0 mV once every 20 s. Stimulation and current recordings were performed with a List EPC-7 amplifier controlled by PClamp 6.0 software (Axon Instruments). Electrodes pulled from borosilicate glass capillaries (flaming/