Guanylyl Cyclase/PSD-95 Interaction
TARGETING OF THE NITRIC OXIDE-SENSITIVE \( \alpha_2\beta_1 \) GUANYLYL CYCLASE TO SYNAPTIC MEMBRANES*

Michael Russwurm‡, Norbert Wittau‡, and Doris Koesling‡

From the ‡Pharmakologie und Toxikologie, Medizinische Fakultät MA N1, Ruhr-Universität Bochum, 44780 Bochum, Germany and the §Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69-73, Berlin 14195, Germany

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The signaling molecule nitric oxide (NO) exerts most of its effects by the stimulation of the NO-sensitive guanylyl cyclase. Two isoforms of the NO receptor molecule exist: the ubiquitously occurring \( \alpha_1\beta_1 \) and the \( \alpha_2\beta_1 \) with a more limited distribution. As the isoforms are functionally indistinguishable, the physiological relevance of these isoforms remained unclear. The neuronal NO synthase has been reported to be associated with PSD-95. Here, we demonstrate the interaction of the so far unnoticed \( \alpha_2\beta_1 \) isoform with PSD-95 in rat brain as shown by coprecipitation. The interaction is mediated by the \( \alpha_2 \) C-terminal peptide and the third PDZ domain of PSD-95. As a consequence of the PSD-95 interaction, the so far considered “soluble” \( \alpha_2\beta_1 \) isoform is recruited to the membrane fraction of synaptosomes, whereas the \( \alpha_1\beta_1 \) isoform is found in the cytosol. Our results establish the \( \alpha_1\beta_1 \) as the cytosolic and the \( \alpha_2\beta_1 \) as the membrane-associated NO-sensitive guanylyl cyclase and suggest the \( \alpha_2\beta_1 \) isoform as the sensor for the NO formed by the PSD-95-associated neuronal NO synthase.

NO-sensitive guanylyl cyclase (GC),¹ the enzyme that catalyzes the conversion of GTP to cGMP, acts as the effector molecule for nitric oxide, thereby playing a crucial role within the NO/cGMP signaling cascade (1, 2). By binding to the prosthetic heme group of GC, NO leads to the up to 200-fold activation of the enzyme (3, 4). The subsequent increase in cGMP formation results in the activation of cGMP effector molecules (cGMP-activated protein kinases, cGMP-regulated phosphodiesterases, cGMP-gated ion channels), which mediate the NO-induced effects. Besides its role in the cardiovascular system, NO acts as a freely diffusible transmitter in numerous pathways in the central and peripheral nervous systems (2, 5). In addition to NO, carbon monoxide (CO) has been proposed as a neuronal messenger that activates GC (5, 6) particularly as CO, carbon monoxide; NMDA, N-methyl-D-aspartate.

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† To whom correspondence should be addressed. Tel.: 49-234-322-6827; Fax: 49-234-321-4521; E-mail: koesling@iname.com.

‡ The abbreviations used are: GC, guanylyl cyclase; NO, nitric oxide; CO, carbon monoxide; NMDA, N-methyl-D-aspartate.
αβ2, GC isoform both present in brain. The in vivo existence of αβ2-PD5 complexes is shown in coprecipitation experiments. In contrast to the αβ2 isoform, the ααβ heterodimer is found in the membrane fraction of synaptosomes demonstrating that the PD5-95 interaction results in the membrane association of this so far soluble-considered GC isoform.

MATERIALS AND METHODS

Antibodies against GC—Antibodies directed against the C-terminal peptides of the α2 subunit (KKDVEEANFLFKAGSIDG, α2 subunit (KKVSYNGTMEFLRTSL), and β2 subunit (SRKNTGTTEETEQDEN) were synthesized as described in Ref. 15. Immune precipitation and Western blotting were performed according to standard protocols using the respective cysteine-tagged antigenic peptides coupled to Sulfolink Coupling Gel (Pierce).

