MacMARCKS interacts with the metabotropic glutamate receptor type 7 and modulates G protein-mediated constitutive inhibition of calcium channels

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Abstract
We have previously shown that the interaction of Ca²⁺/calmodulin with the metabotropic glutamate receptor type 7 (mGluR7) promotes the G-protein-mediated inhibition of voltage-sensitive Ca²⁺ channels (VSCCs) seen upon agonist activation. Here, we performed a yeast two-hybrid screen of a new-born rat brain cDNA library using the cytoplasmic C-terminal tail of mGluR7 as bait and identified macrophage myristoylated alanine-rich c-kinase substrate (MacMARCKS) as a binding protein. The interaction was confirmed in vitro and in vivo by pull-down assays, immunoprecipitation, and colocalization of mGluR7 and MacMARCKS in transfected HEK293 cells and cultured cerebellar granule cells. Binding of MacMARCKS to mGluR7 was antagonized by Ca²⁺/calmodulin. In neurons, cotransfection of MacMARCKS with mGluR7, but not mGluR7 mutants unable to bind MacMARCKS, reduced the G-protein-mediated tonic inhibition of VSCCs in the absence of mGluR7 agonist. These results suggest that competitive interactions of Ca²⁺/calmodulin and MacMARCKS with mGluR7 control the tonic inhibition of VSCCs by G-proteins.

Keywords: calmodulin; metabotropic glutamate receptor; calcium channel; G-protein; protein kinase C; macrophage myristoylated alanine-rich c-kinase substrate/F52.

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In an attempt to identify new mGluR7 interactors and potential PKC targets as molecular components of the mGluR7 signalosome (El Far and Betz 2002), we used the C-terminal tail regions of the mGluR7 splice variants A and B (O’Connor et al. 1999) as baits in a GAL-4 based yeast two-hybrid screen. This screen identified MacMARCKS (macrophage myristoylated alanine rich C kinase substrate, also named F52, or MRp for MARCKS-related-protein), a member of the MARCKS protein family (Kato 1990; Li and Aderem 1992; Ramsden 2000), as novel binding partner of mGluR7. Membrane-associated MARCKS proteins are known to be translocated to the cytosol upon phosphorylation by PKC or binding of Ca\(^{2+}\)/calmodulin (Kim et al. 1994). Here we show that MacMARCKS binds directly to the cytoplasmic tail domain of mGluR7, and that this interaction is antagonized by Ca\(^{2+}\)/calmodulin. In addition, we provide evidence that competitive interaction between MacMARCKS and Ca\(^{2+}\)/calmodulin modulates the mGluR7-mediated tonic inhibition of VSCCs in the absence of receptor agonist.

**Experimental procedures**

**GAL4-based yeast two-hybrid screen**

cDNA fragments encoding the complete tail regions of mGluR7A and mGluR7B were generated by PCR as described elsewhere (O’Connor et al. 1999) and inserted into the pGBT9 bait vector (Clontech, Palo Alto, CA, USA) using EcoR1 and Sal1 restriction sites. 10\(^5\) independent clones of a newborn rat brain cDNA library (Kins et al. 2000) subcloned into the pAD vector (Stratagene, Amsterdam, the Netherlands) were screened with a mixture of both bait vectors by sequential transformation into the Y190 yeast strain. Positive clones were selected based on β-galactosidase expression and their ability to grow on medium containing 25 mM 3-aminotriazole and lacking tryptophan, leucine and histidine. Of 32 positive clones analyzed by PCR, only one proved reproducibly positive and not trans-activating upon re-transformation with the empty pGBT9 vector. This clone contained an in-frame insertion of the full-length rat homologue (AJ301677) of mouse MacMARCKS (NM_010807) with an additional 5\(^\prime\) untranslated sequence of 165 bp lacking any stop codon. Within this additional sequence, only three base pair exchanges were found as compared to the mouse cDNA (guanines at positions 35, 44 and 124 of the mouse sequence are adenines in rat).

