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Phosphorylation of the $GABA_A$ Receptor by cAMP-Dependent Protein Kinase and by Protein Kinase C: Analysis of the Substrate Domain*

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Previous work has shown that the $GABA_A$-receptor ($GABA_A$-R) could be phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and a receptor associated kinase. However, no clear picture has yet emerged concerning the particular subunit\subtypes of the $GABA_A$-R that were phosphorylated by PKA and PKC. In the present report we show that an antibody raised against a 23 amino acid polypeptide corresponding to a sequence in the putative intracellular loop of the β1 subunit of the receptor blocks the in vitro phosphorylation of the purified receptor by PKA and PKC. Moreover, N-terminal sequence analysis of the principal phosphopeptide fragment obtained after proteolysis of the receptor yielded a sequence that corresponds to the β3 subunit of the receptor. Such data provide additional support for our hypothesis (Browning et al., 1990, Proc. Natl. Acad. Sci. USA 87:1315–1317) that both PKA and PKC phosphorylate the β-subunit of the $GABA_A$-R.

INTRODUCTION

The γ-aminobutyric acid receptors ($GABA_A$-R) mediate the majority of inhibitory synaptic transmission in the central nervous system. These receptors are members of a family of ligand-gated ion channels that includes the nicotinic acetylcholine receptors (nACh-R), and the strychnine-sensitive glycine receptor. Significant homologies exist among all of these proteins. The nACh-R and the $GABA_A$-R have been shown to be phosphorylated by cAMP-dependent protein kinase (PKA) and by the Ca^{2+}/phosphatidylserine-dependent protein kinase (PKC) (see (1,2) for pertinent reviews). In addition, this phosphorylation has been shown to enhance the rate of desensitization of the nACh-R (1). There have been a number of recent reports which show that $GABA_A$-R function is altered by factors that activate PKA (3,4). However, we and others have shown that such factors may be producing direct, phosphorylation-*independent* effects on the $GABA_A$-R (5,6). Porter et al, (7) examined the effects of PKA directly by introducing the catalytic subunit of PKA into spinal cord neurons in culture using the whole cell patch technique. They reported that the kinase produced almost total inhibition of GABA evoked chloride current. We have subsequently demonstrated that introduction of PKA into lysed and re-

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sealed microsacs also inhibits muscimol stimulated chloride flux although we have observed only a 25% inhibition (8). In contrast, as discussed in a recent review (2) Yeh et al., using whole cell patch of cerebellar Purkinje cells have shown that PKA *potentiates* GABA-stimulated chloride currents. No explanation of the differences in response seen in those studies has yet appeared. However, we and others have hypothesized that these differences in response to PKA may be due to the multiplicity of $GABA_A$-R subunits and the numerous subtypes of these subunits that have been identified via classical biochemical techniques and through molecular cloning. Indeed recent work suggests that there may be 7 α, 4 β, 3 γ, 1 δ, 1 ρ subtypes of the $GABA_A$-R plus alternative splicing variants of some of these proteins (9-18).

A number of authors have directly examined the ability of the $GABA_A$-R to be phosphorylated in vitro. Sweetnam et al., (19) demonstrated that a kinase associated with the partially purified $GABA_A$-R phosphorylated a polypeptide in the receptor preparation that comigrated on two dimensional electrophoresis with a purified, immunolabeled and photolabeled α-subunit of the $GABA_A$-R. However, this phosphorylation did not seem to be mediated by either PKA or PKC. Kirkness et al. (20) demonstrated that PKA could phosphorylate a subunit of the $GABA_A$-R that comigrated in one-dimensional electrophoresis with a photolabeled β-subunit of the $GABA_A$-R. Subsequently we demonstrated (21) that PKA and PKC could phosphorylate two polypeptides in the purified $GABA_A$-R and these two polypeptides comigrated in one dimensional electrophoresis with two photolabeled β-subunits of the $GABA_A$-R. Finally, Whiting et al., (13) presented evidence that a specific subtype of the γ-subunit (γ2L) of the $GABA_A$-R could be phosphorylated by PKC; however, these authors did not provide data on the stoichiometry of this phosphorylation. Unfortunately, in the studies suggesting that the β-subunit of the receptor was phosphorylated, only comigration in one dimensional gels with muscimol labeled polypeptides was used as evidence. As is so clearly explicated in Dr. Paul Greengard's writing on artifacts associated with phosphorylation research, such data provide only a preliminary form of phosphoprotein identification (see particularly chapter 8 in (22). We have been particularly interested in determining which of the multiplicity of $GABA_A$-R subunits are phosphorylated by PKA and PKC in the hope that such data might contribute to a better understanding of the apparently contradictory reports (see above) concerning the effects of phosphorylation on $GABA_A$-R function. In the present report we attempt to further examine the possibility that the β-subunit(s) of the $GABA_A$-R is phosphorylated in vitro by PKA and PKC.

