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Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site

(alternative splicing/cDNA/polymerase chain reaction)

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ABSTRACT **Diversity of γ-aminobutyrate type A ($GABA_A$) receptors has recently been proposed to be achieved by assembly of receptor subtypes from a multitude of subunits (α1-6, β1-3, γ1-2, and δ) encoded by different genes. Here we report a further mechanism for creating $GABA_A$ receptor diversity: alternative RNA splicing. Two forms of bovine γ2 subunit cDNA were isolated (γ2S and γ2L) that differed by the presence or absence of a 24-base-pair (8-amino acid) insertion in the cytoplasmic domain between the third and fourth putative membrane-spanning regions. Polymerase chain reaction from RNA demonstrated that the two forms of γ2 subunit are expressed in bovine, human, and rat brain. Sequencing of genomic DNA clones encoding the γ2 subunit demonstrated that the 24-base-pair insert is organized as a separate exon. Analysis of the sequence of the 8-amino acid insert revealed that it contains a protein kinase C consensus phosphorylation site. Expression of the large cytoplasmic loop domains of γ2S and γ2L in *Escherichia coli*, followed by phosphorylation of the recombinant proteins by protein kinase C, demonstrated that γ2L, but not γ2S, could be phosphorylated. Thus the two forms of γ2 subunit differ by the presence or absence of a protein kinase C phosphorylation site. This mechanism for creating $GABA_A$ receptor diversity may allow differential regulation of the function of receptor subtypes.**

γ-Aminobutyric acid (GABA) is the major inhibitory amino acid neurotransmitter in the vertebrate nervous system. This fast synaptic inhibition is mediated by the opening of a chloride channel that is intrinsic to the $GABA_A$ receptor macromolecule. $GABA_A$ receptors represent the site of action of several clinically important drugs, such as the widely prescribed benzodiazepine anxiolytics and barbiturates (1–2).

Heterogeneity of the $GABA_A$ receptor-associated benzodiazepine binding sites (BZ1 and BZ2) was initially identified using classic ligand-binding approaches (3). More recently, cloning of cDNAs encoding subunits of $GABA_A$ receptors (α1-6, β1-3, γ1-2, and δ) has demonstrated considerably more diversity than originally thought, and indicated that these receptors are members of a ligand-gated ion-channel gene superfamily (4–13). Expression of various combinations of three subunits (α, β, and γ) individually or together allows formation of GABA-gated chloride channels (4–15), but only when the γ2 subunit is present (bovine cDNAs) is benzodiazepine binding conferred (7, 12, 15). The type of benzodiazepine pharmacology appears to be defined by the type of α subunit: while α1 confers a BZ1-type pharmacology, α2, α3, and α5 appear to define the BZ2 type (12, 15). α6 confers binding of the "alcohol antagonist" Ro15-4513 (13).

Here we report a further mechanism for creating diversity within the $GABA_A$ receptor gene family. We show that alternative RNA splicing results in the expression in brain of two forms of γ2 subunit that differ by the presence or absence of an 8-amino acid insert. This insert contains a protein kinase C (PKC) phosphorylation site, suggesting a novel mechanism of regulating the function of receptor subtypes.†

MATERIALS AND METHODS

Isolation and Sequencing of cDNA Clones. Poly(A)$^+$ RNA was isolated from bovine cortex by standard procedures, and first-strand cDNA synthesis was performed from 1 μg of RNA, using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and hexanucleotide random primers (Boehringer Mannheim) (16). For polymerase chain reaction (PCR; ref. 17), the following oligonucleotide primers (synthesized using an Applied Biosystems 380B synthesizer) derived from the published human γ2 sequence were used, incorporating *Hin*dIII sites before the initiating ATG and after the stop codon: 5′-AAA-AAA-AAG-CTT-GCC-ATG-AGT-TCA-CCA-AAT-ATA-TGG-3′; 5′-TAG-GTC-GAA-GCT-TCT-CAC-AGG-TAG-AGG-TAG-GAG-ACC-C-3′. PCR was performed using 10% of the first-strand cDNA, 1 μg of each primer, and 200 μM deoxynucleoside triphosphates, with 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase and 10× buffer from Cambio (Cambridge, England). Cycling was performed at 94°C (45 sec), 55°C (2 min), and 72°C (5 min) for 30 cycles. A 31st cycle had the elongation step extended for 10 min at 72°C. PCR products were extracted with phenol/chloroform, ethanol-precipitated, digested with *Hin*dIII, and then resolved in a 0.8% agarose gel. The 1400-base-pair (bp) band was excised, the DNA was eluted and subcloned into pBluescript SK(−) (Stratagene), and γ2 cDNA clones were identified by restriction mapping and partial sequencing. Both strands of two clones, γ2S and γ2L, were sequenced from denatured, double-stranded DNA by using Sequenase T7 polymerase (United States Biochemical).