**Expression constructs and protein expression in bacteria**

The MacMARCKS-EGFP expression plasmid used has been described previously (Jess et al. 2002). Glutathione S-transferase (GST) as well as maltose binding protein (MBP) fusion constructs of the mGluR7A and mGluR7B C-termini (GST-7A, GST-7B, MBP-7A and MBP-7B) were generated as detailed elsewhere (El Far et al. 2001). The MacMARCKS cDNA was inserted into pGEX-5×1 (GST-MacMARCKS) using the EcoR1 cloning sites. To this end, the full-length MacMARCKS sequence was excised from the yeast library plasmid using the EcoR1 restriction site present at the 3′ untranslated sequence of the human MacMARCKS gene.
junction between the GAL4 activation domain and the MacMARCKS insert and an endogenous EcoR1 site found in the 3' UTR after the endogenous MacMARCKS stop codon. Flag-tagged (mouse) and myc-tagged (rat) full-length mGluR7 cDNAs were constructed as reported previously (El Far et al. 2000; Perroy et al. 2000). The mGluR7A deletion mutant lacking the interaction site (aa 877–899) for MacMARCKS, mGluR7AΔ(MacMARCKS), was obtained by introducing a silent NruI site in the myc-mGluR7A tail cDNA at the position of the serine 874/arginine 875 codons. Using this NruI and a KpnI site present in the 3' UTR, the wild-type sequence was replaced by a deletion mutant fragment generated by PCR. Construction of the MBP-7A-Δcalmodulin, MBP-N25, MBP-N49 and MBP-7A-F863A plasmids and expression of GST and MBP fusion proteins in *Escherichia coli* BL21 (Stratagene, La Jolla, CA, USA) were performed as previously described (El Far et al. 2001).

**Antibody production**

Purified GST-MacMARCKS fusion protein was used to immunize two New Zealand rabbits following standard procedures using MPL + TDM + CWS emulsion (Sigma-Aldrich, Munich, Germany) as adjuvant. The immune serum (15 mL) was depleted from anti-GST antibodies by overnight incubation at 4°C with a mixture of 1 mg GST immobilized on nitrocellulose and 1.5 mg GST immobilized on glutathione-Sepharose beads (Amersham Biosciences, Freiburg, Germany). IgGs were then precipitated with saturated ammonium sulfate solution and dialyzed against 25 mM HEPES buffer, pH 7.4.

FlagGluR7 antisera were raised in rabbits against a synthetic peptide derived from the mGluR7a sequence SLRSHKPSDR-PNGEAKTELCEVNVDPNSPAKAKKTVYSNNLVI. Rabbits were immunized with 1 mg of peptide attached to bAlA-TENTAGEL beads (Rapp Polymere GmbH, Tübingen, Germany), dispersed in phosphate-buffered saline (PBS) and mixed with Complete Freund’s adjuvant (1.0 mL; Sigma-Aldrich, Prague, Czech Republic). Three successive booster injections were given at 1-month intervals using 0.5 mg of antigen mixed with Incomplete Freund’s adjuvant (0.5 mL). Rabbits were bled by intracardial puncture, and sera were stored in small aliquots at −70°C. Two of four immunized animals produced high titers of mGluR7-specific antibodies. The sera were tested by immunoblotting at dilutions of 1 : 1000 and 1 : 10 000 using rat brain homogenate, HEK293 cells transfected with a mammalian expression vector carrying the mGluR7A cDNA (Corti et al. 1998), or, as a control, untransfected cells. A band corresponding to the mGluR7A was present only in brain lysate and transfected cells.

**GST and MBP pull-down assays, binding studies and competition experiments**

Glutathione-Sepharose beads (Amersham Biosciences) and amylose beads (New England Biolabs, Frankfurt am Main, Germany) were loaded with GST and MBP fusion proteins, respectively. In the presence of 1 mM CaCl2 or 5 mM EGTA, the beads loaded with GST fusion proteins were incubated with either the indicated MBP fusion proteins or with extracts of Flag-tagged full-length mGluR7A or 7B-expressing HEK293 cells. The integrity of the full-length receptor in the HEK293 extracts was verified by purification on a calmodulin agarose (Sigma-Aldrich, Munich, Germany) matrix as described previously (O’Connor et al. 1999). Similarly, the beads loaded with the MBP fusion proteins were incubated with the indicated GST fusion proteins. After washing with binding buffer (BB: PBS, 0.1% Triton X-100 containing either CaCl2 or EGTA), bound proteins were eluted in sodium dodecyl sulfate (SDS) sample buffer and analyzed by western blotting using either anti-MBP polyclonal antibody (New England Biolabs) or the anti-MacMARCKS serum (1 : 1000 dilution). For competition experiments, GST-MacMARCKS (6 μM) immobilized on glutathione-Sepharose beads was incubated in BB with 2 μM MBP-7A and either increasing concentrations of calmodulin (0, 0.4, 0.8 and 2 μM) in the presence of 1 mM CaCl2 or 2 μM calmodulin in the presence of 5 mM EGTA. After washing, bound proteins were analyzed by western blotting using both anti-MBP and anti-calmodulin (Upstate Biotechnology, Dundee, UK) antibodies. Relative band intensities were determined using NIH Image software (https://rsb.info.nih.gov/nih-image/).