EXPERIMENTAL PROCEDURE

The catalytic subunit of PKA was purified from bovine heart as described (23). PKC was purified by the method of Woodgett and Hunter (24). The $GABA_A$ receptor was purified as previously described (15,25,26). Antibodies to the region of the cytoplasmic domain containing the consensus sequence for PKA were prepared as described previously (26-28). ^{125}I-protein A and [*methylene*^{3}H(n)]muscimol(15-31 Ci/mmol (n = nominal) were obtained from Dupont-New England Nuclear. [γ-^{32}P]ATP was obtained from either Amersham or ICN. Phosphatidylserine was obtained from Avanti Polar-Lipids. *Staphylococcus aureus* V8 protease was from Miles. All other chemicals were reagent grade or better.

Phosphorylation Assays. The PKA phosphorylation assay (final volume 100 μl) mixture contained 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, PKA 3 μg/ml, and 50 μM ATP (specific activity 10^7 cpm/nmol). The activity of PKC appeared to be inhibited by a factor in the receptor preparation as the autophosphorylation of PKC was dramatically inhibited in the presence of the receptor. This inhibition was likely due to the presence of detergent and could be diminished by exhaustive dialysis. In addition for PKC phosphorylation, use of the mixed micelle procedure of Hannun et al. (29) was optimal. Briefly, phosphatidylserine and diolein equivalent to 8.0 mol % and 2.5 mol % respectively of the Triton X-100 were dried under a stream of nitrogen in a glass tube. The dialyzed receptor in 0.2% Triton X-100 was then added to the tube and vortexed vigorously for 1 min. Appropriate amounts of the receptor were then added to the PKC reaction mixture which was identical to the PKA reaction except for the following additions (final concentrations):300 μM $CaCl_2$, PKC at 3 μg/ml and PKA was omitted. The concentration of the receptor in the assay was 8-10 pmol/ml. All reactions were carried out at 30°C for 30 min.

Preparation of Phospho-Receptor for Sequence Analysis. $GABA_A$ receptor was purified from whole rat brain as described above. Phosphorylation by PKA of 50 pmol of [3H]muscimol binding sites (120 ug protein) in the presence of [γ-32P] ATP was performed as described above. This was combined with an equal amount of unlabeled receptor and precipitated with chloroform/methanol. After taking up the protein in 125 mM Tris, pH 7.5, containing 1% SDS, the receptor was digested with 5 μg *Staphylococcus aureus* V8 protease at 37°C for 24 h; followed by the addition of 5 μg TLCK chymotrypsin and further digestion for 24 h. Peptide fragments were separated on a 15% gel and transferred using the semi-dry method to PVDF membrane (Immobilon P, Millipore). N-terminal gas phase Edman degradation and sequencing was carried out in the UCLA Microsequencing Service Facility (Director, Dr. Audree Fowler) on an Analytical Biosystems 175A sequencer.

RESULTS

Figure 1 shows a model for the β_1 subunit of the $GABA_A$-R with the four putative membrane spanning regions and the large intracellular loop which contains a consensus sequence for PKA (see excerpted amino acid

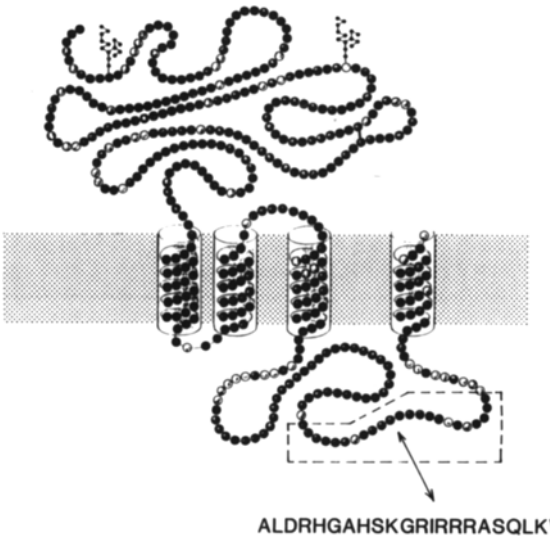

Fig. 1. A model of the β_1 subunit of the GABA$_A$-R. The four putative membrane spanning regions are shown. In addition the double headed arrow indicates the position in the large intracellular loop where a consensus sequence for PKA is located. Also indicated by the arrow is a 23 amino acid sequence which contains this potential site and it is this sequence which was used in the production of the antibodies in this study.