Western Blots—Protein samples were resolved on 7.5% or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking the membranes, the following antibodies were used for detection: immunooaffinity-purified antibodies against the three GC subunits (α1, α2, and β1), anti-α3C-terminal peptide active anti-PD5-95 antibody (Clone 7E3–1B8, Affinity Bioreagents), antiisera against SAP102, SAP97, and PD5-93 (Affinity Bioreagents). Secondary peroxidase-labeled anti-mouse IgG and anti-rabbit IgG antibodies were from Sigma. Detection was performed using ECL and ECL®-Western blotting detection reagents from Amersham Pharmacia Biotech.

Determination of cGMP Forming Activity—GC activity was determined by incubation of the samples (containing 5–15 μg of protein) for 10 min at 37 °C with 1 μM cGMP, 250 μM [α-32P]GTP (about 0.2 μCi), 3 mM MgCl2, 3 mM Na2-dithiotreitol, 0.5 μg bovine serum albumin, 0.25 μg/liter creatine phosphokinase, 5 μm creatine phosphate, 1 mM 3-isobutyl-1-methylxanthine, and 50 mM triethanolamine hydrochloride, pH 7.4, in a total volume of 100 μl. The incubation was started by addition of diethylamine-NO (final concentration 100 μM) and by transferring the tubes from ice to 37 °C. Reactions were stopped by ZnCl2 precipitation, and isolation of the enzymatically formed cGMP was performed using ECL and ECL® Western blotting detection reagents from Amersham Pharmacia Biotech. The identity of the clones was verified by sequencing and expression and was performed in DH5α cells (Life Technologies). After induction of expression with 0.4 μM isopropyl-β-D-thiogalactopyranoside for at least 6 h, the cultures were collected by centrifugation at 7700 × g for 10 min and lyzed in phosphate-buffered saline by sonication. The lysate (1–3 mg/ml) was cleared by centrifugation at 50,000 × g for 15 min.

Preparation of Brain Homogenates for GC Subunit Detection—Rat brains were homogenized in 5 volumes of phosphate-buffered saline containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 1 μM pepstatin A). Subsequently, SDS was added to a final concentration of 1% to solubilize the membranes, and the homogenate was incubated (10 min, 4 °C) and by centrifugation (50,000 × g, 20 min, 4 °C). Protein concentrations were determined by the Bradford protein assay (Bio-Rad) following trichloroacetic acid precipitation. Proteins (40 μg) were analyzed in Western blots.

Precipitations with the Immobilized αC-Terminial Peptide—The cysteine-tagged αC-terminal peptide (C-FLRTESSL-COOH) and, as control, a scrambled peptide (C-SLFRTL-COOH) were coupled to Sulfolink Coupling Gel (Pierce) according to instructions of the manufacturer.

Rat brains were homogenized in 5 volumes of 0.5 M NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40 (Calbiochem), and 25 mM Tris/HCl, pH 7.4, and centrifuged for 20 min at 15,000 × g. The resulting supernatant (1.8 ml containing ~18 mg of protein) was incubated with the respective immobilized peptides (50 μl of resin) on an overhead rotator for 2 h at 4 °C. After extensive washing, interacting proteins were eluted with Laemmli buffer, and aliquots (5%) of the samples were analyzed by Western blotting.

Clone (ATCC) of Escherichia coli expressing the three PDZ domains of PD5-95 as GST fusion proteins prepared as described above were incubated for 30 min with the immobilized peptides (25 μl of resin). After washing, sample buffer was added, and aliquots (5%) of the samples were analyzed in Coomassie Brilliant Blue-stained gels. The identity of the fusion proteins was confirmed in Western blots using anti-GST antibodies (Sigma).

