

GABA_C Receptor Sensitivity Is Modulated by Interaction with MAP1B

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GABA_C receptors contain ρ subunits and mediate feedback inhibition from retinal amacrine cells to bipolar cells. We previously identified the cytoskeletal protein MAP1B as a ρ 1 subunit anchoring protein. Here, we analyze the structural basis and functional significance of the MAP1B- ρ 1 interaction. Twelve amino acids at the C terminus of the large intracellular loop of ρ 1 (and also ρ 2) are sufficient for interaction with MAP1B. Disruption of the MAP1B- ρ interaction in bipolar cells in retinal slices de-

creased the EC₅₀ of their GABA_C receptors, doubling the receptors' current at low GABA concentrations without affecting their maximum current at high concentrations. Thus, anchoring to the cytoskeleton lowers the sensitivity of GABA_C receptors and provides a likely site for functional modulation of GABA_C receptor-mediated inhibition.

Key words: GABA; ρ subunit; MAP1B; EC₅₀; retina; bipolar; uptake

Ionotropic γ -aminobutyric acid receptors are divided into two classes, GABA_A and GABA_C, both of which gate chloride channels but have different properties and expression patterns (Rabow et al., 1995; Lukasiewicz, 1996). GABA_C receptors are present mainly in the retina, with lower levels in the brain and spinal cord (Cutting et al., 1991; Feigenspan et al., 1993; Qian and Dowling, 1993; Enz et al., 1995; Ogurusu et al., 1995). They are enriched on retinal bipolar cell axon terminals where they receive GABAergic input from amacrine cells (Tachibana and Kaneko, 1987; Feigenspan et al., 1993). GABA_C receptor activation inhibits release of glutamate from bipolar cells onto retinal ganglion and amacrine cells and tunes the dynamic range, temporal resolution, and spatial contrast of retinal responses to visual stimuli (Pan and Lipton, 1995; Dong and Werblin, 1998; Euler and Masland, 2000; Shields et al., 2000).

GABA_A receptors are hetero-oligomers, formed from α 1–6, β 1–3, γ 1–3, δ , ϵ , π , and θ subunits (Rabow et al., 1995; Bonnert et al., 1999), whereas GABA_C receptors contain distinct ρ subunits that, when expressed in *Xenopus* oocytes, exhibit properties similar to those of retinal GABA_C receptors (Shimada et al., 1992; Feigenspan et al., 1993; Wang et al., 1994). Three ρ subunits have been cloned that can form functional homo-oligomeric receptors (Cutting et al., 1991; Wang et al., 1994; Shingai et al., 1996) but may also form hetero-oligomers with each other or with some GABA_A receptor subunits (Zhang et al., 1995; Hackam et al., 1997; Qian and Ripps, 1999). Clustering of ρ subunits occurs at synapses on bipolar axon terminals (Enz et al., 1996; Koulen et al., 1998). We previously reported an interaction between ρ 1 and the microtubule-associated protein MAP1B (heavy chain) as a likely mechanism for anchoring GABA_C receptors to the cytoskeleton to maintain this clustered distribution (Hanley et al., 1999).

In addition to interacting with MAP1B, ρ 1 subunits can bind to a glycine transporter splice variant, GLYT-1E/F (Hanley et al., 2000). These interactions suggest similarities to the organization at the postsynaptic density of excitatory synapses, where an array of multimolecular interactions is involved in the anchoring and signaling of AMPA, NMDA, and metabotropic receptors (Ziff, 1997; Kim and Huganir, 1999). For NMDA receptors, interaction with

the cytoskeletal protein α -actinin-2 tethers the receptor and also modulates its inactivation (Wyszynski et al., 1997; Zhang et al., 1998; Krupp et al., 1999). It is unknown whether proteins anchoring inhibitory receptors also modulate channel properties.

Here, we identify the binding site for MAP1B on ρ 1 as a 12 amino acid motif, RI(D/N)THAIDKYSR, at the C-terminal end of the intracellular loop between transmembrane regions three and four. Dialysis of retinal bipolar cells with peptides containing this motif, to competitively disrupt the binding of MAP1B to ρ 1, decreases the EC₅₀ of GABA_C receptors, doubling the amount of current that they produce at low GABA concentrations. These data show, for the first time for an inhibitory synapse, that binding of receptors to the cytoskeleton controls the functional properties of the receptors.

MATERIALS AND METHODS

Antibodies. MAP1B was detected using Anti-MAP1B, clone AA6 (Sigma, St. Louis, MO). ρ 1^{myc} was detected using supernatant from 9E10 hybridoma cells.

Glutathione S-transferase fusion protein production. Glutathione S-transferase-MAP1B is a fusion of GST with amino acids 460–585 of MAP1B [similar to the fusion with residues 460–565 used by Hanley et al. (1999)]. GST-GLYT-1E/F contains the C-terminal 58 amino acids of GLYT-1E/F [GST-clone 6 of Hanley et al. (2000)]. GST- ρ 1 contains the intracellular part of human ρ 1 between transmembrane domains 3 and 4 (amino acids 355–454), and GST- ρ 2, α 1, β 3, and γ 2 were equivalent constructs. GST fusions of ρ 1 truncation mutants were constructed by PCR cloning of cDNA fragments into pGEX-4T3, using primers as follows: ρ 1^{355–444}, 5' sense primer: CGTGGATCCGATCCGATATGCGGCCGTC AAC; 3' antisense primer: GTGGAAATCTCTCAGTCGATTTCATGCT; ρ 1^{355–449}, 5' sense primer: CGTGGATCCGATATGCGGCCGTC AAC; 3' antisense primer: GTAGAATTCTCAATCAATGGCGTGGGT; ρ 2, 5' sense primer: GTGGATCCGAGTATGCGGCTGTCAAC; 3' antisense primer: TATGAATTCACCTAGAGTATTTGTC. Subsequent synthesis and purification of GST fusions was performed as described by Smith and Johnson (1988).

COS cell transfections. COS cells were cultured at 37°C, 5% CO₂, in DMEM (Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum (Life Technologies), 2 mM L-glutamine (Life Technologies), penicillin, and streptomycin. Cells were grown to 50–70% confluency, and transfected by electroporation. Ten milligram cytomegalovirus (CMV) promoter-driven expression constructs were used per transfection.

Affinity purification (pull-down assay). This was performed from retinal extract or from transfected COS cells as described previously (Hanley et al., 1999, 2000). Briefly, retinae or transfected COS cells were lysed in 1% Triton X-100 lysis buffer, and insoluble material was removed by centrifugation. Lysates were incubated with GST fusion proteins bound to glutathione-agarose beads at 4°C with rotation for 2 hr. Beads were then washed three times with lysis buffer and resuspended in SDS-PAGE sample buffer, and bound proteins were detected by Western blotting.