Fig. 2. Comparison of the amino acid sequences containing the consensus sequence for PKA in β-subunits of the GABA$_A$-R from cow, rat and chicken. The shaded areas indicate amino acids identical to the bovine β_1 sequences; overline indicates areas of protein β-like secondary structure. The putative phosphorylation site for PKA is indicated by the circled "P". To facilitate coupling a C-terminal cysteine (C*) was added.

sequence). Figure 2 shows a comparison of a 23 amino acid stretch from this intracellular loop of the β subunits deduced from the cDNA sequences of the rat, cow, and chick GABA$_A$-R. The synthetic peptide representing this sequence (#392-414 from bovine β_1: Figure 2) and containing the consensus sequence for PKA was used to raise the polyclonal antibody used in these studies (15,26). Note the significant homology between the β_1-subunit and other β-subunits of the GABA$_A$-R (Figure 2, see also (11,14,30). However, there is no sequence homology between the β-subunits and the α, γ, δ, and ρ-subunits of the receptor in this region of the large intracellular loop of the GABA$_A$-R (9-12). Polyclonal antibodies produced in rabbits against the synthetic peptide in the cytoplasmic domain of the β_1-subunit shown in Figure 2 were purified by peptide-agarose affinity chromatography of the antiserum. This antibody recognized one band in the purified GABA$_A$-R from bovine cortex on Western blot, corresponding to the major stained band at 57 kDa (26). Microheterogeneity was subsequently demonstrated in this region of the gel, and the antibody recognized four polypeptide bands in the 50-60 kDa region, including most strongly a doublet at 55-58 kDa (27,28) identified as β-subunits by photoaffinity labeling with [^{3}H]muscimol labeling (31) and shown in vitro to be phosphorylated by PKA and PKC (21).

Effects of the β_1-GABA$_A$-R Antibody on Phosphorylation of the Cow GABA$_A$-R by PKA and PKC. We examined the ability of this affinity purified antibody to the β_1-subunit of the cow GABA$_A$-R to inhibit the phosphorylation of the benzodiazepine affinity column purified cow GABA$_A$-R by PKA in vitro. As shown in Figure 3, this antibody produced a concentration dependent inhibition of PKA phosphorylation of the receptor. We next compared the effects of this antibody on the phosphorylation of the GABA$_A$-R by PKC. As is also shown in Figure 3, the antibody was equally potent in inhibiting the PKA and PKC phosphorylation of the GABA$_A$-R.

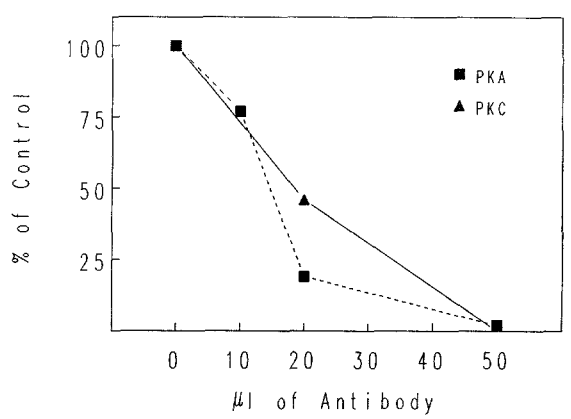

Fig. 3. Concentration response curve showing inhibition of the phosphorylation of the cow GABA$_A$-R by affinity purified antibody raised against a synthetic peptide corresponding to a sequence from the β_1 subunit of the receptor. The receptor was incubated in the absence or presence of various concentrations of antibody and then phosphorylated by PKA or PKC as described in the Methods. The antibody is able to produce comparable concentration dependent inhibition of both PKA and PKC-dependent phosphorylation of the receptor.

In addition, the antibody was able to totally inhibit all phosphorylation of the purified receptor by these two kinases.

