ZIP3, a New Splice Variant of the PKC-ζ-interacting Protein Family, Binds to GABA<sub>C</sub> Receptors, PKC-ζ, and Kvβ2*

Cristina Crocić, Johann Helmut Brandstätters, and Ralf Enz‡*

From the 1Emil-Fischer-Zentrum, Institut für Biochemie, Friedrich-Alexander-Universität Erlangen-Nürnberg, 2Fahrstrasse 17, 91054 Erlangen, Germany and 3Max-Planck-Institut für Hirnforschung, Abteilung Neuroanatomie, Deutscherstrasse 46, 60328 Frankfurt, Germany

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The correct targeting of modifying enzymes to ion channels and neurotransmitter receptors represents an important biological mechanism to control neuronal excitability. The recent cloning of protein kinase C-geta interacting proteins (ZIP1, ZIP2) identified new scaffolds linking the atypical protein kinase PKC-ζ to target proteins. GABA<sub>C</sub> receptors are composed of three ρ subunits (ρ1–3) that are highly expressed in the retina, where they are clustered at synaptic terminals of bipolar cells. A yeast two-hybrid screen for the GABA<sub>C</sub> receptor ρ3 subunit identified ZIP3, a new C-terminal splice variant of the ZIP protein family. ZIP3 was ubiquitously expressed in non-neuronal and neuronal tissues, including the retina. The ρ3-binding region of ZIP3 contained a ZZ-zinc finger domain, which interacted with 10 amino acids conserved in ρ1–3 but not in GABA<sub>C</sub> receptors. Consistently, only ρ1–3 subunits bound to ZIP3. ZIP3 formed dimers with ZIP1–3 and interacted with PKC-ζ and the shaker-type potassium channel subunit Kvβ2. Different domains of ZIP3 interacted with PKC-ζ and the ρ3 subunit, and simultaneous assembly of ZIP3, PKC-ζ and ρ3 was demonstrated in vitro. Subcellular co-expression of ZIP3 binding partners in the retina supported the proposed protein interactions. Our results indicate the formation of a ternary postsynaptic complex containing PKC-ζ, ZIP3, and GABA<sub>C</sub> receptors.

γ-Aminobutyric acid (GABA)¹ is the most important inhibitor neurotransmitter in the mammalian central nervous system, gating GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub> receptors. While GABA<sub>C</sub> receptors form ligand-gated ion channels (1, 2), GABA<sub>A</sub> receptors couple to ion channels via G-proteins (3). Unlike GABA<sub>A</sub> receptors, GABA<sub>C</sub> receptors show no sensitivity for anesthetics and are predominantly expressed in the retina (4–6) where they participate in sharpening the visual image by extracting spatial edges of neuronal representations (7). Furthermore, GABA<sub>C</sub> receptors were detected in the superior colliculus (8–10), hippocampus (11, 12), cerebellum (13, 14), lateral geniculate nucleus (15), and amygdala (16).

Mammalian GABA<sub>C</sub> receptors are composed of three ρ subunits (ρ1–3) that assemble into homo- and hetero-oligomers (17, 18). In the retina, ρ subunits are intensively clustered at bipolar cell terminals (19–21), while expression in other brain areas was significantly lower (22–25). N and C termini of ρ subunits are extracellular (26) and held in position by four transmembrane regions (TM1–TM4). Between TM3 and TM4, a long intracellular loop contains consensus sites for modulatory proteins, such as protein kinase C (PKC), and activation of PKC down-regulated GABA-gated chloride currents by receptor internalization (27–31).

Recently, two proteins interacting with the TM3–TM4 loops of GABA<sub>C</sub> receptor ρ1 and ρ2 subunits were identified. A new C-terminal splice variant of the glycine transporter GLYT-1 bound to ρ1 (32), while the microtubule-associated protein 1B (MAP-1B) interacted with ρ1 and ρ2 and was co-localized with GABA<sub>C</sub> receptors in the retina (33–35). Although MAP-1B binds microtubuli, the protein was not essential to anchor GABA<sub>C</sub> receptors at the cytoskeleton of bipolar cell synapses since GABA<sub>C</sub> receptor expression in MAP-1B-deficient mice was indistinguishable from wild-type animals (36). Importantly, neither GLYT-1 nor MAP-1B interacted with the GABA<sub>C</sub> receptor ρ subunit or GABA<sub>A</sub> receptors.