**Comununoprecipitation**

HEK293 cells coexpressing MacMARCKS-EGFP and either myc-mGluR7A or myc-mGluR7AΔ(MacMARCKS) were solubilized in PBS containing Triton X-100 (1.5% w/v), protease inhibitors and 5 mM EDTA and incubated overnight with 5 μg of a polyclonal antimmun antibody (Upstate Biotechnology). Immune complexes were recovered by incubating protein-A-agarose (Amersham Biosciences). Input and immunoprecipitated complexes were resuspended in SDS sample buffer and incubated for 30 min at 60°C under reducing conditions prior to 10% SDS-polyacrylamide gel electrophoresis (PAGE). Immunoprecipitated MacMARCKS-EGFP was detected by western blotting using a monoclonal anti-GFP antibody (Roche, Meylan, France). Immunoprecipitation of myc-mGluR7A and myc-mGluR7AΔ(MacMARCKS) was monitored by probing the western blot with a monoclonal antimmun antibody (9E10, Sigma).

Cerebellar granule cell cultures were lysed using a protocol similar to that described above. Polyclonal anti-mGluR7 antibody was covalently coupled to protein A-conjugated magnetic beads (Ademtech, Pessac, France). Twenty microliters of the anti-mGluR7 coated beads (or beads only as control) were added to 500 μL of cell lysate overnight at 4°C. Immunocomplexes were washed with the following buffer: Na2HPO4 (10 mM), NaCl (200 mM), EDTA (10 mM), NP-40 (0.5% w/v) and SDS (0.1% w/v), resuspended and analysed as described above. Immunoprecipitated endogenous MacMARCKS was detected by western blotting using a polyclonal anti-MacMARCKS antibody (Chemicon, Hampshire, UK). These experiments were repeated three times.

**Neuronal cell culture and transfection**

Mouse cultured cerebellar granule cells were prepared as previously described (Perroy et al. 2002). Briefly, 8-day-old mice were decapitated, and the cerebella dissected and mechanically dissociated using fire-polished Pasteur pipettes. The cell suspension was centrifuged at 600 × g, and the cells were resuspended in serum-free Dulbecco’s modified Eagle’s medium (DMEM) : F12 medium (1 : 1, Gibco, Gergy Pontoise, France) supplemented with 30 mM glucose, 2 mM glutamine, 13 mM sodium bicarbonate and 5 mM HEPES, pH 7.4. cDNAs (1 μg each for 2.5 × 10^5 cells) were transfected using the lipid-based transfection reagent TransFast (Promega, Mannheim, Germany) (Ango et al. 1999). Cells were then plated on 35 mm dishes coated with poly-L-ornithine and poly-L-lysine. The cells were then incubated for 3 days at 37°C and used for all experiments.
maintained for 8–10 days in vitro in medium supplemented with 10% semisynthetic calf serum (Biowhittaker, Verviers, Belgium), antibiotics and KCl (25 mm).

**Immunocytochemistry**

HEK293 cells grown on fibronectin-coated coverslips were transfected with the expression constructs. Forty-eight hours after transfection, cells were washed with PBS and fixed with ice-cold 4% (w/v) paraformaldehyde in PBS at 22°C for 5 min. The cells were treated with PBS containing 5% bovine serum albumin and to 0.25% (w/v) Triton X-100 before exposure to antimyc purified mouse monoclonal (1 : 1000 dilution, Sigma) antibody to detect myc-tagged mGluR7 constructs. mGluR7 immunostaining was then revealed using Alexa-594 conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Hamburg, Germany).

Cultured cerebellar granule cells were washed and fixed as described for HEK293 cells and incubated overnight at 4°C with antimyc purified mouse monoclonal antibody (as above). They were then washed and incubated for 1 h at 22°C in the presence of Alexa-594-conjugated secondary antibody (as above) to detect mGluR7 constructs. Confocal images were taken using a Leica TCS-SP microscope equipped with Leica TCS-NT 16551 software.

