Phosphorylation of γ -aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase

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Preparations of γ -aminobutyrate (GABA)/benzodiazepine receptor from pig cerebral cortex are composed of three major bands of polypeptides (51, 55 and 57 kDa) which are purified in a ratio of approx. 2:1:1 respectively. Treatment of purified receptor preparations with cyclic AMP-dependent protein kinase resulted in major incorporation of ^{32}P into the 55 kDa band only. The maximum incorporation achieved was 0.6 mol of ^{32}P /mol of 55 kDa polypeptide. The phosphorylated receptor subunit (β -subunit) displays the same apparent M_r as a band labelled irreversibly with the GABA receptor agonist [^3H]muscimol. The two non-phosphorylated subunit polypeptides (51 and 57 kDa) are each labelled irreversibly with [^3H]flunitrazepam and are recognized by anti-peptide antibodies specific for α -subunits.

INTRODUCTION

Chloride channels which are gated by the inhibitory neurotransmitter γ -aminobutyrate (GABA), and are subject to modulation by anxiolytic benzodiazepines, have been termed GABA/benzodiazepine GABA_A receptors. Benzodiazepine-affinity chromatography has been applied to purify these receptors from several species and brain regions [1]. In most studies, purified receptor preparations have been found to consist of two major bands of polypeptides, termed α - and β -subunits. Recently, additional α -like subunits have been detected using α -subunit-specific monoclonal [2] or antipeptide [3] antibodies. In purified receptor preparations, distinct α -like subunits may result from differential post-translational modification, although their existence is also consistent with the recent detection of distinct α -like mRNAs in bovine brain [4]. Of the four bovine receptor subunits ($\alpha 1$ – $\alpha 3$, β) for which complete amino acid sequences have been deduced [4, 5], only the β -subunit contains a consensus sequence for phosphorylation by cyclic AMP-dependent protein kinase. However, this enzyme has not yet been shown to phosphorylate the receptor complex.

Several protein kinases have been implicated in the regulation of receptors and ion channels [6, 7]. With respect to the GABA_A receptor, protein kinase C has been implicated in the down-regulation of receptors [8], while a distinct phosphorylating enzyme may be required for maintenance of receptor function [9]. A role for cyclic AMP-dependent protein kinase in the functional regulation of GABA_A receptors has yet to be established. However, this enzyme has been implicated in the regulation of a structurally related receptor, the nicotinic acetylcholine receptor of *Torpedo* electric organ [10] and rat myotubes [11].

Recently, a partially purified preparation of rat brain GABA_A receptor was found to contain a protein kinase activity which appears to phosphorylate the α -subunit of

the receptor [12]. This kinase activity was insensitive to several regulators of known protein kinases (e.g. cyclic AMP-dependent). Here we report that receptor preparations, purified from pig cerebral cortex, contain a phosphorylation site on the β -subunit accessible to cyclic AMP-dependent protein kinase. The stoichiometry of subunit labelling was determined and the relative abundance of α - and β -like subunits was quantified.

EXPERIMENTAL

Materials

Reconstituted GABA_A receptor preparations were prepared from pig cerebral cortex as described previously [3] except that vesicles were formed by dialysis against 20 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/0.02% (w/v) NaN_3 . ATP, bovine heart protein kinase catalytic subunit (1 unit transfers 1.0 pmol of phosphate from ATP to casein/min at pH 6.5, 30 °C) and cyclic AMP-dependent protein kinase inhibitor (Type III) [13] were from Sigma. [γ - ^{32}P]ATP (3000 Ci/mmol) and [*methyl*- ^3H]flunitrazepam (82 Ci/mmol) were from New England Nuclear. [*methylamine*- ^3H]muscimol (21 Ci/mmol) was from Amersham International. Affinity-purified anti-($\alpha 1$ –101–109) antibodies and other antisera were prepared as described previously [3].