Screening a rat brain cDNA-library for proteins binding to the GABA<sub>C</sub> receptor ρ3 subunit, we identified ZIP3, a new C-terminal splice variant of a protein family interacting with the ζ-isofrom of protein kinase C (PKC-ζ; Refs. 37, 38). ZIP proteins link PKC-ζ to shaker-type potassium channel β subunits (39) and play an important scaffold role in the activation of the transcription factor NF-κB (40, 41). This study identified a new interaction between ZIP3 and GABA<sub>C</sub> receptors in vitro and postulates the formation of a PKC-ζ/ZIP3/GABA<sub>C</sub> receptor macromolecular complex.

EXPERIMENTAL PROCEDURES

All experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany, the National Institutes of Health, and the Max Planck Society.

Yeast Two-hybrid Techniques—Unless specified otherwise, all reagents were parts of the MATCHMAKER GAL4 Two-hybrid System 3 (Clontech, Palo Alto, CA). The large intracellular loop between TM3 and TM4 of the rat GABA<sub>C</sub> receptor ρ3 subunit (TM3-TM4, amino acids 344–445) was PCR-amplified and subcloned in-frame into the EcoRI-BamHI site of the bait vector pGKT7-BD for expression as a GAL4 fusion protein. Yeast AH109 cells were sequentially transformed with the ρ3 bait vector and 1 mg of a rat brain MATCHMAKER cDNA-library cloned in pGADT7-AD using the lithium acetate method (42) and plated on media selecting for reporter gene activations containing 10 mM 3-amino-1,2,4-triazole (3-AT, Sigma) to suppress background growth. Yeast colonies were incubated for 4 days at 30 °C and transferred to plates containing 5-bromo-4-chloro-3-indoxyl-β-d-galac-
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Preparation of Protein Extracts from HEK-293 Cells and Rat Brain—

Human embryonic kidney cells (HEK-293, ATCC CRL1573) were transfect-(45)ed with cDNAs encoding for T7-tagged ZIP3, the β3 TM3-TM4 loop fused to GST, or GST alone. Proteins were expressed under control of the Rous sarcoma virus promoter. Cells were lysed in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline, pH 7.4) containing 1 mM dithiothreitol (Sigma), 250 units/ml Benzonase, and a protease inhibitor mixture (Roche Molecular Biochemicals) and sedimented for 30 min at 13,200 × g at 4 °C. The supernatant was diluted 1:4 in phosphate-buffered saline supplemented with 1 mM dithiothreitol and mixed with gluthathione-Sepharose beads (pre-incubated as above) for 5 h at 4 °C. Subsequent washing, elution, and Western blot detection of interacting proteins was performed as described above.

Adapted rat brains were homogenized on ice in 10 ml/mg tissue of homogenization buffer I (0.32 M saccharose, 20 mM Tris-HCl, pH 7.4) containing 10 mg/ml DNaseI and protease inhibitors (Roche Molecular Biochemicals) using a glass/Teflon homogenizer and centrifuged at 30,000 × g for 30 min, and then the resulting crude membrane pellet was homogenized again in 5 ml/mg tissue of hypotonic homogenization buffer II (20 mM Tris-HCl, pH 7.4, plus protease inhibitors) to release proteins inside cytosolic vesicles. After centrifugation at 30,000 × g for 30 min, the supernatant was separated completely from the pellet, which was solubilized in 5 ml/mg tissue of buffer III (20 mM Tris-HCl, 200 mM NaCl, 1% Triton X-100, pH 7.4, plus protease inhibitors) for 2 h. The clearing of this suspension at 100,000 × g for 30 min was the supernatant was saved (P2). For binding assays, ~5 mg of S1 and P2 protein fractions were incubated with beads coated with GST-ZIP3, the His-tagged β3 TM3-TM4 loop, or GST and His tags as negative controls for 5 h under slow agitation. Bound proteins were washed, eluted and visualized as described above. All protein preparation steps were carried out on ice at 4 °C.

Primary antibodies were used as follows for Western blotting: rabbit anti-PKC-ζ (1:10,000, Sigma), mouse anti-Kvβ2 (1:500, Upstate Biotechnology, Lake Placid, NY), goat anti-PICK1 (1:500, N-18, Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-PP1y (1:2000, C-19, Santa Cruz Biotechnology).