Site-directed mutagenesis. Mutations were made in a CMV promoter-driven ρ 1^{myc} construct (full-length ρ with the myc tag EQKLISEEDL between amino acids 4 and 5 of the mature peptide) by the Transformer

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Site-Directed Mutagenesis Kit (Clontech, Cambridge, UK), using the following mutagenic primers: VSM→KKT: AGCAGCTATAAGAAGAC-TAGAATCGATACC; RID→FNS: GTGAGCATGTTTAACTCGAC-CCACGCCATT; THA→VSK: ATGGAATCGATGTATCAAAAATT-GATAAATAC; KY→RL: CACGCCATTGATCGTCTATCCAGGATC.

Peptide competition assays. Peptides were synthesized by Altabioscience and stored at -20°C in DMSO at 40 mg/ml. Pull-down assays were performed as above in a volume of 1 ml, followed by washing beads once in lysis buffer. Beads were resuspended in 400 ml lysis buffer, and peptide was added to varying final concentrations. After rotation at 4°C for 15 min, the beads were washed an additional three times in lysis buffer and then resuspended in SDS-PAGE sample buffer, and bound proteins were analyzed by Western blotting. The normal peptide used containing the (rat) binding site amino acids had the sequence X-RINTHAIDKYSR (where X was the antennapedia sequence RQIKIWFQNRMRKWKK, biotinylated at the N terminus; X promotes peptide entry into cells, but this property was not used in the experiments reported here). The peptide with the scrambled version of the binding site had the sequence X-HRTSKINIYRDA. The N-terminally truncated peptide used, THAIDKYSR, did not have the antennapedia sequence added.

Isolated bipolar cells. Approximately one-quarter of an isolated retina from an adult [postnatal day 35 (P35)] rat was incubated at 34°C for 20 min in 2 ml of solution containing (in mM): NaCl 101, KCl 3.7, NaHCO₃ 25, NaH₂PO₄ 10, Na-pyruvate 1, glucose 15, DL-cysteine 10, plus papain (Sigma P3125, 15 μl/2 ml), then washed four times in extracellular solution and triturated through a Pasteur pipette, before plating into the recording chamber.

Retinal slices. Retinal slices from adult (P35) rats were prepared as described by Werblin (1978) for salamander retina. Briefly, retinal pieces ~2 mm square were laid on Millipore filter paper, ganglion cell side down; the sclera was gently removed, and the tissue was covered with normal external solution (lacking CoCl₂). The retina was then cut into 200-μm-thick slices, each still attached to a strip of Millipore, with a hand-operated razor blade. The slices were rotated through 90°, and the attached Millipore was embedded in lines of Vaseline to hold the slice so that all cell types were visible for patch-clamping using an upright, fixed-stage microscope. ON-bipolar cells were identified, both before whole-cell clamping and after filling with Lucifer yellow from the patch pipette, by the location of their soma in the inner nuclear layer close to the outer plexiform layer, with dendrites ascending to the outer plexiform layer and an axon descending to terminate in the inner part of the inner plexiform layer. Most were probably rod bipolars, or the morphologically similar type 8 and 9 cone ON bipolars in the classification of Euler and Wässle (1998). Experiments were at room temperature (21–25°C).

Electrodes. Electrodes had a resistance of 5–10 MΩ in external solution. The series resistance in whole-cell mode was ~25 MΩ, giving series resistance voltage errors <3 mV for currents <0.1 nA.

Solutions for electrophysiology. Extracellular solution contained (in mM): NaCl 130, KCl 2.5, MgCl₂ 2, HEPES 10, glucose 10, CaCl₂ 2, bicuculline 0.3 (to block GABA_A receptors), pH set to 7.4 with NaOH, bubbled with O₂. For experiments on slices, CaCl₂ was replaced by CoCl₂ (4 mM) to block synaptic transmission (Euler et al., 1996). Intracellular solution contained (in mM): KCl 135, CaCl₂ 0.5, Na₂EGTA 5, HEPES 10, MgCl₂ 2, MgATP 2, pH set to 7.2 with KOH, and Lucifer yellow (di-potassium salt) 0.2%. In addition, the solution contained 100 μM of either a peptide including the binding site for MAP1B on ρ1, X-RINTHAIDKYSR, or a peptide containing a scrambled version of the binding site X-HRTSKINIYRDA (see above). In some experiments, the peptidase inhibitors bestatin (10 μM), leupeptin (100 μM), and pepstatin (1 μM) were included to prevent peptide breakdown, with no significant effect on the results.

Peptide diffusion time to bipolar cell axon terminal. The diffusion constant of the peptides was estimated as $D = 1.07 \times 10^{-10} \text{ m}^2/\text{sec}$ by scaling the value for somatostatin (Holladay and Puett, 1976) in proportion to the square root of the ratio of its molecular weight (1638) to that of the peptides used here (3945). For an axon of length $L = 50 \text{ μm}$, the diffusion time from the soma to the axon terminal is $\sim L^2/2D = 12 \text{ sec}$. Thus, once inside the cell soma, the peptides used should equilibrate through the cell relatively quickly. The time constant for the peptide to equilibrate between the patch pipette and the cell volume is given by $\tau = VR_s/(D\rho)$ (Marie and Attwell, 1999), where the cell volume V is $\sim 850 \text{ μm}^3$ (for a 10-μm-diameter soma, 100 μm total length of axon plus dendrites of diameter 2 μm, and a synaptic terminal of diameter 3 μm), the series resistance R_s is 25 MΩ, and the resistivity of the pipette solution ρ is 0.8 Ωm. With these numbers, $\tau = 248 \text{ sec}$.

Effect of uptake on GABA_C receptor EC₅₀ outside bipolar cells in retinal slices. [GABA]_o, below the concentration applied in the bulk solution, [GABA]_B, we treat a simplified model of the slice in which the bulk solution is separated from the extracellular space by a diffusion barrier of permeability P . GABA enters the extracellular space at a rate:

$$P([GABA]_B - [GABA]_o), \quad (1)$$

and at equilibrium this must equal the rate of uptake into cells:

$$U[GABA]_o/(K_U + [GABA]_o), \quad (2)$$

where U and K_U are the maximum rate and Michaelis-Menten constant of uptake. From Equations 1 and 2:

$$[GABA]_B = [GABA]_o + (U/P)/\{1 + (K_U/[GABA]_o)\}. \quad (3)$$

Thus, when the bulk solution [GABA]_B is at a value that makes [GABA]_o equal the EC₅₀, so a half-maximal current is generated:

$$[GABA]_B = EC_{50} + (U/P)/\{1 + (K_U/EC_{50})\}. \quad (4)$$

Because [GABA]_o = EC₅₀ = 11 μM (measured in isolated cells in cobalt/zero Ca²⁺ solution; see Results), when the applied [GABA]_B = 40.4 μM (mean EC₅₀ measured for the GABA concentration applied to slices; see Results), and $K_U \sim 30 \text{ μM}$ for GAT-1 and GAT-3 in rat (Borden et al., 1994), Equation 4 gives $U/P = 109.6 \text{ μM}$.