Standard phosphorylation assay

The standard incubation mixture (35 μl) contained 25 mM-Tris/HCl (pH 7.5), 10 mM-MgSO₄, 0.5% (w/v) Triton X-100, 12.5 units of protein kinase catalytic subunit, 10 mM-dithiothreitol, 2.6 μM -[γ - ^{32}P]ATP (17.5–28.1 Ci/mmol) and 10 μl of reconstituted receptor preparation (250 μg of protein/ml). The mixture was preincubated at 37 °C for 2 min in the absence of ATP. The phosphorylation reaction was initiated by the addition of [γ - ^{32}P]ATP (5 μl) and terminated by addition of 35 μl of 'SDS-stop solution' containing 60 mM-Tris/HCl (pH 6.8), 2%

Abbreviation used: GABA, γ -aminobutyrate.

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(w/v) SDS, 10 mM-dithiothreitol, 10% (w/v) glycerol and 0.005% (w/v) Bromophenol Blue. The mixture was heated at 100 °C for 2 min and a 50 μ l aliquot was subjected to SDS/polyacrylamide-gel electrophoresis (see below).

Photoaffinity labelling

Samples of reconstituted receptor containing 1.0 pmol of [³H]flunitrazepam binding sites or 4.5 pmol of [³H]muscimol binding sites were photolabelled [3,14] with 20 nM-[³H]flunitrazepam and 100 nM-[³H]muscimol respectively. Following sample concentration by precipitation in trichloroacetic acid [14] and SDS/polyacrylamide-gel electrophoresis (see below), gels were treated with PPO [15], dried and subjected to fluorography at -70 °C.

SDS/polyacrylamide-gel electrophoresis, immunoblotting and quantification

SDS/polyacrylamide-gel electrophoresis was performed using the system of Laemmli [16] with a 7–13% (w/v)-polyacrylamide gradient. In order to obtain optimum resolution of receptor subunits [3], M_r marker proteins of 20 kDa were run off the end of the gels. Following electrophoresis, gels were Coomassie-stained, silver-stained [17] or blotted on to nitrocellulose filters [18]. Filters were first treated with affinity-purified anti-(α 1-101-109) antibodies (1:1000) followed by ¹²⁵I-labelled goat anti-(rabbit IgG) IgG (10⁶ c.p.m./ml). All blocking and washing steps were as described previously [18].

Autoradiography of dried gels or nitrocellulose filters was performed at -70 °C. Quantification of ³²P-labelling was determined by cutting labelled bands from dried gels and solubilizing the radioactivity with 1 ml of 30% (w/v) H₂O₂ (100 °C, 2 h). Samples were counted for radioactivity in 4 ml of Optiphase Safe (LKB).

Protein concentration was determined by a micro-Lowry method [19] or, where noted, by gel scanning of Coomassie-stained proteins at 550 nm. In both cases, bovine serum albumin was used as a standard.

RESULTS

As shown previously [3], SDS/polyacrylamide gel electrophoresis resolved three major bands of polypeptides in preparations of the GABA_A receptor purified from pig cerebral cortex (Fig. 1a, lane 2). Gel-scanning revealed that these bands, with apparent molecular masses of 51, 55 and 57 kDa, comprised over 95% of the total stained protein and were stained with relative intensities of $1:0.44 \pm 0.02:0.46 \pm 0.02$ respectively (mean values $\pm$ S.D.; $n = 3$ from two separate receptor preparations). Although these values may be subject to some residual error if the subunits are stained differentially by Coomassie Blue, such differences are likely to be small in view of the structural similarity of the subunits [5]. Similar assumptions were made by Mamalaki *et al.* [20] in relation to the bovine receptor.

When present alone in the phosphorylation assay medium, preparations of receptor complex or protein kinase catalytic subunit were not labelled with detectable amounts of radioactivity (Fig. 1a, lanes 3, 4, 10 and 11). Incubation of the receptor with the catalytic subunit resulted in a time-dependent incorporation of ³²P into a polypeptide corresponding to the 55 kDa subunit of the

Fig. 1. Time course for phosphorylation of the GABA/benzodiazepine receptor by cyclic AMP-dependent protein kinase catalytic subunit

(a) Purified receptor alone (lanes 2, 3 and 10), catalytic subunit alone (lanes 4 and 11) or a combination of receptor and catalytic subunit (lanes 5–9) were incubated for various times in phosphorylation media, as described in the Experimental section. The samples, together with marker proteins (lane 1) were subjected to SDS/polyacrylamide-gel electrophoresis, stained with Coomassie Blue (lanes 1 and 2) and incorporation of [³²P]phosphate was detected by autoradiography (lanes 3–11). (b) The incorporation of ³²P into samples described in (a) was quantified following excision of the 55 kDa region from dried gels. The gel slices were derived from assay mixtures containing receptor alone (□), catalytic subunit alone (■) or a combination of receptor and catalytic subunit (●). Each point represents the mean $\pm$ S.D. of three separate determinations.