Immunocytochemistry—Adult Wistar rats were anesthetized with halothane and decapitated. The posterior eye cups with the retinas attached were immersion fixed for 15–30 min in 4% (w/v) paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). The retinas were dissected and cryoprotected in 40% (w/v) sucrose in PB overnight at 4 °C. Pieces of retinas were mounted in freezing medium (Beichert-Jung, Germany), sectioned vertically at a thickness of 12 μm with a cryostat and processed for immunocytochemistry.

The following antibodies were used: a rabbit polyclonal antiserum (1:1000) recognizing the p1, p3, and β3 subunits of the rat GABA<sub>C</sub> receptor (19), a mouse monoclonal antibody against PKC-α (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), a monoclonal antibody against PKC-ζ (1:500, Upstate Biotechnology, Lake Placid, NY), and a rabbit polyclonal antiserum against PKC-ζ (1:10,000; Sigma). In double-labeling immunocytochemical experiments, co-expression of PKC-α and PKC-ζ and of PKC-α and GABA<sub>C</sub> receptor was examined. The binding sites of the primary antibodies were revealed by the secondary antibodies Alexa<sub>488</sub> (488 fluorescent) goat anti-mouse or goat anti-rabbit IgG (H + L) conjugates (1:500; Molecular Probes, Eugene, Oregon). Double-labeled sections were examined and analyzed with a confocal laser-scanning microscope (LSM 5 Pascal, Zeiss, Oberkochen, Germany), and resulting images were adjusted in brightness and contrast using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA).

The Transcription of RNA and Reverse Transcription—PCR—Total RNA was extracted from brain, lung, liver, kidney, and spleen using the TRIZOL Reagent according to the manufacturer’s protocol (Invitrogen) and from cerebellum, cortex, hippocampus, olfactory bulb, thalamus, and spinal cord of an adult rat following a method described by Chomczynski and Sacchi (46). For cDNA-synthesis, 3 μg of total RNA were used. The reaction mixture was incubated with Moloney murine leukemia virus reverse transcriptase (M-MLV), 3 mM dNTPs, 75 KCl, 10 dithiothreitol, pH 8.3, 0.5 μM of each dNTP (Amersham Biociences), 250 ng of p(dN)₆ (Boehringer, Mannheim, Germany), 20 Units RNasin (Roche Molecular Biochemicals) and 400 units of SuperscriptII RNaseH⁻ reverse transcriptase (Invitrogen). Incubation times were 15 min at room temperature followed by 2 h at 42 °C. PCR amplification of 25 cycles was performed in a final volume of 50 μl of PCR-buffer (in mM: 20 Tris-Cl, 50 KCl, 1.5 MgCl₂, 0.2 dNTPs, and 0.5 μM each ZIP3 primer (sense 5′-CAGCAAGCTCATCTTCTCCAC-3′, nt 480–501; antisense 5′-CATCATTGACGATCTAAAGCA-3′, nt 684–705), 5 units Taq-polymerase (Invitrogen), pH 8.0) in a thermocycler.
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RESULTS

ZIP3 Represents a New C-terminal Splice Variant of the PKC-ζ-interacting Protein Family—To identify proteins that bind to the GABAC receptor ρ3 subunit, a rat brain cDNA library was screened against the intracellular TM3-TM4 loop of this subunit. Of \(1.9 \times 10^7\) transformed yeast cells, 100 yeast colonies were isolated on selection plates and analyzed further. Upon sequencing, clones 56 and 72 were of specific interest since they represented two independent clones coding for an unknown C-terminal splice variant of the PKC-ζ-interacting proteins ZIP1 and ZIP2 (38). Therefore, the protein was termed ZIP3. The N-terminal part of ZIP3 was identical to ZIP1 and ZIP2 and contained a recently characterized acidic putative protein-binding motif described as cdc-homology domain (37, 39), a ZZ-zinc finger domain that also has been associated with protein-protein interactions (48), and two consensus sequences for phosphorylation by PKC (Fig. 1A). However, due to the new C terminus, ZIP3 did not contain the PEST and ubiquitin-associated domains present in ZIP1 and ZIP2 (39). Interestingly, the ZIP3-specific C terminus started at the same splice site that is used to generate the ZIP1-specific cassette missing in ZIP2 (Fig. 1A; Refs. 39, 49).