For a 32% reduction (from 40.4 μM) of the [GABA]_B generating a half-maximal current, as seen experimentally with the peptide disrupting the MAP1B-ρ interaction (see Results), solving Equation 4 gives an EC₅₀ value of 6.9 μM, i.e., reduced from 11 μM by 37%. Thus, fractional changes in the bulk solution EC₅₀ underestimate fractional changes of the real EC₅₀ by a factor of 32/37 = 0.86, i.e., a 14% underestimate. The presence of uptake therefore has no effect on the conclusion (see Results) that disrupting the ρ-MAP1B interaction approximately doubles the current at low GABA concentrations.

Data analysis

The Hill equation (Eq. 5) was fitted to dose-response data using SigmaPlot. Data are presented as mean ± SEM.

RESULTS

MAP1B binds at the C-terminal end of the ρ1 subunit intracellular domain

We have previously demonstrated that MAP1B binds to the C-terminal quarter of the TM3-TM4 loop of the human ρ1 subunit, between amino acid residues S⁴³⁴ and R⁴⁵⁴ (Hanley et al., 1999). To analyze the binding site further, we constructed two C-terminal truncations of the GST fusion protein with the full-length TM3-TM4 loop (GST-ρ1); we have shown previously that the GST fusion with the full loop binds MAP1B (Hanley et al., 1999, their Fig. 2e). These polypeptides, which start at E³⁵⁵ and terminate at I⁴⁴⁹ or I⁴⁴⁴, were tested for interaction with MAP1B from retinal extract in pull-down assays. The 10 amino acid truncation (E³⁵⁵-I⁴⁴⁴) showed no MAP1B binding (Fig. 1B), indicating that crucial residues for the interaction are located within the region D⁴⁴⁵-R⁴⁵⁴. The five amino acid truncation (E³⁵⁵-I⁴⁴⁹) showed an extremely low level of MAP1B binding, but it was still higher than the GST control (Fig. 1C). Taken together, these data indicate that the residues required for strong binding to MAP1B are present within the final five amino acids (D⁴⁵⁰KYSR⁴⁵⁴) of the intracellular loop and that there are also contributing residues among amino acids D⁴⁴⁵-THAI⁴⁴⁹.

Identification of residues required for MAP1B binding on the ρ1 subunit

To investigate the importance of specific amino acids in ρ1 for interaction with MAP1B, we performed site-directed mutagenesis of a mammalian expression construct encoding full-length human myc-tagged ρ1. COS cells transfected with these constructs were lysed, and binding to GST-MAP1B [containing amino acids 460–585 of MAP1B (Hanley et al., 1999)] was analyzed in pull-down assays. Figure 1 demonstrates that residues at the extreme C terminus of the intracellular ρ1 loop are crucial for the interaction, so we mutated residues in this region adjacent to TM4. We have previously demonstrated that the α1, β3, and γ2 subunits of GABA_A receptors do not interact with MAP1B in pull-down assays (Hanley et al., 1999), so we chose to mutate residues in ρ1 to those in α1. Mutations were performed in groups of three residues as shown in Figure 2A. Of the six most C-terminal residues of this region, IDKYSR, four are identical in α1 (and also homologous to a range of other ionotropic GABA receptor subunits), so only two of these six amino acids were mutated (KY→RL).

The most N-terminal mutation, VSM→KKT, did not affect binding of ρ1^{myc} to GST-MAP1B, indicating that these residues are outside the critical binding site. The RID→FNS and THA→VSK mutants both display a reduced interaction with GST-MAP1B, indicating that these residues are important contributors to the interaction. The most C-terminal mutation, KY→RL, abolished binding to undetectable levels. Thus, of the five most

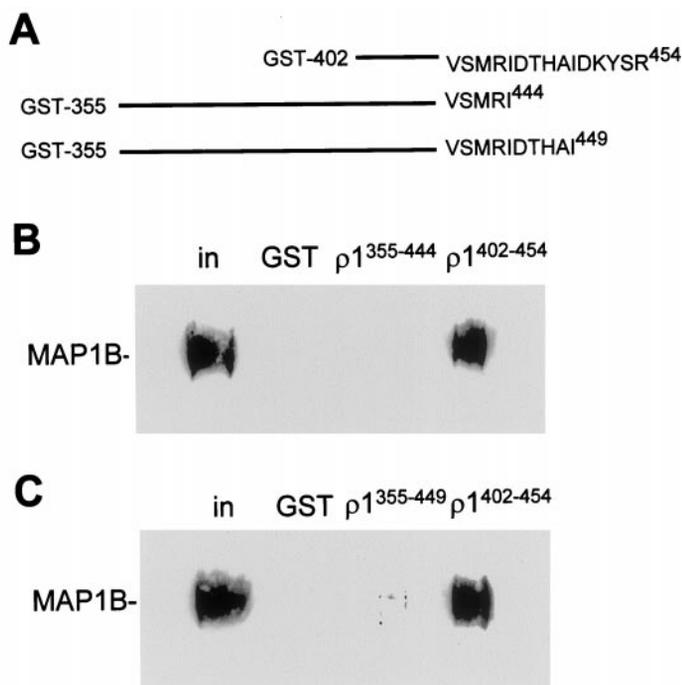


Figure 1. MAP1B binds to the extreme C terminus of the $\rho 1$ TM3–TM4 intracellular loop. Immobilized GST fusion proteins corresponding to C-terminal truncations of the $\rho 1$ intracellular loop were incubated with retinal extract in pull-down assays. MAP1B binding was determined by Western blotting. *A*, Sequences of the GST fusions with the C-terminal half of $\rho 1$ intracellular loop ($\rho 1^{402-454}$), with a 10 amino acid truncation (355–454) and a five amino acid truncation (355–449). *B*, Binding of MAP1B to the 10 amino acid truncation fusion protein, compared with binding to the C-terminal half of the intracellular loop $\rho 1^{402-454}$. Other lanes show lack of binding to GST and the input to the assay. *in* represents the MAP1B present in 1% of the input. *C*, Same as *B* but for the five amino acid truncated fusion protein.