**Electrophysiology**

Mature (8–10 days in vitro) cerebellar granule cells expressing either the transfection marker EGFP alone or the MacMARCKS-EGFP protein were used for whole-cell patch-clamp recording at room temperature (20–24°C). The neurons were continuously perfused with an extracellular solution containing 20 mm BaCl2, 10 mm HEPES, 9 mM tetraethylammonium acetate, 130 mm Na-acetate, 15 mm glucose, and 0.3 mm tetrodotoxin, adjusted to pH 7.4 with NaOH and to 330 mosm with Na-acetate. Recording pipettes were pulled from borosilicate glass and had a resistance of 3–5 MΩ when filled with a solution containing 100 mm Cs-acetate, 20 mm CsCl, 2 mm MgCl2, 10 mm HEPES, 20 mm EGTA, 1 mm cAMP, 2 mm Na2-ATP, 15 mm glucose, adjusted to pH 7.2 with CsOH and to 300 mosm with Cs-acetate. Barium currents were recorded using an Axopatch-200 amplifier (Axon Instruments, Molecular Device Corp., Sunnyvale, CA, USA). Cell capacitance and series resistance compensation were applied electronically. Data were filtered at 1 kHz, digitized at 5 kHz and analyzed using PCLAMP 8 (Axon Instruments) and ORIGIN 5.0 (Microcal, Northampton, MA, USA). Records were leak subtracted using the PCLAMP software (p/4 protocol). Barium currents were evoked by 500-ms pulses at 0 mV from an holding potential of −60 mV. Facilitation of the evoked currents was assessed by a double-pulse protocol (Bertaso et al. (2003)). A first 30-ms step (P1) to −20 mV was followed by a 300 ms period of repolarization to −80 mV. To assess the voltage-dependence of current inhibition, a strong depolarizing prepulse (PP) of 30 ms to +100 mV was delivered prior to a second pulse (P2) to the same voltage as the first test pulse (P1). PP and P2 were separated by a 10-ms repolarization time to −80 mV. Pulses were delivered every 10 s. Currents were measured 10 ms after the onset of P1 or P2, and the average over a 2-ms period was calculated and used for subsequent analysis. The amount of voltage-dependent facilitation of barium current induced by PP stimulation was expressed as the ratio of amplitude of the current evoked by P2 over the one evoked by P1.

**Results**

MacMARCKS interacts with the cytoplasmic tail region of mGluR7

To identify new intracellular proteins that interact with the C-terminus of mGluR7, we performed a GAL4-based yeast two-hybrid screen using a mixture of bait cDNAs encoding both the mGluR7A and mGluR7B tail regions. From 10^6 colonies screened, a single positive clone was isolated with the mGluR7B tail sequence. This clone contained the entire open reading frame of the rat MacMARCKS cDNA and 165 bp of the 5′ untranslated sequence.

To confirm an interaction of MacMARCKS with the mGluR7 tails, we generated a rabbit polyclonal serum directed against a GST-MacMARCKS fusion protein. As shown in Fig. 1, this serum recognized this fusion protein, as well as MacMARCKS-enhanced green fluorescent protein fusion (MacMARCKS-EGFP) expressed in HEK293 cells. Only weak unspecific staining was seen with GST-containing samples. In cultured mouse cerebellar granule cells transfected with the MacMARCKS-EGFP expression construct, the serum stained the recombinant fusion protein (68-kDa band) and two bands of 40 kDa and 36 kDa, which probably represent endogenous MacMARCKS protein.