To analyze the distribution of ZIP3, total RNA from different tissues was reverse-transcribed and subjected to PCR amplification. ZIP3 was present at similar levels in all organs analyzed, including the brain (Fig. 1B). Within the central nervous system, ZIP3 was abundantly expressed in spinal cord, thalamus, cortex, and the retina (Fig. 1B, right panels). PCR products of similar intensity for β-actin indicated that approximately equal amounts of cDNA from each tissue were used.

Mapping of Interacting Domains between ZIP3 and GABAC Receptor ρ Subunits—The highest sequence diversity between GABAC receptor subunits is found in their TM3-TM4 loops. Thus we analyzed whether these and the ρ3 subunit additional subunits of the GABAC receptor were able to interact with ZIP3. PCR products representing TM3-TM4 loops were cloned in the bait vector of the yeast two-hybrid system and expressed fusion proteins were analyzed for their ability to interact with ZIP3. Protein-protein interaction was monitored by the ability of transformed yeast colonies to grow on selective media, containing 3 µg or 3-AT. To our surprise, TM3-TM4 loops of all three ρ subunits bound ZIP3, with the ρ3 loop showing the highest binding affinity (Fig. 2A, left panel). In contrast to GABAC receptor ρ subunits, no interaction was observed between ZIP3 and subunits of the GABAC receptor (Fig. 2A, middle panel).

The specific interaction of ZIP3 with the ρ subunits of the GABAC receptor allowed the identification of amino acids important for the binding. An alignment of the TM3-TM4 loops of the rat ρ1–3 subunits identified two regions of high similarity, located at the very N- and C-terminal ends of the loops (Fig. 2B, upper half). To determine the involvement of these regions in ZIP3 binding, subsequent N- and C-terminal deletions were generated in the ρ3 TM3-TM4 loop, and the resulting protein fragments were tested directly for their ability to interact with ZIP3 in yeast cells (Fig. 2B, lower half) as described above. While amino acid regions located at the C-terminal part of the ρ3 loop showed no binding to ZIP3, 10 amino acids at the very N-terminal part of the loop were sufficient for the interaction (gray background in Fig. 2B). To analyze if the deletions changed the ρ3 binding affinity for ZIP3, the relative binding strength was estimated using a semi-quantitative β-galactosidase assay. Compared with the binding strength of the complete ρ3 TM3-TM4 loop, the relative ZIP3 binding affinities of the ρ3 constructs decreased slightly with subsequent deletions. Thus, construct ρ3-D3 contained a minimal ZIP3 binding region; however, additional amino acids present in constructs ρ3-D1 and ρ3-D2, which are absent in the TM3-TM4 loops of ρ1 and ρ2, support the interaction. This result is consistent with our finding that all three ρ subunits did interact with ZIP3 and that ρ3 showed a higher binding strength than ρ1 and ρ2 (Fig. 2A, left panel).