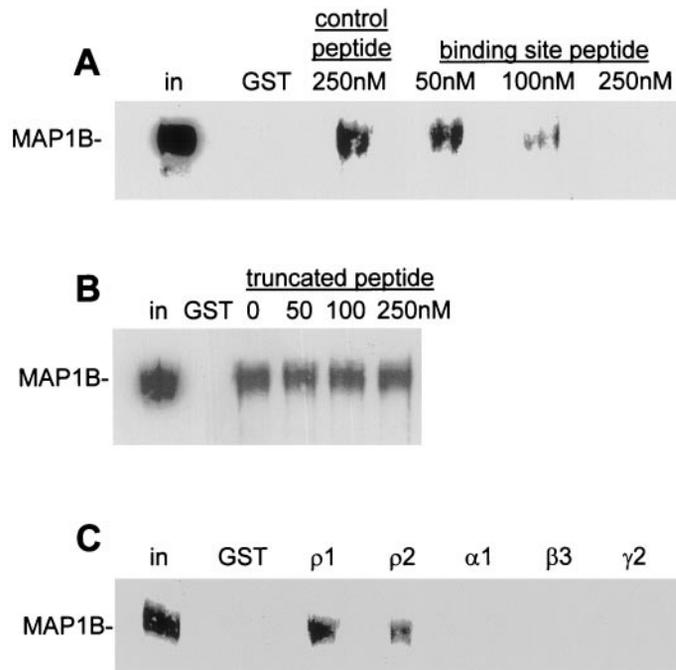


Figure 3. Competitive inhibition of MAP1B binding to ρ by peptides. The peptide containing the motif RINTHAIDKYSR (*A*) but not that containing THAIDKYSR (*B*) competes for MAP1B binding in retinal pull-down assays. *A*, Immobilized GST- $\rho 1$ was incubated first with retinal extract, followed by 50–250 nM MAP1B binding site peptide or 250 nM scrambled control peptide. MAP1B bound to beads after this treatment was determined by Western blotting. *B*, Same as in *A*, but with truncated binding-site peptide. *C*, Pull-down assay from retinal lysate using immobilized GST alone or GST fusions of $\rho 1$, $\rho 2$, $\alpha 1$, $\beta 3$, and $\gamma 2$ subunit intracellular loops. For all panels, lane labeled *GST* shows no binding of MAP1B to GST alone, and *in* represents MAP1B present in 1% of input.

C-terminal amino acids shown in Figure 1 to be important for binding to MAP1B, the lysine–tyrosine motif makes a crucial contribution.

Although, theoretically, the mutations and truncations described above could be preventing the binding of MAP1B to $\rho 1$ by altering the conformation of a binding site in a distant part of the $\rho 1$ protein, the results of the experiments described in the next section make this extremely unlikely: a peptide mimicking the C-terminal part of the $\rho 1$ intracellular loop competes with $\rho 1$ for binding to MAP1B.

Block of MAP1B binding to $\rho 1$ by competition with a binding site peptide

The data above suggest that MAP1B binds to the region RI(D/N)THAIDKYSR in $\rho 1$ (where D/N denotes the human/rat sequence). To further confirm that this sequence represents the binding site, we synthesized a peptide containing this sequence (see Materials and Methods; rat version, as it would be used for biochemical and electrophysiological experiments on rat tissue, as described below). To investigate whether this peptide is sufficient to bind to MAP1B, we performed pull-down assays from rat retinal extract using GST- $\rho 1$. If the peptide is capable of binding to MAP1B, then it will compete with GST- $\rho 1$ for the binding site on MAP1B and result in a reduced level of MAP1B binding to GST- $\rho 1$ beads. The peptide was added to the assay at varying concentrations, and the levels of MAP1B were analyzed by Western blotting (Fig. 3*A*). Peptide at 50 nM had little effect on the amount of MAP1B bound, but 100 nM significantly reduced the binding, and at 250 nM no binding was detected to GST- $\rho 1$. This competitive inhibition of binding of the human $\rho 1$ sequence to MAP1B using a peptide with the rat $\rho 1$ sequence shows that the one amino acid difference in these sequences (D/N) does not prevent binding. A peptide containing a scrambled version of the binding site, HRTSKINIYRDA, was used as a control (see Ma-

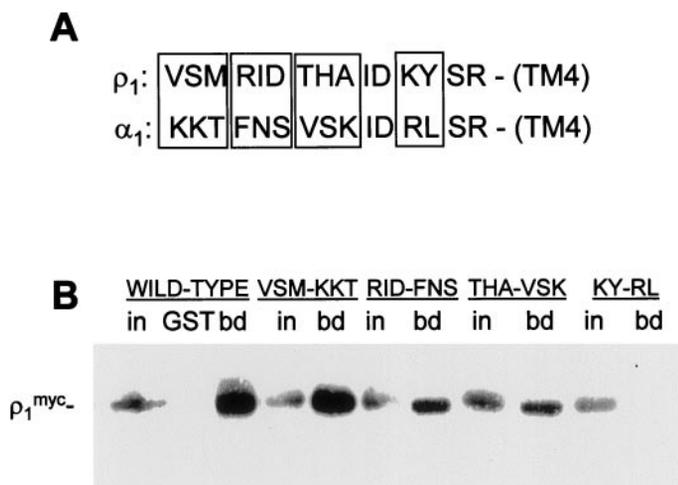


Figure 2. Identification of amino acid residues in the $\rho 1$ intracellular loop important for MAP1B binding. Immobilized GST fusion protein corresponding to the $\rho 1$ -binding region of MAP1B was incubated with extracts of COS cells transfected with mutants of full-length $\rho 1^{myc}$ in pull-down assays. Groups of residues in $\rho 1$ were mutated to the equivalent sequence of the $\alpha 1$ subunit of GABA_A receptors. *A*, Alignment of extreme C-terminal regions of intracellular TM3–TM4 loop of $\rho 1$ and $\alpha 1$ subunits. Amino acid substitutions are shown in boxes; identical amino acids were not mutated. *B*, Binding of $\rho 1^{myc}$ mutants to GST-MAP1B as determined by Western blotting. For each construct, *in* represents the $\rho 1^{myc}$ present in 5% of the input, and *bd* represents protein bound to GST-MAP1B; *GST* shows lack of binding of $\rho 1^{myc}$ to GST alone.