Using this antiserum it was possible to confirm the results from the two-hybrid screen by pull-down assays with GST-
MacMARCKS and recombinant MBP-7A and MBP-7B tail fusion proteins, respectively. Figure 2a shows binding experiments with MBP, MBP-7A and MBP-7B immobilized on amylose beads and incubated with GST-MacMARCKS, in the presence of either 1 mM CaCl2 or 5 mM EGTA. The GST-MacMARCKS fusion protein bound MBP-7A and MBP-7B, but not MBP, in a Ca2+-independent manner. In a second set of experiments, GST-MacMARCKS immobilized on glutathione beads was incubated with MBP, MBP-7A or MBP-7B (Fig. 2b). Western blot analysis with anti-MBP polyclonal serum revealed that MBP-7A and MBP-7B, but not MBP, bound specifically to GST-MacMARCKS. These results indicate that MacMARCKS interacts directly with the C-terminal tails of mGluR7. We then examined whether MacMARCKS binding can also be demonstrated with the detergent solubilized full-length receptor. To this end, detergent extracts of HEK293 cells expressing recombinant Flag-tagged mGluR7A and mGluR7B were incubated with GST-MacMARCKS immobilized on glutathione-Sepharose beads. Both full-length mGluR7 isoforms bound specifically to GST-MacMARCKS, but not to GST alone (Fig. 2c). However, and in contrast to the results obtained with the mGluR7 tail fusion proteins, recombinant full length mGluR7 binding to GST-MacMARCKS was detected only in the presence of 5 mM EGTA, not in the presence of 1 mM Ca2+ (Fig. 2c). The molecular integrity of Flag-mGluR7A and B expressed in HEK293 cells and offered for binding to GST-MacMARCKS was verified by purifying these receptors from transfected HEK293 cells on a calmodulin agarose matrix (Fig. 2c, ‘input’ lane).

Mapping of the mGluR7 interaction site with MacMARCKS

As MacMARCKS bound to both the mGluR7A and mGluR7B tails, the MacMARCKS interaction site of mGluR7 was likely to reside within the common C-terminal region conserved between these splice variants. The mGluR7A and mGluR7B sequences are identical throughout the first 899 amino acids (aa), including the proximal C-terminal 49 aa of the tail region (Fig. 3a). To further delineate the interaction borders within this domain, we used GST-fused mutant and deletion constructs (Fig. 3a). First, we tested an MBP fusion protein, MBP-7-N49, harboring the first proximal 49 aa of mGluR7 tail, which are shared by isoforms. This construct supported MacMARCKS binding. In contrast, a fusion protein including only the first 25 aa, MBP-N25, which binds both Ca2+/calmodulin and Gβγ (O’Connor et al. 1999; El Far et al. 2001), failed to interact with MacMARCKS (Fig. 3b). These data suggested that the MacMARCKS interaction site of mGluR7 is distinct from the Ca2+/calmodulin and Gβγ binding sites. Hence, we examined whether determinants of the Ca2+/calmodulin and Gβγ binding sites of mGluR7 are important for MacMARCKS interaction. Both deletion constructs, MBP-7A-Calmodulin, which does not bind to Ca2+/calmodulin (O’Connor et al. 1999), and an MBP fused tail mutant, MBP-7A-F863A, which displays reduced affinity for Ca2+/calmodulin (El Far et al. 2001), still bound to GST-MacMARCKS (Fig. 3b). Together these results indicate that the MacMARCKS binding site of mGluR7 lies within the 23 aa peptide sequence positioned between serine 877 and asparagine 899. Thus, the structural determinants of Ca2+/calmodulin binding do not overlap with the MacMARCKS binding site.

To further confirm that the 23 aa sequence delineated above is necessary for MacMARCKS binding to mGluR7, we generated a myc-tagged full-length mGluR7A that lacked this putative binding region (mGluR7 ΔMacMARCKS)). Upon coexpression in HEK293 of myc-tagged wild-type or mGluR7(ΔMacMARCKS) with MacMARCKS-EGFP, antimyc antibodies coimmunoprecipitated MacMARCKS-EGFP.
only from extracts containing wild-type mGluR7A but not mGluR7(ΔMacMARCKS) (Fig. 3c). This further indicated that aa 877–899 are required for MacMARCKS binding to mGluR7.