Preparation of Synaptosomal Cytosol and Membranes—Crude synaptosomes were prepared by combination of the methods described by Cohn et al. (29) and Mayer et al. (30). Rat brains were homogenized in 4 volumes of buffer A (0.32 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.4 containing 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 1 μM pepstatin A, and 2 mM Na2-dithiotreitol) by 15 strokes with a glass-Teflon Potter-Elvehjem homogenizer. The homogenate was diluted 1:10 in buffer A, and a low speed pellet was removed by centrifugation (750 × g, 10 min, 4 °C). The resulting supernatant was recentrifuged (17,300 × g, 10 min, 4 °C) to pellet the crude synaptosomal fraction. The crude synaptosomes were lysed by hypotonic shock and ultrasound in 1 mM EDTA and 50 mM triethanolamine hydrochloride, pH 7.4, containing protease inhibitors and Na2-dithiotreitol as in buffer A. The lysate was then centrifuged at 1.8 mg of protein for 15 min at 4 °C, and subsequently the NaCl concentration was increased to 150 mM to remove loosely associated proteins of the membrane. The lysate was then centrifuged (32,000 × g, 15 min, 4 °C). The resulting synaptosomal cytosol and membranes were analyzed in Western blots, and cGMP-forming activity in the fractions was determined as described above. Protein concentrations were determined by the Bradford protein assay (Bio-Rad) after trichloroacetic acid precipitation of the proteins.

Immunoprecipitation of PD5-95—Crude synaptosomes were prepared as described above and resuspended in buffer A. Triton X-100 was added to a final concentration of 1% to extract a fraction of PD5-95 and associated proteins. After a 10-min incubation at 4 °C, the Triton X-100 extract was centrifuged (32,000 × g, 10 min, 4 °C) to remove the cell debris. PD5-95 immunoprecipitation was carried out as follows: the synaptosomal extract (4.2 ml containing ~10 mg of protein) was incubated for 4 h at 4 °C with monoclonal anti-PD5-95-antibody (15 μl, Clone 7E3–1B8, Affinity Bioreagents) and 250 μl of Dynabeads Mouse IgG (Dynal); as control, the incubation was performed without the monoclonal anti-PD5-95 antibody. Dynabeads were precipitated using a magnet, and the beads were extensively washed with buffer A. Precipitated proteins were eluted with 65 μl of SDS-containing sample buffer, and 20 μl of the samples were analyzed in Western blots.

Precipitation of GC with GST-PD5 Fusion Proteins—The three PDZ-GST fusion proteins prepared as described above were loaded onto glutathione-Sepharose CL4B (Amersham Pharmacia Biotech) as suggested by the manufacturer. The amounts of loaded GST fusion proteins were checked by Bradford protein assay (Bio-Rad). Rat brains were homogenized in 5 volumes of a buffer containing 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 1 μM pepstatin A, 2 mM Na2-dithiotreitol, and 50 mM triethanolamine hydrochloride, pH 7.4, and centrifuged for 1 h at 220,000 × g to obtain a cytosolic fraction. Cytosolic proteins (10 mg of protein in 2.5 ml) were incubated with each of the three immobilized PDZ-GST fusion proteins (200 μl of fusion protein with ~1.2 mg of GST fusion protein) for 30 min at 4 °C. After extensive washing, 2.5 μl of the Sepharose were used to detect bound GC by measuring NO-stimulated cGMP-forming activity as described above. For Western blots, PDZ interacting proteins were eluted with SDS-containing sample buffer and analyzed in Western blots (2.5 μl of resin/lane).

RESULTS

Occurrence of the α2 Subunit in Rat Brain—To date, the existence of the α2 subunit of GC on protein level has been demonstrated only in human placenta (12). As the occurrence of the α2 subunit in brain was a prerequisite for a possible PD5-95/α2 interaction, we studied the expression of this subunit in rat brain using immunoadfinity-purified antibodies generated against the C-terminal peptide of the α2 subunit. As can be seen on the Western blot (Fig. 1), a protein with the appropriate molecular mass was detected in brain homogenates showing the expression of the α2 subunit in rat brain. In addition, the presence of the α1 and β2 subunits was demonstrated with the respective affinity-purified antibodies. The antibody against the α1 subunit detected an additional high molecular mass band; however the nature of this band remains to be clarified.