Isolation and Sequencing of Genomic DNA Clones. A human genomic library (constructed with λ DASH; obtained from Stratagene) was screened (5×10^5 phage) with a ^{32}P-labeled probe sequence (labeled by random priming; ref. 16) encompassing the putative large cytoplasmic domain of γ2L. The probe sequence was excised by digestion with *Nde* I and *Hin*dIII from the vector pRSET5a (18), into which it had been

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Abbreviations: GABA, γ-aminobutyric acid; PKC, protein kinase C; PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M55563).

engineered for bacterial expression (see below). Nylon filters (Hybond, Amersham) with the transferred phage were hybridized overnight at 42°C in 40% (vol/vol) formamide/5× Denhardt's solution (1× is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/5× SSPE (1× is 0.18 M NaCl/0.01 M sodium phosphate, pH 7.4/1 mM EDTA)/0.5% SDS containing denatured salmon sperm DNA at 15 μg/ml and were washed at 60°C (twice, 15 min, 5× SSPE; twice, 10 min, 0.3× SSPE). Positive clones were plaque-purified and larger amounts of DNA were prepared from plate lysates (16). The DNA inserts were restriction-mapped and fragments were subcloned into pBluescript SK(−) by standard DNA cloning techniques (16). Subclones were sequenced using oligonucleotide primers (16-mers, synthesized on an Applied Biosystems 380B synthesizer) located in and around the putative large cytoplasmic domain sequence.

Analysis of the Expression of Subunit mRNA by PCR. Poly(A)$^+$ RNA was isolated from brain tissue (human brain RNA was purchased from Clontech) and first-strand cDNA synthesis was performed as described above. PCR used oligonucleotide primers derived from the published sequence of human γ2: forward primer (starting at bp 1051, Fig. 1), 5′-GTG-GAG-TAT-CAT-ATG-TTG-CAT-TAT-TTT-GTC-3′; reverse primer (starting at bp 1341, Fig. 1), 5′-GAT-CCA-AGG-TTA-GGA-CTA-CAT-TTT-GGC-3′. PCR was performed essentially as described above, with the exception that 4 μCi of [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) was included and cycling was performed at 94°C (45 sec), 60°C (2 min), and 72°C (2 min). The 31st cycle had an extension time of 4 min. The PCR products were extracted with phenol/chloroform and precipitated with ethanol. The products were then resolved in a denaturing 6% polyacrylamide gel and visualized by autoradiography. Size was determined by using a sequencing "ladder" resolved in the same gel.

Expression of Putative Large Cytoplasmic Loop Domains in *Escherichia coli*. The DNA sequences of γ2L and γ2S encompassing the putative large cytoplasmic loop domain were amplified from 1 μg of plasmid DNA by PCR using oligonucleotide primers and conditions described immediately above. PCR products were digested with *Nde* I and *Hin*dIII, purified in 0.8% agarose gels, and ligated into *Nde* I/*Hin*dIII-digested pRSET5a, a T7 polymerase bacterial expression vector (18, 19). For expression of protein, expression vectors were transformed into *E. coli* strain BL21(DE3) (Lys S). Bacteria were grown in LB medium containing ampicillin (20 μg/ml) to an OD_{600} of 0.6, and then expression of recombinant protein was induced by addition of 1 mM isopropyl β-D-thiogalactoside. After 3 hr of induction, cells were harvested by centrifugation (1000 × *g*, 10 min) and the pellet was homogenized (three times, 20 sec, setting 10, Ultra Turrax homogenizer) in 10 mM Tris·HCl/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride, pH 7.5. Insoluble material, containing the recombinant proteins, was recovered by centrifugation (20,000 × *g*, 15 min) and washed twice further by repeated homogenization and centrifugation. Recombinant γ2L and γ2S proteins were further purified by preparative SDS/15% PAGE and recovered by electroelution. Proteins were analyzed by SDS/15% PAGE and staining with Coomassie blue, and the protein sequence was confirmed by N-terminal amino acid analysis.