**Ca2+ dependence of MacMARCKS–mGluR7 interaction and effect of Ca2+/calmodulin**

Although the Ca2+/calmodulin and MacMARCKS binding sites on mGluR7 are topologically distinct (Fig. 3a), their proximity could result in steric hindrance. Moreover, Ca2+ has opposite effects on the binding of calmodulin and MacMARCKS to mGluR7, facilitating the former and inhibiting the latter. These observations suggested that Ca2+/calmodulin and MacMARCKS may compete in binding to mGluR7, facilitating the former and inhibiting the latter. The deduced MacMARCKS binding region is boxed. The binding sites for Gβγ subunits and calmodulin (calmodulin) are also indicated. (b) GST-MacMARCKS immobilized on glutathione-Sepharose was incubated with MBP alone, the full length C-terminal constructs MBP-7A and MBP-7B, the deletion mutants MBP-N25, MBP-N49, and MBP-7A(Δcalmodulin), and the point mutation MBP-7A-F863A. Bound proteins were eluted and analyzed by western blotting using anti-MBP antibody. (c) Wild-type myc-mGluR7 or mutant myc-mGluR7(ΔMacMARCKS) were immunoprecipitated with monoclonal α-myc antibodies from transfected HEK293 cells coexpressing the corresponding receptor and MacMARCKS-EGFP. Immunoprecipitated material was analyzed by western blotting using monoclonal α-myc and α-EGFP. Input represents 1% of the cell extract used for immunoprecipitation. *1, *2 and *3 indicate, respectively, mGluR7 multimers, monomers and MacMARCKS-EGFP migration levels. CaM, calmodulin.
explains the calcium sensitivity of MacMARCKS binding to mGluR7 observed in Fig. 2c, as the HEK293 cell extract contained both Ca\(^{2+}\) ions and calmodulin.

Coimmunoprecipitation and localization of mGluR7 and MacMARCKS in living cells

To verify that the interaction of MacMARCKS with the C-terminus of mGluR7 takes place in living neurons, coimmunoprecipitation experiments were performed with extracts from non-transfected cultured cerebellar granule cells. Figure 5a shows that endogenous mGluR7-MacMARCKS complexes could be immunoprecipitated from the extracts using beads coated with our mGluR7 polyclonal antibody.

The interaction between MacMARCKS and mGluR7A in living cells was further confirmed by studies on the cellular localization of the complex in HEK293 cells and cerebellar neurons. In HEK293 cells, MacMARCKS-EGFP expressed alone showed a reticular distribution in 28 of 40 cells analyzed (Fig. 5b). In the remaining 12 cells, MacMARCKS displayed a more homogeneous (including membrane) distribution (not shown). Consistent with previous reports (El Far et al. 2000), expression of myc-tagged mGluR7A alone produced a clear surface staining in all 50 cells analyzed (Fig. 5c), indicative of plasma membrane insertion of the receptor. When coexpressed with MacMARCKS-EGFP, the myc-tagged mGluR7A was predominantly retained in MacMARCKS-EGFP rich intracellular structures in 42 of 45 cells observed (Fig. 5d). In contrast, in 39 of 50 cells analyzed, the myc-tagged mGluR7(\(\Delta\)MacMARCKS) mutant lacking the MacMARCKS binding site did not show this intracellular sequestration (Fig. 5e).

As the anti-MacMARCKS antibody was not optimal for immunofluorescent labeling, localization of endogenous MacMARCKS in neurons could not be studied. As an alternative approach, we cotransfected cultured cerebellar granule cells with MacMARCKS-EGFP and myc-tagged mGluR7A. Both proteins localized in cell body and dendrites, but with a predominant localization at the periphery of the soma in 36 of 40 neurons; Fig. 5f). On the other hand, when MacMARCKS-EGFP was cotransfected with the myc-tagged mGluR7(\(\Delta\)MacMARCKS) mutant, mGluR7(\(\Delta\)MacMARCKS) localized on the plasma membrane, whereas MacMARCKS-EGFP stayed in the cytosol (in 21 of 25 cells; Fig. 5g). These observations suggest that mGluR7A recruits MacMARCKS at the plasma membrane and corroborate our biochemical data showing interaction between these two proteins.

MacMARCKS binding to mGluR7 blocks G\(\beta\gamma\)-mediated tonic inhibition of voltage-sensitive Ca\(^{2+}\) channels

As mGluR7 inhibits VSCCs in cerebellar granule cells (Perroy et al. 2002), we examined the role of MacMARCKS in this receptor function. Barium currents were recorded in cerebellar granule cells using the whole-cell patch-clamp configuration. This technique allows current recording mainly in the somatic region, whereas cerebellar granule cells express endogenous mGluR7 only at presynaptic sites (Perroy et al. 2002). However, we have previously shown
that transfected mGluR7A is localized on the somatic plasma membrane of these neurons and induces inhibition of VSCCs (Perroy et al. 2002). Here, myc-mGluR7A and MacMARCKS-EGFP were coexpressed in cerebellar neurons (Fig. 5f). This did not significantly change the mGluR7A-mediated inhibition of Ba\(^{2+}\) currents seen upon application of the agonist, D,L-AP4 (600 \(\mu\)M; 34 ± 5% inhibition in the absence of MacMARCKS, \(n = 15\) [Fig. 6a1] and 36 ± 6% inhibition in the presence of MacMARCKS, \(n = 15\) [Fig. 6a2]). Thus, MacMARCKS did not affect the functional expression of mGluR7A at the neuronal plasma membrane. However, the following experiments showed that MacMARCKS modifies an agonist-independent inhibitory action of mGluR7 on VSCCs.