Fig. 2. Labelling of GABA/benzodiazepine receptor subunits with [3 H]receptor ligands, 32 P and antipeptide antibodies

Purified receptor was photoaffinity labelled with [3 H]flunitrazepam (lane 1) or [3 H]muscimol (lane 2) and subjected to SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. Purified receptor (2.5 μ g) was phosphorylated with catalytic subunit under standard conditions and, following electrophoresis, was silver-stained for protein (lane 3) and subjected to autoradiography (lane 4). Purified receptor (3 μ g) was subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose filters and treated with affinity-purified anti-(α 1-101-109) antibodies (lane 5) as described in the Experimental section.

receptor preparation (Fig. 1a, lanes 5-9; Fig. 1b). Using 12.5 units of catalytic subunit, maximal incorporation of 32 P into approx. 2.5 μ g of receptor protein was achieved in less than 10 min (Fig. 1b). By gel scanning and determination of the 32 P content of the 55 kDa band, it was estimated that maximum incorporation (30 min incubation) was 0.60 ± 0.08 mol of 32 P/mol of polypeptide (mean $\pm$ s.d.; $n = 3$). This value is reduced only slightly (0.56 ± 0.08) when taking into account the molecular mass of deglycosylated β -subunit (51400 Da; [5]).

A partially purified cyclic AMP-dependent protein kinase from a synaptic membrane fraction [21] caused major phosphorylation of the 55 kDa subunit only in the presence of cyclic AMP. This activity, and the activity of purified catalytic subunit on the 55 kDa polypeptide, were blocked by a cyclic AMP-dependent protein kinase inhibitor (results not shown).

In order to compare the receptor polypeptide which is phosphorylated by cyclic AMP-dependent protein kinase

with other markers of receptor subunits, the same receptor preparation was probed with photoaffinity ligands and with α -subunit-specific antibodies (Fig. 2). Covalent labelling of the receptor with [3 H]flunitrazepam results in major incorporation of radioactivity into 51 and 57 kDa bands (Fig. 2, lane 1). In contrast, [3 H]muscimol labels predominantly a 55 kDa band of polypeptides (Fig. 2, lane 2). This band comigrates with the phosphorylated band of polypeptides (Fig. 2, lane 4) but is not recognized by antibodies raised against a nonapeptide sequence contained within the three known bovine α -subunits. These antibodies only recognized polypeptides in the region of 51 and 57 kDa (Fig. 2, lane 5).

Preliminary studies indicate that the purified GABA $_A$ receptor complex is not phosphorylated significantly by protein kinase C, Ca^{2+} /calmodulin-dependent, cyclic GMP-dependent or 3-phosphoglycerate-dependent protein kinases [22] (results not shown). However, detergents which were used to maintain the receptor in solution (Triton X-100 or sodium cholate) caused partial inhibition of these enzymes, and determination of optimum assay conditions will be necessary before their activity at the receptor can be defined conclusively.

DISCUSSION

GABA $_A$ receptor preparations derived from pig cerebral cortex contain three distinct bands of polypeptides (51, 55 and 57 kDa) which are present in a ratio of approx. 2:1:1. In contrast, the receptor complex purified from bovine cerebral cortex has been reported to consist of two subunits α and β (53 and 57 kDa respectively) estimated to be present in a ratio of 1:1 [20]. It is possible that bovine cerebral cortex also contains distinct 55 and 57 kDa polypeptides which were not resolved in the β -subunit region of the gel during electrophoresis. If so, the apparent immunological cross-reactivity of α and β subunits [20] could also be explained by antibodies recognizing only α -like subunits, some of which are now known to possess apparent molecular masses similar to those of β -subunits [2, 3].