Interaction of the α2 Peptide with PD5-95—The existence of the α2 subunit in brain encouraged us to investigate whether the C-terminal peptide of the α2 subunit was capable of interacting with the PDZ domain-containing protein PD5-95 and related proteins. For that reason, we incubated the immobilized C-terminal α2 peptide with brain extracts and subse-
Figure 1. Expression of GC subunits in rat brain. Rat brain homogenate (40 µg per lane) was analyzed in Western blots using immunopurified antibodies against the three GC subunits. The most prominent signals with molecular masses of ~83 kDa, ~82 kDa, and ~66 kDa correspond to the α1, α2, and β1 subunits, respectively.

Figure 2. Affinity precipitation of PSD-95 and PSD-95 related proteins by the immobilized α2 C-terminal peptide. α2 C-terminal peptide or with a scrambled peptide as control. After washing, interacting proteins were separated on SDS-gels. The Western blots performed with antibodies against PSD-95, PSD-93, SAP102, and SAP97 demonstrated the specific precipitation of these proteins by the immobilized α2 C-terminal peptide. In the input, 6% of the brain extracts used for the precipitation experiments were applied.

Subcellular Distribution of GC Subunits—In vivo interaction of αβ1/GC and PSD-95 should lead to the colocalization of the αβ1 isoform and PSD-95 in synaptic membranes. As PSD-95 has been shown to be enriched in membranes of synaptosomal preparations (22), we proceeded to study the subcellular distribution of GC subunits in this material. All GC subunits were detected in crude synaptosomes (not shown); however, after disruption of the synaptosomes, the α1 subunit was almost exclusively found in the synaptosomal cytosol, whereas the majority of the α2 subunit was detected in the membrane fraction (Fig. 3A). The β1 subunit was distributed almost equally between membranes and cytosol. This finding is in accordance with our former observation that the α1 subunit represents the dimersizing partner for both the α2 and α1 subunits. The immunological evidence of a cytosolic and a membrane-associated NO-sensitive GC was further supported by determination of NO-stimulated cGMP formation in the respective fractions (Fig. 3B). Enzyme activities in the presence of NO amounted to ~4 and ~1 nmol × mg⁻¹ × min⁻¹ in cytosolic and membrane fractions, respectively. Non-stimulated cGMP-forming activities were below the detection limit. These results show that the membrane-associated isoform of GC was responsible for a substantial amount of the NO-stimulated cGMP-forming activity in synaptosomes and established the α1β1/GC as the cytosolic and the α2β1/GC as the membrane-associated isoform of GC. Moreover, the results demonstrate the presence of the α2β1 heterodimer in the PSD-95-containing compartment.

αβ1/GC Coprecipitates with PSD-95—To study the existence of in vivo formed αβ1/PSD-95 complexes in rat brain, we performed immunoprecipitation experiments. To be able to precipitate PSD-95, synaptosomes had to be extracted with Triton X-100 to solubilize at least a portion of the otherwise membrane-associated PSD-95. The Triton extracts were incubated with monoclonal antibodies against PSD-95, and precipitated proteins were analyzed in Western blots using immunopurification-purified antibodies against each of the GC subunits. In the PSD-95 precipitate, the α1 antibodies recognized a protein with the appropriate molecular mass clearly showing that the α2 subunit is associated with PSD-95 (Fig. 4). The dimerizing partner of the α1 subunit, the β1 subunit, was found in the PSD-95 precipitate, too. In contrast, the α1 subunit was not detected in the PSD-95 precipitate, indicating that only the α2 subunit but not the α1 subunit is able to interact with PSD-95 family.