To map the location of amino acids that would act in combination with the 10 amino acids of the ρ3-D3 construct, we generated three additional deletions of the ρ3 TM3-TM4 loop. Upon deletion of the first 10 amino acids (construct ρ3-D6), the binding affinity for ZIP3 was reduced by about 50%, indicating that this domain is an important but not the only mediator for the interaction between ρ3 and ZIP3. When the first 10 amino acids were used in combination with more C-terminal domains of the ρ3 TM3-TM4 loop (constructs ρ3-D7 and ρ3-D8), the binding strength increased to values comparable with the wild-type sequence.
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Next, regions of ZIP3 were analyzed for their capability to bind the β3 TM3-TM4 loop, again using a strategy of subsequent deletions. The ZIP3/β3 interaction was mediated by the ZZ-zinc finger and a C-terminal adjacent protein region (amino acids 119–221, gray background in Fig. 2C), while the ZIP3-specific C terminus and the cdc-homology region were not involved in the binding. Dividing the interacting protein region in two parts resulted in a reduction in binding affinities (constructs ZIP3-D2 and ZIP3-D7). However, interaction with β3 was still present, indicating that amino acids in both protein regions contribute to the binding site in a synergistic manner. Indeed, the ZZ-zinc finger domain alone (construct ZIP3-D9) was able to bind the β3 TM3-TM4 loop at a binding intensity similar to those of constructs ZIP3-D2 and ZIP3-D7. ZIP3 Is Able To Dimerize and To Interact With GABAC Receptor β Subunits, Kvβ2, and PKC-ζ—Several proteins have been described in the literature to physically interact with ZIP1 or ZIP2, including PKC-ζ, shaker-type potassium channel β subunits, or the ZIP proteins themselves (37, 39). Therefore, we analyzed the binding characteristics of the newly identified member of the ZIP protein family, ZIP3. Immobilized GST fusion proteins were incubated with E. coli protein extracts and bound proteins were analyzed in Western blots. Unspecific interactions were excluded using immobilized GST. ZIP3 bound specifically to ZIP1, ZIP2, and ZIP3 and to the TM3-TM4 loops of the p1, p2, and β3 subunits (Fig. 3A), consistent with the results obtained from the yeast two-hybrid experiments. Furthermore, ZIP3 interacted with Kvβ2 (Fig. 3A) and with PKC-ζ (Fig. 3B). Since the N-terminal regions of ZIP1, ZIP2, and ZIP3 are identical, our data indicate that the ZIP dimerization domain and the binding regions for PKC-ζ and Kvβ2 are located in the N termini. Indeed, the region important for dimerization and binding of ZIP1 to PKC-ζ was mapped to the cdc-homology region (see Fig. 1; Refs. 37, 39, 41, 50).

In a next step, we analyzed the capability of native proteins to interact with ZIP3. Immobilized GST fusion proteins were incubated with cytosolic (S1) or membrane (P2) protein fractions prepared from adult rat brains. Consistent with our pre-
Previous results, ZIP3 interacted specifically with PKC-ζ and Kvβ2 (Fig. 3C). Nonspecific interactions were excluded using antibodies against protein phosphatase 1C (PP1C; 51) and against a protein interacting with C-kinase (PICK1; Ref. 52) as negative controls for cytosolic (S1) and membrane (P2) protein preparations, respectively. So far, no report described a physical interaction between ZIP3 and the GABAC receptor. To analyze whether the interaction between ZIP3 and the GABAC receptor ρ3 subunit in mammalian cells, HEK-293 cells were co-transfected with ZIP3 and GST or ZIP3 and GST fused to the TM3-TM4 loop of the ρ3 subunit. Cell lysates were incubated with glutathione-Sepharose beads, and bound proteins were analyzed using a monoclonal anti-T7 immunserum (Invitrogen). Bound proteins were detected on Western blots using specific immunsera as indicated on the right. Antibodies against PP1C and PICK1 served as negative controls to ensure specificity of the assay. The concentration of GST and GST fusion proteins bound to Sepharose beads is shown on Coomassie-stained SDS-PAGE (arrowheads). In addition, we tested whether the full-length ZIP3 subunit (Fig. 3D, lower panels). A, to analyze the binding of native proteins to ZIP3, cytosolic (S1) or membrane (P2) protein preparations of rat brain were incubated with GST or GST-ZIP3 immobilized on glutathione-Sepharose. Bound proteins were detected on Western blots using specific immunsera as indicated on the right. Antibodies against PP1C and PICK1 served as negative controls to ensure specificity of the assay. The concentration of GST and GST fusion proteins bound to Sepharose beads is shown on Coomassie-stained SDS-PAGE (arrowheads). B, to analyze whether the interaction between ZIP3 and PKC-ζ bound to Sepharose beads is shown on Coomassie-stained SDS-PAGE (arrowheads). C, to analyze the binding of native proteins to ZIP3, cytosolic (S1) or membrane (P2) protein preparations of rat brain were incubated with GST or GST-ZIP3 immobilized on glutathione-Sepharose. Bound proteins were detected on Western blots using specific immunsera as indicated on the right. Antibodies against PP1C and PICK1 served as negative controls to ensure specificity of the assay. The concentration of GST and GST fusion proteins bound to Sepharose beads is shown on Coomassie-stained SDS-PAGE (arrowheads). D, to verify the interaction between ZIP3 and the GABAC receptor ρ3 subunit in mammalian cells, HEK-293 cells were co-transfected with ZIP3 and GST or ZIP3 and GST fused to the TM3-TM4 loop of the ρ3 subunit. Cell lysates were incubated with glutathione-Sepharose beads, and bound proteins were analyzed using a monoclonal anti-T7 antibody. E, to verify the interaction between ZIP3 and the full-length ρ3 subunit, ρ3 was synthesized in vitro using radioactive methionine in the presence of microsomal membranes. The lysate was incubated with immobilized GST-ZIP3, and the bound ρ3 subunit was detected radiographically. In all panels, the Benchmark prestained protein ladder (Invitrogen) or calculated sizes of interacting proteins are indicated in kDa.