Effects of the Cow β_1-GABA$_A$-R Antibody on Phosphorylation of the Rat GABA$_A$-R Receptor. We next compared the effects of this antibody on phosphorylation of rat and cow GABA$_A$-R by PKA. The data in Figure 4 show that the antibody to the cow receptor was able to produce only a modest (25-30%) inhibition of PKA phosphorylation of the rat GABA$_A$-R at a concentration of antibody that produced complete inhibition of the cow receptor.

N-terminal sequence analysis of V8 proteolytic fragment of PKA phosphorylated GABA$_A$-R. As described in the Experiment Procedure, the receptor which had been phosphorylated followed by *staphylococcus aureus* V8 protease and chymotrypsin digestion was electrophoresed on a 15% SDS gel and then the fragments were transferred to PVDF membrane. Autoradiography of the Coomassie-stained PVDF membrane revealed two major phosphorylated fragments of molecular weights 9kD and 11kD (Figure 5A). N-terminal sequence analysis of the 11kD peptide (Figure 5B) yielded a sequence corresponding to the published rat β3 gene product (11). This sequence begins at Met353 and follows a V8 protease cleavage site (Glu352). Only a few residues of sequence data were obtained for the 9 kD fragment and this bore no resemblance to any known GABA$_A$-R subunit.

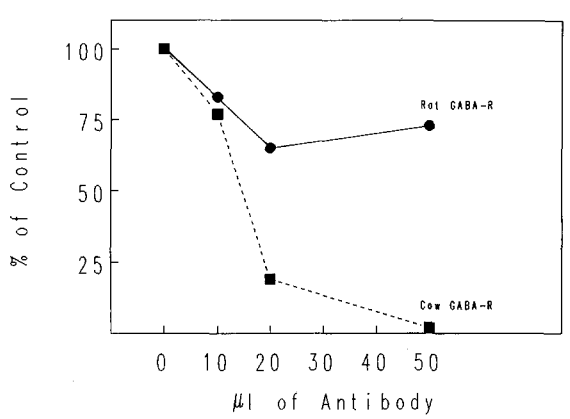

Fig. 4. Concentration response curve comparing antibody inhibition of cow and rat receptor phosphorylation by PKA. Either the rat or the cow receptor were incubated in the absence or presence of various concentrations of antibody and then phosphorylated by PKA as described in the Methods. The antibody to the β_1 peptide produced substantial inhibition of cow receptor phosphorylation but only marginal inhibition of the rat receptor phosphorylation.

DISCUSSION

In a previous study we demonstrated that PKA and PKC could phosphorylate the GABA$_A$-R with good stoichiometry (21). In addition, we also suggested that these kinases had phosphorylated β-subunits of the receptor. We based this hypothesis simply on the fact that the polypeptides that were phosphorylated by PKA and PKC comigrated in one dimensional SDS-PAGE with polypeptides that were photoaffinity labeled with ^{3}H-muscimol. Given the possibility that other subunits besides the β-subunits could migrate similarly in SDS gels, we have been particularly interested in obtaining more evidence concerning the identity of the GABA$_A$-R subunits phosphorylated by PKA and PKC.

The data presented here provide additional support for our hypothesis that β-subunits of the GABA$_A$-R are substrates for phosphorylation by both PKA and PKC. We raised an antibody against a synthetic peptide within the cytoplasmic domain sequence in the β_1-subunit of the GABA$_A$-R that has significant homology with the other β-subunits of the receptor but which has little or no homology with any of the other subunits of the GABA$_A$-R. The resultant antibody totally blocks phosphorylation of the receptor by both PKA and PKC. Thus, the data presented here are consistent with our previous report that PKA and PKC phosphorylated polypeptides in the GABA$_A$-R that comigrated in one dimensional SDS gel electrophoresis with β-subunits of the receptor. Moreover, given the fact that our antibody was raised against a 23 amino acid sequence of the β_1-subunit of the receptor, we hypothesize that it is this region in the receptor that contains the phosphorylation sites for both PKA and PKC. It should be emphasized however, that it is possible that the antibody molecule could bind to this 23 amino acid region and block the phosphorylation of other sites due to steric hindrance. We next attempted to directly identify the sequence(s) of the polypeptide(s) in the purified GABA$_A$-R that was phosphorylated by PKA. We therefore used *staphylococcus aureus* V8 protease and TLCK chymotrypsin to cleave the receptor that had been phosphorylated by PKA into small fragments. As shown in Figure 5A we obtained a number of phosphopeptides in this procedure. However, the most prominent phosphopeptide had an M_r of approximately 11,000. N-terminal sequence of this peptide yielded a sequence corresponding to the published rat β3 gene product (Figure 5B). The phospho-fragment sequenced has a predicted molecular weight of 11080 Daltons if it extends up to the C-terminus. This agrees with the molecular weight as determined by SDS-PAGE. In addition, the