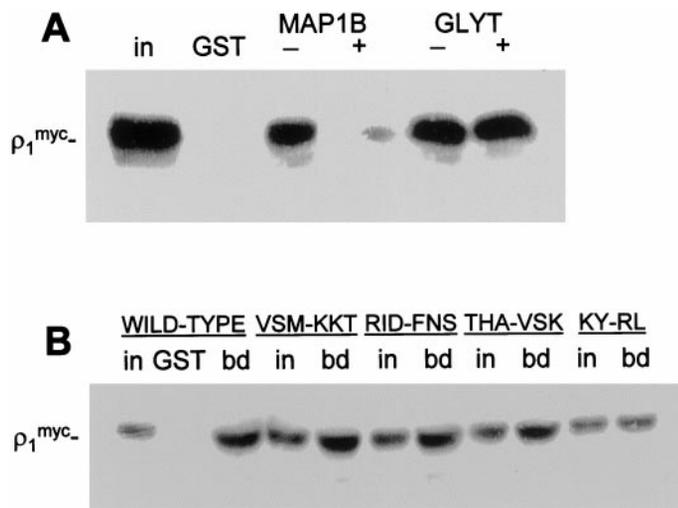


Figure 4. GLYT-1E/F and MAP1B interact with different regions of the $\rho 1$ intracellular loop. **A**, Immobilized GST-GLYT-1E/F or GST-MAP1B were incubated first with extract of COS cells transfected with $\rho 1^{myc-}$, followed by 250 nM of the MAP1B binding site peptide (+) containing RINTHAIDKYSR, or of the peptide containing a scrambled version (-) of the binding site. $\rho 1^{myc-}$ bound to beads after this treatment was determined by Western blotting. GST shows lack of binding of $\rho 1^{myc-}$ to GST alone, and *in* represents the $\rho 1^{myc-}$ present in 5% of the input. **B**, Immobilized GST-GLYT-1E/F was incubated with extracts of COS cells transfected with mutants of $\rho 1^{myc-}$ subunit in pull-down assays. Groups of residues in $\rho 1$ were mutated to the equivalent sequence of $\alpha 1$ subunit of GABA_A receptors (Fig. 2). For each construct, *in* represents the $\rho 1^{myc-}$ present in 5% of the input, and *bd* represents protein bound to GST-MAP1B; GST shows lack of binding of $\rho 1^{myc-}$ to GST alone.

terials and Methods); this did not compete for MAP1B binding, even at the highest concentration (250 nM). We also synthesized an N-terminally truncated peptide corresponding to the sequence THAIDKYSR and tested this in a similar pull-down assay (Fig. 3B). This peptide was insufficient to compete for MAP1B binding, demonstrating that, in addition to the KY pair identified above, one or more of the amino acid residues RIN are required for the interaction with MAP1B (interaction of $\rho 2$ with MAP1B, described below, suggests that it is the N and not the RI that is important).

We previously reported that MAP1B does not interact with the $\alpha 1$, $\beta 3$, or $\gamma 2$ subunits of GABA_A receptors, nor does it interact with the $\rho 2$ subunit of GABA_C receptors in the yeast two-hybrid system (Hanley et al., 1999). The sequence of $\rho 2$ at the extreme C terminus of the TM3–TM4 loop is FQNTHAIDKYSR, which is identical to $\rho 1$ with the exception of FQ. To test the possibility that $\rho 2$ can interact with MAP1B, we constructed a GST fusion protein with the TM3–TM4 loop of $\rho 2$ and analyzed binding to MAP1B from retinal extract using a pull-down assay (Fig. 3C). MAP1B bound to GST- $\rho 2$ at similar levels to $\rho 1$ in this assay. This apparent discrepancy with the previous negative result for $\rho 2$ in yeast (Hanley et al., 1999) is likely to reflect limitations of the yeast two-hybrid system, because the pull-down assay is performed under more native conditions. Confirming earlier pull-down and immunoprecipitation data (Hanley et al., 1999), in which $\rho 2$ was not tested, MAP1B did not interact with $\alpha 1$, $\beta 3$, and $\gamma 2$ (Fig. 3C).

The glycine transporter GLYT-1E/F and MAP1B bind to different regions of $\rho 1$

To investigate whether GLYT-1E/F binds to the same region of $\rho 1$ as MAP1B or has a different binding site, we performed assays pulling down myc-tagged $\rho 1$ from COS cells using GST-MAP1B and GST-GLYT-1E/F [containing the 58 most C-terminal amino acids of the bovine transporter (Hanley et al., 2000)]. In each case, the reactions were treated with 250 nM of the peptide corresponding to the MAP1B binding site, or of the scrambled control peptide, and the binding of $\rho 1$ to GST-MAP1B and GST-GLYT-1E/F was determined by Western blotting (Fig. 4A). The binding site

peptide competed for binding to GST-MAP1B, confirming that this $\rho 1$ sequence is the site of interaction with MAP1B. However, the peptide did not compete for binding to GST-GLYT-1E/F, indicating that the transporter binds to a different region of $\rho 1$. To confirm this result, the MAP1B binding site mutants of $\rho 1^{myc-}$ were tested for binding to GST-GLYT-1E/F in pull-down assays from transfected COS cell lysate (Fig. 4B). All four mutants show a similar level of binding compared with input, indicating that they bind equally well to GST-GLYT-1E/F and implying that the GLYT-1E/F binding site differs from that for MAP1B.

Disrupting the ρ -MAP1B interaction decreases the EC₅₀ of GABA_C receptors

To determine whether the interaction of ρ subunits with MAP1B affects the function of GABA_C receptors, we whole-cell-clamped rat retinal bipolar cells. Included in the pipette solution, and thus dialyzed into the cells, were peptides (100 μ M) including the sequence of the MAP1B-binding domain on $\rho 1$ or a scrambled version of it (see Materials and Methods). The aim, as in the experiments of Figures 3A and 4A, was for the binding site peptide to compete with endogenous $\rho 1$ for binding to MAP1B and thus displace MAP1B from $\rho 1$, allowing the effect of MAP1B binding on GABA_C receptor-generated currents to be investigated. This strategy has been used successfully previously to disrupt interactions of endogenous proteins with AMPA receptors and glutamate transporters (Nishimune et al., 1998; Marie and Attwell, 1999). Experiments were performed at -60 mV, with the Nernst potential for Cl⁻ near 0 mV, so GABA-evoked currents are inward.

Applying GABA either to isolated bipolar cells or to bipolar cells in retinal slices (see Materials and Methods), in the presence of bicuculline (300 μ M) to block GABA_A receptors, generated a current that was blocked by the GABA_C receptor blocker (1,2,5,6-tetrahydropyridine-4-yl)methylphosphonic acid (TPMPA) (Fig. 5A). Block by TPMPA was only slowly reversible. The response to 30 μ M GABA was reduced by 97 ± 2% (*n* = 4 cells in slices) by 200 μ M TPMPA, whereas 2 mM TPMPA reduced the current generated by 300 μ M GABA by 93 ± 3% (*n* = 2).