Specific immunsera to detect native ZIP3 or the GABAC receptor ρ3 subunit on Western blots do not exist, which prevented us from analyzing the interaction between these two proteins in native tissue. To circumvent this fact, we analyzed whether the interaction between ZIP3 and the GABAC receptor ρ3 subunit occurred when the binding partners were synthesized in mammalian cells. For this purpose, HEK-293 cells were co-transfected with T7-tagged ZIP3, and the ρ3 subunit TM3-TM4 loop fused to GST or T7-ZIP3 and GST as a control. Proteins interacting with the ρ3 loop were precipitated using glutathione-Sepharose beads and analyzed on a Western blot using an anti-T7 immunserum. In agreement with our previous data, ZIP3 was able to bind specifically to the ρ3 subunit, while no interaction could be observed with GST (Fig. 3D) or in cells co-transfected with the green fluorescent protein instead of ZIP3 (data not shown).

In addition, we tested whether the full-length ρ3 subunit would be able to bind to ZIP3. The ρ3 subunit was synthesized in vitro using a rabbit reticulocyte lysate in the presence of canine pancreatic microsomal membranes and subsequently incubated with immobilized GST-ZIP3 fusion proteins. Consistent with our data, we observed an interaction between the full-length ρ3 subunit and ZIP3, but not between ρ3 and GST (Fig. 3E).

Indication for a PKC-ζZIP3/GABA<sub>C</sub> Receptor-containing Macromolecular Complex—Our findings demonstrated that ZIP3 was able to dimerize with ZIP family members and to interact with GABAC receptor ρ subunits, PKC-ζ and Kvβ2, in vitro. We mapped the ZIP3 binding site for ρ subunits to a region different from the cdc-homology domain, that has been shown to mediate the interaction with PKC-ζ (see Fig. 2C; Ref. 37). Therefore, ZIP3 could bind to ρ subunits and PKC-ζ at the same time, acting as a scaffold, physically linking PKC-ζ to GABAC<sub>C</sub> receptors. To analyze whether the ρ3 subunit and PKC-ζ were indeed able to bind simultaneously to ZIP3, competition experiments were performed. Glutathione-Sepharose beads coated with the TM3-TM4 loop of the ρ3 subunit were first incubated with 500 μl (equivalent to 100% of the total volume) of ZIP3 containing E. coli protein extract, washed extensively, and subsequently mixed with increasing amounts of PKC-ζ containing protein extract. PKC-ζ was not able to displace ZIP3 from binding to the ρ3 subunit (Fig. 4A), indicating that PKC-ζ and the ρ3 subunit interacted with different regions of ZIP3, consistent with our previous data (Fig. 2C). Furthermore, this experiment directly demonstrated that ZIP3, PKC-ζ, and the ρ3 subunit were able to form a ternary complex in vitro, suggesting the possibility that ZIP3 could serve as a scaffold protein at synapses expressing GABAC<sub>C</sub> receptors.
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The binding of ZIP3 to GABA<sub>C</sub> receptors, PKC-ζ, and Kvβ2 was investigated in vitro using the GST/p3 + ZIP3 + PKC-ζ ternary complex. A, to analyze if PKC-ζ and the ρ3 subunit would interact simultaneously with ZIP3, competition experiments were performed. Equal amounts of GST/p3TM3-TM4 immobilized on Sepharose beads were first incubated with constant quantities of ZIP3-containing E. coli protein extracts, washed, and subsequently mixed with increasing concentrations of PKC-ζ extracts (12–100% of the total volume). Different volumes of PKC-ζ protein extracts were adjusted to 500 μl (defined as 100% of the total volume) by adding protein extracts of non-transfected E. coli cells. Bound proteins were detected on Western blots as described in Fig. 3A. B, to test for a direct interaction between PKC-ζ and the ρ3 subunit, cytosolic protein preparations of rat brain were incubated with the His-tagged TM3-TM4 loop of the ρ3 subunit immobilized on Ni-NTA beads. Bound proteins were detected using a PKC-ζ specific immunosum. In all panels, the Benchmark prestained protein ladder (Invitrogen) or calculated sizes of interacting proteins are indicated in kDa.