αβ1/GC Preferentially Interacts with the Third PDZ Domain of PSD-95—To study the molecular basis for the αβ1/PSD-95 interaction, we generated GST fusion proteins of each of the PDZ domains of PSD-95 and tried to precipitate these fusion proteins with the immobilized α2 C-terminal peptide. As can be seen on the Coomassie Brilliant Blue-stained gel (Fig. 5), all three PDZ domains were precipitated by the α2 peptide showing that the PSD-95/α2 subunit interaction was mediated by the PDZ domains of PSD-95 and the C-terminal peptide of the α2 subunit. However, in this experimental setting no binding preference of the α2 peptide to one of the three PDZ domains was detectable. We attributed this lack of differential binding to the fact that only the minimal parts of both binding partners required for interaction were present in the experiment. Therefore, we used the immobilized PDZ-GST fusion proteins to precipitate the complete αβ1 enzyme of brain cytosol and quantified the amount of PDZ domain-bound GC by measuring GC activity. Here, the third PDZ domain of PSD-95 precipitated substantially more NO-stimulated cyclic GMP-forming activity than the first and second PDZ domains (Fig. 6A) indicating a higher affinity of the complete αβ1 enzyme to the third PDZ domain. To ensure that PDZ domain-bound cGMP-forming activity was solely due to the α1β1 isoform, we performed a Western blot analysis of the PDZ precipitates. As shown in Fig. 6B, only the α2 and β1 subunits but not the α1 subunit is associated with PSD-95.
subunit were detected, again demonstrating that the α₂β₁ but not the α₁β₁ isoform interacted with PSD-95.

**DISCUSSION**

Nitric oxide has been implicated to be involved in synaptic transmission since the late 1980’s, and a role in neuronal plasticity has been proposed. Although other targets for NO might exist, the NO-sensitive GC is the best studied NO receptor. However, little information about NO-sensitive GC in brain is available.

In the current report, we studied the so far unnoticed guanylyl cyclase isoform α₂β₁ and present evidence that the special quality of this isoform is defined by the α₂ C-terminal peptide which is able to mediate the interaction with PDZ domains. In particular, we studied the α₂β₁/PSD-95 interaction, thereby demonstrating for the first time the existence of the α₂β₁ heterodimer in brain and addressing the so far unattended aspect in the GC research, i.e. the membrane association of the until now considered cytosolic enzyme.

PDZ domains are present in a plethora of proteins and have been reported to act as molecular adaptors to direct the subcellular distribution of proteins thereby mediating the formation of functional complexes. In brain, PSD-95 and related proteins have been proposed to play important roles e.g. by clustering NMDA receptors at excitatory synapses and by anchoring the receptor to downstream effector proteins like the neuronal NO synthase. Mice deficient in PSD-95 showed unaffected synaptic morphology and NMDA-receptor function but featured impaired learning and enhanced long-term potentiation (31). These results emphasize the importance of the formation of “transducisomes” downstream the NMDA receptor for synaptic plasticity and learning.

In most cases, PDZ domains bind to the C-terminal sequence of their binding partners, although in the case of the NO-
synthase an internal stretch of amino acids has been reported to account for the interaction with PSD-95 (32). A C-terminal consensus sequence (S/T)X(V/I) for proteins interacting with the PDZ domains of PSD-95 has been deduced from the known binding partners of PSD-95 (18). Despite the fact that the α2 peptide (RETLSL) contains a leucine residue at the C-terminal position, we were able to show that the C-terminal α2 peptide does bind to the PDZ domains of PSD-95. The relevance of this finding is corroborated by the demonstration that the recombinant PDZ domain-containing protein. Further experiments have to show whether this membrane association of the α2β1 isoform of GC via PDZ domains represents a general biological principle also found in other tissues. Up to this date, GCs have been divided in membrane-bound and soluble enzymes. This categorization may be the reason why this membrane-associated NO-sensitive GC has been overlooked for the past 20 years. The classification in membrane-bound and soluble guanylyl cyclase enzymes is incompatible with the physiological existence of receptor-linked membrane-bound GCs, a membrane-associated NO-sensitive GC (α2β1), and a soluble NO-sensitive GC (α1β1). Rather, we suggest the classification of the enzyme family according to the regulatory properties of their members, i.e. receptor-linked GCs and NO-sensitive GCs.

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