A tetrameric quaternary structure ($\alpha_2\beta_2$) has been suggested for the GABA $_A$ receptor [20], although two related ligand-gated ion channels, *Torpedo* nicotinic acetylcholine receptors and glycine receptors, have been proposed to exist as pentamers [23, 24]. The subunit ratio of the pig cortical preparation (2:1:1) is consistent with a tetrameric structure. However, it is also possible that combinations of two or three subunits could exist in either tetrameric or pentameric structures. The possibility that distinct combinations of subunits occur is supported by evidence demonstrating a lack of detectable 57 kDa α -subunit in pig cerebellar receptor preparations [3] and the selective expression of distinct α -subunit mRNAs in different brain regions [4]. A recent report demonstrating the ability of individual subunits to form GABA-gated chloride channels [25] also raises the possibility of homooligomeric receptors in brain tissue.

Of the four bovine receptor subunits ($\alpha 1$ - $\alpha 3$, β) for which complete amino acid sequences have been derived, only the β -subunit has a consensus sequence for phosphorylation by cyclic AMP-dependent protein kinase. As only the 55 kDa band of pig cortical preparations was phosphorylated by this enzyme, it is likely that this polypeptide is a β -subunit. The 55 kDa

band was phosphorylated to a ratio of approx. 0.6 mol of phosphate/mol of polypeptide. Presently it is not known whether this submaximal incorporation is due to an overestimation of protein content, due to additional polypeptides which comigrate with the β -subunit, or results from the purification of receptor preparations which are already partially phosphorylated. If, for example, the receptor was phosphorylated in response to receptor occupancy, the release of large amounts of GABA during the preparation of membranes and soluble extracts could result in phosphorylation of a significant proportion of receptor molecules.

Partially purified preparations of rat brain receptor have been found to contain a receptor-associated protein kinase which phosphorylates the α -subunit of the receptor [12]. Using assay conditions appropriate for this enzyme [12], a similar kinase activity in pig receptor preparations was not observed, although the β -subunit remained a substrate for phosphorylation by cyclic AMP-dependent protein kinase (data not shown). It is probable that the thorough washing procedure (including 1 M-urea) employed during preparation of the pig receptor results in dissociation of any associated kinase molecules.

As noted previously [3], reconstituted receptor preparations appear to incorporate [3 H]flunitrazepam and [3 H]muscimol into distinct polypeptides (Fig. 2, lanes 1 and 2). As [3 H]muscimol is incorporated predominantly into a 55 kDa band which comigrates with the phosphorylated band, it is likely that [3 H]muscimol labels the β -subunit preferentially. However, recent reports have described [3 H]muscimol-labelling of multiple subunits [26], and the detection of GABA-gated chloride channels formed by receptors comprised of α -subunits alone [25]. This would suggest that GABA-binding sites may not be located exclusively on β -subunits although predominant incorporation of [3 H]muscimol may occur at high-affinity binding sites which are only associated with β -subunits. Phosphorylation by cyclic AMP-dependent protein kinase therefore represents a more reliable marker than [3 H]muscimol labelling for the β -subunit polypeptide in purified preparations of receptor. The polypeptides of the pig GABA_A receptor of 51 kDa and 57 kDa which are recognized by anti-(α -101-109) antibodies and are labelled with [3 H]flunitrazepam are likely to represent α 1 and α 3 respectively on the basis of their regional distribution in the brain [3, 4].

Phosphorylation by cyclic AMP-dependent protein kinase appears to modulate the activity of a number of receptors and ion channels, e.g. by increasing the rate of desensitization of the nicotinic acetylcholine receptor [10]. Preliminary studies using cultured neurons suggest that stimulation of the enzyme [27] or introduction of the catalytic subunit (R. L. Macdonald, N. Porter, R. E. Twyman & M. Uhler, personal communication) also reduces GABA-gated currents. Here we have provided biochemical evidence which demonstrates that GABA_A receptor preparations possess an accessible phosphorylation site for the enzyme. With the increasing complexity of subunit identification arising from the detection of additional α -like subunits, cyclic AMP-dependent protein kinase may also serve as a tool to label the β -subunit of the GABA_A receptor selectively and so help distinguish between receptor subunits.

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