ZIP3 might serve as a linker, bringing PKC-ζ in close vicinity of ρ3-containing GABA<sub>C</sub> receptors. The ρ3 TM3-TM4 loop contains a consensus sequence for phosphorylation by PKC; however, a direct binding of PKC-ζ to the ρ3 subunit would be needed for its phosphorylation. Indeed, we could show a direct interaction between PKC-ζ and the ρ3 TM3-TM4 loop in native protein preparations (Fig. 4B). Non-specific interactions between ρ3 and the protein extract were excluded using antibodies against PP1C (51), that has not been reported to bind GABA<sub>C</sub> receptors. In addition to the native proteins, PKC-ζ and the ρ3 loop did also interact when recombinant proteins synthesized in E. coli were used (data not shown).

**PKC-ζ and GABA<sub>C</sub> Receptor ρ Subunits Are Co-expressed in the Same Cellular Compartments in the Mammalian Retina**—A prerequisite for any protein-protein interaction is the co-expression of the binding partners in the same cellular compartments. As said before, ZIP3-specific immunosera needed to detect the protein in native tissues were not available. Therefore, we analyzed whether proteins that physically interact with ZIP3, namely PKC-ζ and GABA<sub>C</sub> receptor ρ subunits, were co-expressed in the rat retina.

In the rat central nervous system, the highest concentration of GABA<sub>C</sub> receptor ρ subunits was detected in the retina, where they are clustered at synaptic terminals of bipolar cells (22–25). Vertical cryostat sections of adult rat retinas were double-labeled with antibodies recognizing GABA<sub>C</sub> receptor ρ subunits/PKCα and PKα/PKC-ζ. Stained sections were analyzed using confocal laser-scanning microscopy. Co-expression of PKC-ζ and the ρ subunits of the GABA<sub>C</sub> receptor had to be shown indirectly as the antisera are generated in the same species (rabbit). Fig. 5A shows that at the rod bipolar cell terminals that stratify deep in the inner plexiform layer and are stained with the antibody against PKCα, GABA<sub>C</sub> receptor ρ subunits are clustered. Importantly, in the terminals of the PKα-labeled rod bipolar cells, PKC-ζ is co-expressed (Fig. 5B). Co-expression can be clearly seen in the merge of the stainings, showing the terminals of the rod bipolar cells in a higher power view (broken lines mark the region of the inner plexiform layer shown). The results of the staining experiments suggest, although indirectly, the co-expression of GABA<sub>C</sub> receptor ρ subunits and PKC-ζ in rod bipolar cell terminals.

**DISCUSSION**

Increasing evidence underlines the importance of macromolecular signaling complexes containing ion channels, neurotransmitters receptors, kinases, and phosphatases for the specific regulation of neuronal excitability. The modulation of neurotransmitter receptors by kinases and phosphatases represents an important mechanism to regulate neuronal activity (54). However, factors controlling the specific targeting of these enzymes are largely unknown. The recently discovered ZIP proteins (38) form a new protein family and physically link the atypical protein kinase C isoform PKC-ζ to target proteins, such as potassium channels (39) or the death-domain kinase RIP, a protein involved in the activation of the transcription factor NF-κB (40, 41). In this study, we identified ZIP3 as a new member of the ZIP protein family, which is abundantly expressed in the brain and retina. ZIP3 was able to form homo- and heterodimers and bound to PKC-ζ and Kvβ2. Furthermore, ZIP3 interacted with GABA<sub>C</sub> receptor ρ subunits using a different binding site, allowing simultaneous binding of ρ subunits and PKC-ζ in vitro. These results suggest a possible formation of a PKC-ζ ZIP3/GABA<sub>C</sub> receptor containing macromolecular complex.