Tonic inhibition of VSCCs by G\(\beta\gamma\) is observed in many different neuronal cell types in the absence of G-protein-coupled receptor activation (Jeong and Ikeda 1998; Park and Dunlap 1998; Brody and Yue 2000). This inhibition is voltage-dependent, as it can be relieved by strong membrane depolarization. In cerebellar granule neurons transfected with mGluR7A, a prepulse depolarisation from –80 to +100 mV induced a 60% facilitation of Ba\(^{2+}\) currents evoked by depolarization pulses at –30, –20 or –10 mV (Fig. 6b1,b2). This effect was observed in the absence of agonist and is consistent with a G\(\beta\gamma\)-mediated tonic inhibition of VSCCs. Co-transfection of mGluR7A and MacMARCKS, but not of MacMARCKS alone (not shown), strongly reduced (down to <20%) the facilitation evoked by the prepulse depolarization (Fig. 6b1, b2). This reduction was not seen with mGluR7A(ΔMacMARCKS) (Fig. 6b2), although this mutant was functionally expressed at the cell surface, as assessed by both membrane immunostaining of non-permeabilized cerebellar granule cells (Fig. 5g) and D,L-AP4-induced inhibition of Ba\(^{2+}\) current in the transfected neurons (25 ± 7% inhibition, \(n = 11\); Fig. 6a3). These results are consistent with an agonist-independent
mGluR7A-mediated inhibition of VSCCs by Gβγ that is antagonized by the interaction of MacMARCKS with the receptor.

Interestingly, transfection of the mGluR7A(Δcalmodulin) mutant alone significantly reduced the voltage-sensitive tonic inhibition of Ba2+ currents (20 ± 10% inhibition; n = 8; Fig. 6b2), suggesting that binding of Ca2+/calmodulin to mGluR7 is required for receptor-dependent tonic inhibition of VSCCs.

Discussion

In this study, we identify MacMARCKS as a novel interaction partner of the cytoplasmic C-terminal tail domain of mGluR7. The rat, mouse, rabbit and human MacMARCKS sequences are 95% identical and share 52% identity with bovine and chicken MARCKS (Li and Aderem 1992). In particular, MacMARCKS and MARCKS display high sequence homology in the following regions of the molecule:

- the N-terminal myristoylation consensus site;
- the MH2 domain (MARCKS homology domain 2);
- the PKC substrate and central ‘effector domain’ that binds actin and Ca2+/calmodulin (Li and Aderem 1992).

Actin has been suggested to bind to the MH2 domain, which is required for the actin cross-linking activity of MARCKS (Hartwig et al. 1992).

The use of mutated and truncated mGluR7 C-terminal tail fusion proteins allowed us to delineate the MacMARCKS binding region of the mGluR7 tail. This region encompasses 24 amino acids (aa 877–899), starts immediately after the consensus Ca2+/calmodulin binding region and terminates at the end of the sequence conserved between the mGluR7A and mGluR7B splice variants. Although the MacMARCKS binding domain of mGluR7 is positioned outside the core Ca2+/calmodulin binding region, Ca2+/calmodulin and MacMARCKS were found to bind in a mutually exclusive manner to the recombinant mGluR7 tail region in pull-down experiments. This suggests that MacMARCKS binding may be regulated by changes in intracellular Ca2+ concentrations.

Endogenous mGluR7 and MacMARCKS proteins could be coimmunoprecipitated from mouse cerebellar granule cells extract, consistent with these proteins interacting in living neurons. Moreover, we found that mGluR7A can recruit MacMARCKS in HEK293 cells and neurons. This effect required the aa 877–899 MacMARCKS binding motif of mGluR7, suggesting that it depended on the physical interaction between the two proteins. In cotransfected HEK293 cells, mGluR7 immunoreactivity was mainly seen intracellularly, whereas in neurons it was found mainly at the membrane. The different localization of MacMARCKS in HEK293 cells and neurons when transfected with mGluR7 might be explained by a greater efficiency of expression of the transfected MacMARCKS by the cell line, which could result in a greater proportion of MacMARCKS not binding to the receptor. Alternatively, one might hypothesize that endogenous proteins not present in HEK cells are targeting receptor/MacMARCKS complex to neuronal membranes. In
mGluR7 expressing cerebellar granule cells MacMARCKS did not significantly alter the agonist-induced inhibition of Ca\(^{2+}\) currents, suggesting that MacMARCKS does not interfere with surface targeting of the receptor in neurons (Boudin et al. 2000; Perroy et al. 2002).