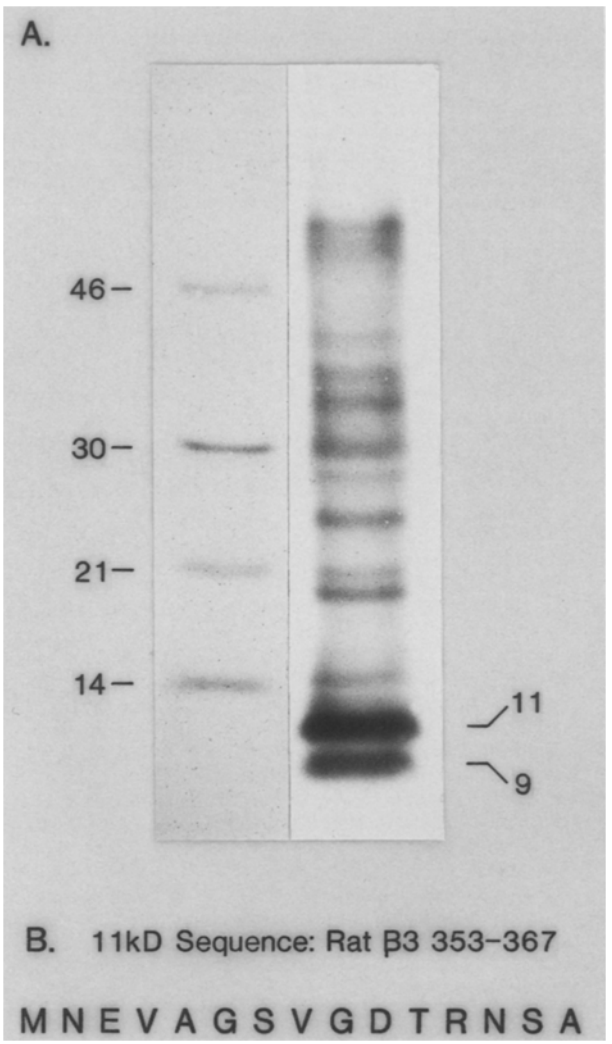

Fig. 5. A. Autoradiograph showing that sequential digestion of PKA phosphorylated rat receptor by *staphylococcus aureus* V8 protease and chymotrypsin produced two major phosphopeptide fragments of 11kD and 9kD. B. N-terminal sequence analysis of the 11kD phosphopeptide yielded the sequence MNEVAGSVGDTRNSA which corresponds to rat β3 beginning at Met353. Analysis of the 9kD band gave a sequence not found in the published clones.

fragment would contain the consensus sequence for PKA-dependent phosphorylation, although our sequencing did not proceed far enough into the polypeptide to read the putative phosphorylated serine/threonine residue. It is therefore likely that the β3 subunit is a substrate for this in vitro phosphorylation. However, the *staphylococcus aureus* V8 protease cleavage site at Glu352 of the β3 sequence is also conserved among β1 and β2 subunits and the fragments thus generated would be predicted to have similar molecular weight and contain the phosphorylation site. Thus, it is possible that these subunits are also substrates for PKA but that they were not present in sufficient amounts for sequencing. Indeed it is quite likely that the β_3 subunit of the receptor would be a prominent β subtype in the purified receptor given that in situ hybridization (32,33) and immunolabeling (Endo and Olsen, unpublished observations) indicate that the β3 subtype of the receptor is very prominent in brain. Lastly it is also possible that the β3 fragment is not a substrate but was present in stoichiometrically higher amounts than the actual substrate, leading to its false identification. This is not likely, however, since no minor sequence suggesting the presence of other subunits was observed.

In sum, the present data provide additional support for our original hypothesis that both PKA and PKC phosphorylate the β-subunit. Clearly additional sequencing work needs to be done to conclusively identify the substrates and sites on the $GABA_A$-R that are phosphorylated by PKA and PKC.

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