In isolated bipolar cells, GABA_C receptors showed an EC₅₀ for GABA of 3.0 ± 0.2 μ M (*n* = 7) (Fig. 5B), similar to the 4.2 μ M obtained by Feigenspan and Bormann (1994a). However, as found previously (Karschin and Wässle, 1990), whole-cell-clamped isolated cells stayed healthy for only a few minutes, which is not long enough to dialyze into the cell the peptide disrupting the MAP1B- $\rho 1$ interaction. We therefore studied the effect of disrupting this interaction in bipolar cells in retinal slices, which could be clamped for up to 30 min with no deterioration in health. Slice bipolars were also preferred because the cytoskeleton with which the GABA_C receptors interact is likely to be less disrupted than in the isolated cells that have been enzyme-treated and triturated. Cobalt chloride (4 mM) was included in the extracellular solution, and calcium was omitted, to block synaptic transmission and ensure that the GABA-evoked currents seen were generated by the recorded cell (Euler et al., 1996).

In bipolar cells *in situ* in slices, the EC₅₀ for GABA applied in the superfusate was larger than in isolated cells. Dose–response curves for the GABA-evoked current, *I*, could be fitted approximately by the Hill equation:

$$I = I_{max} [GABA]^N / ([GABA]^N + EC_{50}^N), \quad (5)$$

where *I*_{max} is the maximum current at saturating [GABA], the Hill coefficient, *N*, was 1.6, and the EC₅₀ varied between cells (range 26–80 μ M) with a mean value of 40.4 ± 1.9 μ M in 14 cells. Blocking the mainly neuronal GABA transporter GAT-1 with SKF89976A (100 μ M) (Borden et al., 1994) decreased the EC₅₀ by fourfold (Fig. 5C), making it much closer to the value in isolated cells, showing that the presence of GABA uptake (Johnson et al., 1996), which lowers the GABA concentration around the cells within the slice, is partly responsible for the higher EC₅₀ in slices. The residual difference in EC₅₀ between slice bipolar cells with GAT-1 blocked and isolated bipolar cells, may reflect the use of solution containing

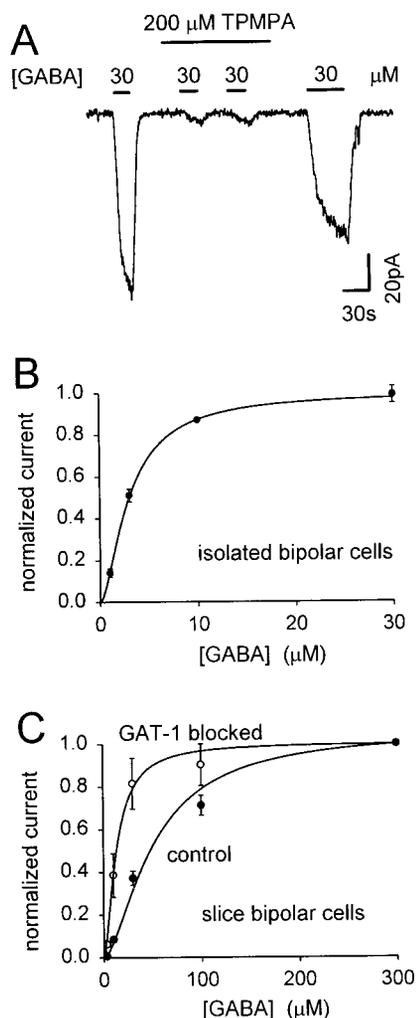


Figure 5. GABA_C receptor-evoked currents in retinal bipolar cells with no peptide included in the pipette solution. *A*, Membrane current of a cell in a slice, clamped to -60 mV, during repeated application of 30 μ M GABA in the presence of 300 μ M bicuculline to block GABA_A receptors. The GABA_C receptor blocker TPMPA (200 μ M) greatly reduces the response to GABA. This block was only slowly removed on washing out TPMPA. *B*, Dose–response curve for the application of GABA to isolated bipolar cells in the presence of 300 μ M bicuculline (normalized to the response to 10 μ M GABA in each cell and then rescaled to a saturating response of 1). Curve is a Hill equation (Eq. 5) with $n = 1.6$ and a mean EC_{50} of 3 μ M. Data from seven cells. *C*, Dose–response curves for five bipolar cells in slices, in the presence of 300 μ M bicuculline and 4 mM $Co^{2+}/0$ mM Ca^{2+} , initially in normal conditions (control) and then in the presence of 100 μ M SKF89976A (GAT-1 blocked). Hill equations through the data have $n = 1.6$ and an EC_{50} of 51 μ M in control conditions (mean value was 51.0 ± 3.7 μ M) and 13.4 μ M with GAT-1 blocked (mean value was 13.4 ± 2.2 μ M).

cobalt and zero calcium to block synaptic transmission in the slice (Euler et al., 1996); in five isolated bipolar cells this solution increased the EC_{50} to 11 ± 2 μ M [data not shown; cf. Kaneda et al. (1997)]. Mathematical analysis (see Materials and Methods) shows that, although uptake increases the EC_{50} measured in the slice, the fractional change of EC_{50} induced by disrupting the MAP1B- $\rho 1$ interaction is similar to that which would be measured if uptake were absent (the fractional change is underestimated by 14%).

When the peptide mimicking the MAP1B binding site was introduced into slice bipolar cells, the GABA responses changed with time after starting whole-cell clamping, with low GABA doses producing an increased current at later times but the response to high doses changing little (Fig. 6*A,B*). This corresponds to a decrease of EC_{50} of the dose–response curve (Fig. 6*C*). By contrast, including the scrambled peptide in the pipette resulted in either no time-dependent change of the GABA responses or a reduction of the fractional response to low [GABA] (Fig. 6*D*).