Another novel finding of this study is that MacMARCKS antagonizes the mGluR7-dependent tonic inhibition of VSCC by G\(\beta\gamma\) in the absence of agonist. Tonic inhibition of VSCCs by G\(\beta\gamma\) is found in different neuronal preparations (De Waard et al. 1997; Zamponi et al. 1997; Canti et al. 2000). Several studies have shown an agonist-independent activation of trimeric G-proteins by constitutively active receptors (for review see de Ligt et al. 2000)). A constitutive activity of mGluR7 has not been demonstrated yet and therefore cannot be excluded. The contribution of AGS (Activator of G-protein Signaling) proteins in generating a free G\(\beta\gamma\) pool that tonically inhibits VSCC should also be considered (Blumer et al. 2005). We found that MacMARCKS blocks the tonic inhibition of VSCCs, and that this effect depends on mGluR7, but not on mGluR7 agonist. Such an agonist-independent tonic inhibition has been previously shown for other GPCRs (Beedle et al. 2004).

Recently, the G-protein-coupled receptor ORL1 has been found to bind VSCCs, thereby forming a signalling complex in which the bound VSCC is inhibited by G\(\beta\gamma\). This inhibition was shown to be agonist-independent and receptor concentration-dependent (Beedle et al. 2004). Similarly, Kitano et al. (2003) have demonstrated that mGluR1 can inhibit Cav2.1 in the absence of agonist via an interaction between the cytoplasmic domains of the receptor and the channel proteins. In the case of mGluR7, no direct interaction of the receptor with Ca\(^{2+}\) channels has yet been found. Therefore, the mode of action of this receptor on Ca\(^{2+}\) channels may be different. Our electrophysiological results obtained with the mGluR7A(\(\Delta\)calmodulin) mutant suggest that modulation requires Ca\(^{2+}\)/calmodulin binding to the receptor’s tail region. Therefore, a possible explanation could be that, in the absence of agonist, the binding of Ca\(^{2+}\)/calmodulin to mGluR7 displaces G\(\beta\gamma\) from the receptor and thus inhibits VSCCs. Accordingly, the receptor would act as a source of G\(\beta\gamma\). Alternatively, binding of MacMARCKS could displace Ca\(^{2+}\)/calmodulin, and hence more receptor would be available to bind G\(\beta\gamma\) (Fig. 7). Our data do not allow us to distinguish between these alternatives.

We have previously shown that Ca\(^{2+}\)/calmodulin and G\(\beta\gamma\) binding to mGluR7 is abolished upon PKC phosphorylation of the C-terminal serine 862 (Airas et al. 2001). In the present study, the effect of PKC phosphorylation on mGluR7 binding of MacMARCKS has not been addressed. In case phosphorylation should affect MacMARCKS binding, a complex regulation of MacMARCKS, Ca\(^{2+}\)/calmodulin and G\(\beta\gamma\) interactions with mGluR7 might be achieved through dynamic phosphorylation of MacMARCKS and mGluR7 by PKC. In addition, as both the interactions of calmodulin with mGluR7 and MacMARCKS and PKC activation are strongly Ca\(^{2+}\)-dependent, mGluR7 signaling is likely to be dynamically regulated by changes in the intracellular Ca\(^{2+}\) concentration, and thus neuronal activity. According to the model proposed in Fig. 7, low intracellular Ca\(^{2+}\) concentrations and MacMARCKS binding would facilitate G\(\beta\gamma\) recruitment by mGluR7 and thereby allow for efficient VSCCs activation. In contrast, increases in intracellular Ca\(^{2+}\), and hence calmodulin binding to mGluR7, should reduce MacMARCKS interactions and facilitate VSSC inhibition. In conclusion, this regulation of mGluR7 signaling would play an important role in the fine-tuning of synaptic transmission and provide an intrinsic protective pathway that prevents excessive Ca\(^{2+}\) influx through VSCCs during high-frequency synaptic activity.

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