Mapping of protein regions mediating the ZIP3/GABA<sub>C</sub> receptor binding identified 10 amino acids that are conserved in the TM3-TM4 loops of ρ1–3 subunits. Compared with the complete ρ3 TM3-TM4 loop, subsequent C-terminal deletions of the ρ3 loop resulted in a slight decrease in ZIP3 binding affinity, indicating that the 10 amino acids represent a minimal binding motif, while additional ρ3 sequences support the interaction. Indeed, when these 10 amino acids were deleted, the binding affinity for ZIP3 was reduced by nearly 50%, while deletion of internal regions of the loop did not significantly alter the binding strength compared with the wild-type. Therefore, we propose at least two binding domains in the ρ3 TM3-TM4 loop contacting ZIP3, one present in the first 10 amino acids that acts in combination with a more C-terminally located motif. TM3-TM4 loops of GABA<sub>C</sub> receptor subunits were not able to bind ZIP3. This is consistent with the fact that the identified 10 amino acids of the ρ subunits are not conserved in GABA<sub>C</sub> receptor subunits.

In this study, we identified a region of ZIP3, including the ZZ-zinc finger domain that has been associated with protein-protein interactions (48), to be important for the binding to the ρ3 TM3-TM4 loop. This protein region is identical between ZIP3 and the protein extract was excluded using antibod-
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Fig. 5. Co-expression of GABA<sub>C</sub> receptors, PKC<sub>α</sub> and PKC-ζ, in the rat retina. Confocal micrographs of vertical sections through adult rat retinas double-immunolabeled for PKC-α and GABA<sub>C</sub> receptor ρ subunits (A) and for PKC-α and PKC-ζ (B). A, rod bipolar cells are immunoreactive for PKC-α (green) and their axon terminals are decorated with GABA<sub>C</sub> receptor immunoreactive puncta (red). This can be clearly seen in the merge of the two stainings, showing a higher power view of rod bipolar cell terminals (broken lines mark the region of the IPL shown). GABA<sub>C</sub> receptors present on PKC-α labeled terminals appear orange-yellow. B, PKC-α immunoreactive axon terminals of rod bipolar cells (green) also express PKC-ζ (red), shown as a yellowish color in the merge of the two stainings (broken lines mark the region of the IPL shown) (OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer). Scale bars, 10 μm.

Earlier studies that analyzed the retinal distribution of PKC-ζ showed contradictory results. While one study found that PKC-ζ co-localized with PKC-α in bipolar cells (55), another study showed PKC-ζ expression exclusively in the inner segments of photoreceptors (56). Here we confirm the co-localization of PKC-ζ and PKC-α in rod bipolar cells. Cerebellar Purkinje cells also express GABA<sub>C</sub> receptors (23) and, similar to the results reported in this study, co-express ZIP proteins and PKC-ζ (39).

PKC consensus sequences are present in the TM3-TM4 loops of rat ρ1–3 subunits, and GABA<sub>C</sub> receptor currents are modulated by PKC (27–29). This suggests a functional link between GABA<sub>C</sub> receptors and PKC-ζ. Indeed, we could show a direct interaction between PKC-ζ and the ρ3 TM3-TM4 loop in recombinant and native protein preparations, which would be needed for a protein phosphorylation. However, mutation of PKC consensus sequences of the ρ1 subunit did not prevent its modulation by PKC (30, 31, 57), indicating that residues other than those of the consensus sequences are phosphorylated (58). On the other hand, proteins other than the ρ1 subunit might be the target of the PKC activity. So far, no similar studies have been performed for the ρ3 subunit, and therefore it remains elusive whether the PKC consensus sequences of this protein are used. Alternatively, two consensus sequences for PKC present in ZIP3 might represent new players in the modulation of GABA<sub>C</sub> receptors. Furthermore, the synaptic clustering of GABA<sub>C</sub> receptors might be influenced by its interaction with ZIP3 and PKC-ζ since PKC-ζ interacts with tubulin and the actin cytoskeleton (60–61).

In summary, we present ZIP3 as a new member of the PKC-ζ-interacting protein family that highly expressed in the mammalian retina and demonstrated in vitro its interaction with PKC-ζ, GABA<sub>C</sub> receptor ρ subunits and Kvβ2. Therefore we suggest the formation of a postsynaptic macromolecular protein complex at GABA<sub>C</sub> receptor-containing synapses using ZIP3 as a scaffold.
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Cristina Croci, Johann Helmut Brandstätter and Ralf Enz

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