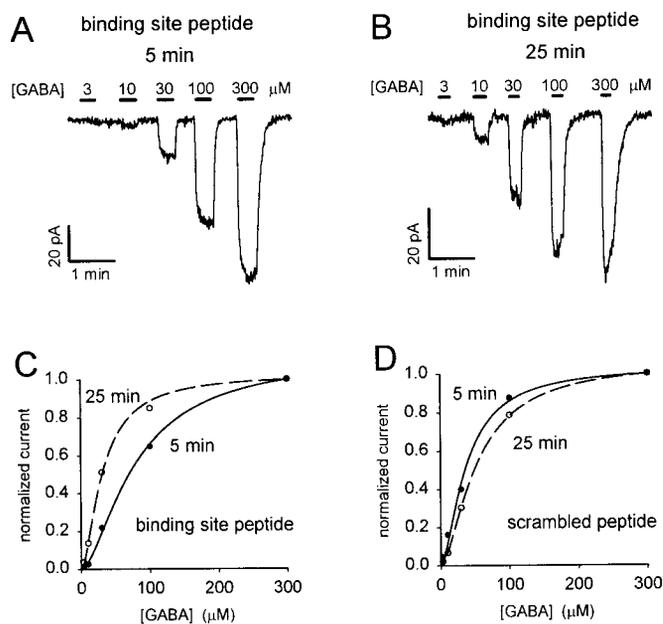


Figure 6. Effect of dialysis with MAP1B binding site peptide on the GABA dose–response curve in retinal bipolar cells. *A*, Specimen current responses to different GABA concentrations (in the presence of bicuculline) 5 min after going to whole-cell mode, with the binding site peptide in the pipette. *B*, Dose–response curve in the same cell as *A*, 25 min after starting whole-cell clamping. Responses in *A* and *B* have been scaled to be the same for 300 μ M GABA, to compensate for a slight decline with time (Fig. 7*A*), and to facilitate comparison of the dose dependence of the responses. The relative responses to low doses of GABA are much larger in *B* than in *A*. *C*, Dose–response data from *A* and *B*, normalized to the current produced by 300 μ M GABA, fitted with the Hill equation (Eq. 5) with a Hill coefficient of 1.6. At 5 min the EC_{50} is 79 μ M; at 25 min it is 31 μ M. *D*, Specimen dose–response data as in *C*, but from a cell clamped with the scrambled peptide in the pipette. Fitting the Hill equation (with a Hill coefficient of 1.6) gives an EC_{50} of 37 μ M at 5 min and 53 μ M at 25 min after starting whole-cell clamping.

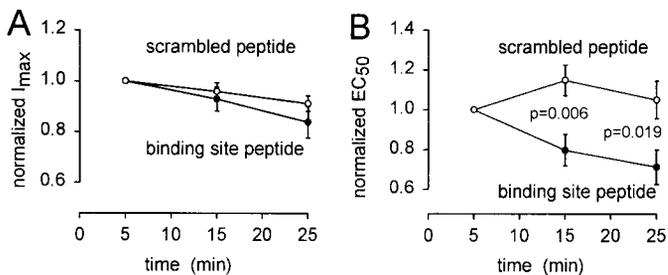


Figure 7. Time-dependent changes in the properties of GABA_C receptors induced by competitive removal of MAP1B binding. Maximum current (*A*) and EC_{50} (*B*) derived from fitting the Hill equation (Eq. 5) to dose–response data measured at different times after starting whole-cell clamping with pipette solution containing the MAP1B binding site peptide (●) or a scrambled version of it (○). Data from each cell were normalized to their values measured 5 min after starting whole-cell clamping, before averaging for these graphs. Data in *A* are not significantly different for the two peptides. The p values in *B* are from two-tailed t tests. Eight to thirteen cells contribute to each data point.

To quantify the change of GABA_C receptor properties, we fitted Equation 5 to dose–response data obtained at different times after starting whole-cell clamping, to derive the EC_{50} and I_{max} for the receptors. The maximum current showed no significant difference in behavior when the active or scrambled peptides were introduced (Fig. 7*A*). Both showed a slow decrease with time that may reflect an alteration of internal milieu by the non-peptide components of the pipette solution (cf. Feigenspan and Bormann, 1994b). To quantify the change of receptor sensitivity, for each cell we normalized the EC_{50} values by the EC_{50} measured at the start of whole-cell recording. This procedure was adopted to remove vari-

ability in the initial value of the EC₅₀ (see above) and thus allow us to monitor solely the time-dependent change of EC₅₀ produced by the peptide. Figure 7B shows that the binding site peptide led to a decrease of EC₅₀, whereas the scrambled peptide produced no significant change. The data for the two peptides differ significantly after 15 and 25 min recording. Furthermore, this difference is underestimated by our data, because the “initial” value of EC₅₀ by which the subsequent data were normalized was measured 5 min after starting whole-cell clamping, by which time the binding site peptide may already have had some effect. The time constant for filling of the cell by the dialyzed peptides is estimated to be ~4 min (see Materials and Methods), so the time course of the changes seen with the binding site peptide in Figure 7B may reflect both this filling time and the time needed for competitive displacement of endogenous $\rho 1$ from MAP1B by the peptide.

The mean data in Figure 7B show that the binding site peptide reduces the EC₅₀ at 25 min after starting whole-cell clamping to 68% of its value with the scrambled peptide, implying a 1.9-fold increase of the current evoked by low doses of GABA (e.g., see specimen data in Fig. 6C). Thus, in the absence of the dialyzing peptide, the interaction with MAP1B roughly halves the size of the current that low doses of GABA generate.

Glycine does not alter GABA_C responses

Although the cellular location of GLYT-1E/F transporters is not known, their interaction *in vitro* with $\rho 1$ suggests that, if these transporters are present in bipolar cells, the rate of glycine uptake could alter GABA_C receptor properties (or vice versa). However, we found that applying 300 μ M glycine (in the presence of 10 μ M strychnine to block glycine receptors) had no effect on the GABA_C response of retinal bipolar cells to 30 μ M GABA (subsaturating so that we might observe changes in either EC₅₀ or maximum current; five cells; data not shown). No glycine uptake current was detectable in the bipolar cells, so we could not test the effect of GABA_C receptor activation on glycine uptake.

DISCUSSION

Our analysis of the binding site for MAP1B on the $\rho 1$ subunits of GABA_C receptors suggests that MAP1B binds to the sequence RI(D/N)THAIDKYSR, where D/N denotes the human/rat sequence. Of these residues, the section KY is crucial, because mutating it prevents binding (Fig. 2B) but is not sufficient, because the truncated peptide THAIDKYSR does not prevent binding (Fig. 3B) and because mutating THA or RID to the homologous residues in $\alpha 1$ subunits also reduces binding, suggesting that these residues also contribute to binding. Binding occurs whether the residue present at the D/N position is either D or N, because the rat sequence peptide (N) competes well with human $\rho 1$ (D) for binding to MAP1B (Fig. 3A). This may be allowed because although D is charged whereas N is polar, they both have small side chains. The more C-terminal residues ID and SR may contribute to binding but are present in α and β subunits of GABA_A receptors, which do not bind MAP1B. The binding site may not extend N-terminally beyond R⁴⁴³ (in the human sequence) because $\rho 1^{\text{myc}}$ binds efficiently to GST-MAP1B even when V⁴⁴⁰SM⁴⁴² are mutated to KKT.

The motif RI(D/N)THAIDKYSR in its entirety is not found in any other proteins in the database, but the $\rho 2$ subunit has an equivalent 12 amino acid motif of FQNTHAIDKYSR, which also binds MAP1B in pull-down assays (Fig. 3C). The first two amino acids of these sequences differ, suggesting a minimal binding motif of NTHAIDKYSR. The $\rho 3$ subunit, which was not analyzed, contains the sequence LENNHVIDTYSR. The major GABA_A receptor subunits do not show high homology with ρ in this region, the most similar being the β subunits (e.g., $\beta 3$: LTDVNAID-RWSR). The absence of MAP1B binding to GABA_A receptors, which has been demonstrated by pull-down assay and immunoprecipitation from retina (Hanley et al., 1999), is consistent with these differing sequences. GABA_A receptors may be anchored to the cytoskeleton through the tubulin binding proteins gephyrin and GABARAP (Essrich et al., 1998; Wang et al., 1999), although

direct binding of gephyrin to GABA_A receptor subunits has not been shown. The existence of the different tethering molecules for GABA_A and GABA_C receptors may help to explain their specific location at different synapses and their lack of colocalization in bipolar cells (Koulen et al., 1998). Interestingly, GABARAP shows 31% identity to light-chain-3 of MAP1A and MAP1B, and it binds to $\gamma 2$ subunits in a C-terminal region of their TM3–TM4 loop at an equivalent position to the MAP1B binding site on $\rho 1/\rho 2$.

Dialyzing retinal bipolar cells with a peptide mimicking the MAP1B binding motif, which we have shown to competitively disrupt MAP1B- $\rho 1$ binding (Fig. 3A), led to a 32% decrease in the EC₅₀ of the cells' GABA_C receptors, which is sufficient to approximately double the GABA-evoked current at low GABA concentrations (Fig. 6C). At the same time, there was no change of the maximum GABA-evoked current, suggesting that binding of MAP1B to ρ does not alter the number of receptors in the membrane (in contrast to the effect of binding of NSF to AMPA receptors) (Nishimune et al., 1998; Luscher et al., 1999). It is likely that the binding of MAP1B to ρ alters the energetics of the conformation changes that occur when the receptor binds GABA and opens its channel, and in this way MAP1B alters the EC₅₀. A similar phenomenon has been observed for glutamate transporters (Marie and Attwell, 1999). Because MAP1B aggregates $\rho 1$ subunits (Hanley et al., 1999), it is possible that, in addition to changing the EC₅₀, disruption of the MAP1B- ρ interaction may allow GABA_C receptors to disperse to a more extrasynaptic location; however, it is likely that the change in EC₅₀ occurs as soon as the ρ subunits detach from MAP1B.

In the retina, the feedback inhibition of bipolar cells by amacrine cells, mediated largely by GABA_C receptors, increases the dynamic range of the ganglion cells and produces temporal and spatial shaping of the visual signal, making signals more transient and enhancing edge detection (Dong and Werblin, 1998; Jacobs and Werblin, 1998; Euler and Masland, 2000; Roska et al., 2000; Shields et al., 2000). GABA_C receptors produce long duration IPSCs in bipolar cells when amacrine cells release GABA (Lukasiewicz and Shields, 1998; Shields et al., 2000). Lowering the EC₅₀ of GABA_C receptors is expected to increase the duration of the synaptic current in the following circumstances. First, if after the peak of the IPSC the extracellular GABA concentration falls slowly compared with the receptor and channel gating kinetics, then if the EC₅₀ is lower the GABA concentration will have to fall to a lower level before the receptors can deactivate, which will take longer. Alternatively, if the GABA concentration falls to zero rapidly (compared with the GABA unbinding/channel gating kinetics), then a lower EC₅₀ is predicted to prolong the IPSC decay provided that the effect of MAP1B on the EC₅₀ is mediated by an alteration of the rate constant for GABA unbinding or for channel opening or closing [but there will be no effect on the IPSC decay if the EC₅₀ is lowered by increasing the GABA binding rate (cf. Jones et al., 1998)]. To illustrate this quantitatively, we performed calculations based on the GABA_C receptor kinetic scheme of Chang and Weiss (1999) with channel opening occurring when three GABA molecules are bound. Individual rate constants were altered to decrease the EC₅₀ by 32%, as we found [in the Chang and Weiss (1999) model, we used an absolute change from 0.83 μ M, when interacting with MAP1B, to 0.56 μ M (their standard parameters) in the absence of MAP1B]. Producing this EC₅₀ change by decreasing the GABA unbinding rate, decreasing the channel closing rate, or increasing the channel opening rate led to the IPSC decay time constant being increased by 40, 114, or 89%, respectively.

A prolongation of the IPSC will increase the spatiotemporal filtering mediated by this feedback synapse. In addition, doubling the GABA_C receptor-mediated current at the low GABA concentrations likely to be continually present in the retina, where most synapses mediate graded potentials and are tonically active, would produce an extra tonic inhibition of glutamate release from bipolar cell synaptic terminals and thus decrease ganglion cell firing. For such an alteration of information processing to occur, for example during light adaptation, the interaction between MAP1B and ρ

subunits would have to be modulated. This could potentially occur by phosphorylation of either of the serine residues in the $\rho 1$ sequence, which are within or flank the MAP1B binding site defined above (S⁴⁴¹ and S⁴⁵³ in the human sequence; S⁴⁴¹ is a consensus site for PKC phosphorylation).

The MAP1B binding site peptide does not compete for binding of $\rho 1$ to the C-terminal tail of the glycine transporter GLYT-1E/F, and none of the MAP1B binding site mutations in $\rho 1^{\text{myc}}$ affected binding to GST-GLYT-1E/F (Fig. 4), indicating that the transporter binds to a region on $\rho 1$ distinct from the MAP1B binding site. This suggests that MAP1B and GLYT-1E/F may be able to interact with $\rho 1$ simultaneously, perhaps allowing $\rho 1$ and GLYT-1E/F to be anchored to the cytoskeleton as a complex. The location of GLYT-1E/F in the retina has not yet been determined: previous studies of glycine transporter location have used antibodies raised against transporter C and N termini, which are not present in GLYT-1E/F. Because some bipolar cells accumulate glycine, but not via GLYT-1A or -B (Pow and Hendrickson, 1999), it is possible that GLYT-1E/F is colocalized in bipolar cell synaptic terminals with $\rho 1$ subunits. Application of glycine had no effect on GABA_C receptor-mediated currents, implying that if GLYT-1E/F transporters are present, their linkage to $\rho 1$ subunits does not result in transporter activity modulating GABA_C receptor properties [it also shows that the potentiation of homomeric $\rho 1$ receptor activity by glycine seen in oocyte expression experiments (Calvo and Miledi, 1995) does not occur for bipolar cell GABA_C receptors]. We have been unable to test whether activation of GABA_C receptors modulates glycine uptake into bipolar cells, because the glycine uptake present in these cells is too small to generate a detectable current.

In summary, by identifying a motif for MAP1B binding to ρ subunits, we have shown for the first time that inhibitory GABA_C receptors have their EC₅₀ modulated by attachment to the cytoskeleton and that they can potentially interact simultaneously with MAP1B and with a glycine transporter. This suggests that the postsynaptic density at inhibitory synapses is likely to comprise as complicated a web of interacting receptors and signaling proteins as occurs at excitatory synapses (Ziff, 1997; Kim and Haganir, 1999).

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