PKC Phosphorylation. For phosphorylation by PKC, recombinant proteins, either as crude, insoluble material or as protein purified by preparative SDS/PAGE, and synthetic peptide (Pro-Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys-Ala, synthesized by Peninsula Laboratories) were incubated at 30°C for 4 min with 1 mM [γ-^{32}P]ATP (30 Ci/mmol), PKC (containing α, β1, and γ isoforms, gift of Peter Parker, Ludwig Institute, London) at 1 unit/ml, 0.7 mM $CaCl_2$, 0.125 mM $MgCl_2$, and 50 mM Hepes (pH 7.5) in the presence or absence of lipids (phosphatidylserine, 125 mg/ml; phorbol 12-myristate 13-acetate, 1.25 μg/ml). ^{32}P incorporation was measured by binding of protein to P81 ion-exchange paper (Whatman), washing three times for 10 min with 30% (vol/vol) acetic acid, and counting of Cerenkov radiation.

RESULTS

Isolation of cDNAs Encoding Two Forms of Bovine γ2 Subunit. In preliminary studies, oligonucleotide primers derived from the human γ2 sequence (7) flanking the large putative cytoplasmic loop domain of the γ2 subunit (Leu^{317}–Met^{402}) were used to isolate cDNAs from bovine brain mRNA by PCR. PCR products were subcloned, and when one of the subclones was sequenced the deduced amino acid sequence was found to be identical to the human γ2 sequence except for an insertion of 8 amino acids (Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys) between Pro^{337} and Ala^{338} (data not shown). Full-length cDNAs encoding the bovine γ2 subunit were then isolated by using oligonucleotide primers derived again from the published human γ2 sequence, but flanking the start and stop codons (see *Materials and Methods*). Isolated γ2 cDNA clones were initially characterized by sequencing the region containing the additional 8 amino acids.

Five out of 14 cDNA clones contained the additional sequence. Two clones, one that had the insertion (γ2L) and one that did not (γ2S), were completely sequenced. Not including the 8-amino acid insertion, the two cDNAs were identical apart from base 687 (G → A, Gly → Ser in γ2L) and base 897 (C → T, Ser → Phe in γ2L) (Fig. 1). These two base changes may represent polymorphism or, more likely, errors incorporated in the PCR. Bovine γ2S has 98.5% amino acid sequence identity with human γ2 (7). It thus contains the motifs of the ligand-gated ion-channel gene family, including four putative transmembrane regions and a large putative cytoplasmic loop domain between the third and fourth transmembrane regions, where the 8-amino acid insert is located. As discussed in more detail below, this insert contains a PKC consensus phosphorylation site.

The 8-Amino Acid Insertion Is Encoded by a Separate Exon. To characterize further the basis of the 8-amino acid insertion, a human genomic library was screened with a cDNA probe derived from the sequence encompassing Leu^{317}–Met^{402} of γ2L. A clone containing a 14-kilobase (kb) insert was isolated and a 6.5-kb *Xba* I fragment, which hybridized to an oligonucleotide probe just 5′ to the 24-bp insert, and a 4.8-kb *Eco*RI–*Xba* I fragment, which hybridized to an oligonucleotide probe just 3′ to the 24-bp insert, were subcloned. By using sequencing primers located around and within the large putative cytoplasmic loop domain, the intron/exon organization of this part of the gene was determined (Fig. 2). The 24-bp insertion, which was identical to the corresponding bovine sequence, was found to exist as a separate exon.

The base sequence around the downstream intron/exon boundaries corresponds well to consensus donor–acceptor splice sequences (20). This correspondence was less obvious for the upstream donor–acceptor splice sequences, suggesting that splicing at this site involves a different mechanism, which could perhaps be differentially regulated. No other introns were found in the genomic sequence corresponding to Leu^{317}–Met^{402}. These data strongly suggest that the two forms of γ2 subunit arise by alternative splicing of RNA derived from a single gene leading to the inclusion, or omission, of the 24-bp sequence in the mature mRNA.

Both γ2S and γ2L mRNA Are Expressed in Nervous Tissue. PCR using oligonucleotide primers flanking the large putative cytoplasmic loop of the γ2 subunit was used to examine the expression of γ2S and γ2L mRNA. Two products were visualized, 314 bp (corresponding to γ2L) and 290 bp (corresponding to γ2S), in bovine brain and also in rat and human brain (Fig. 3). Previous *in situ* hybridization studies using an

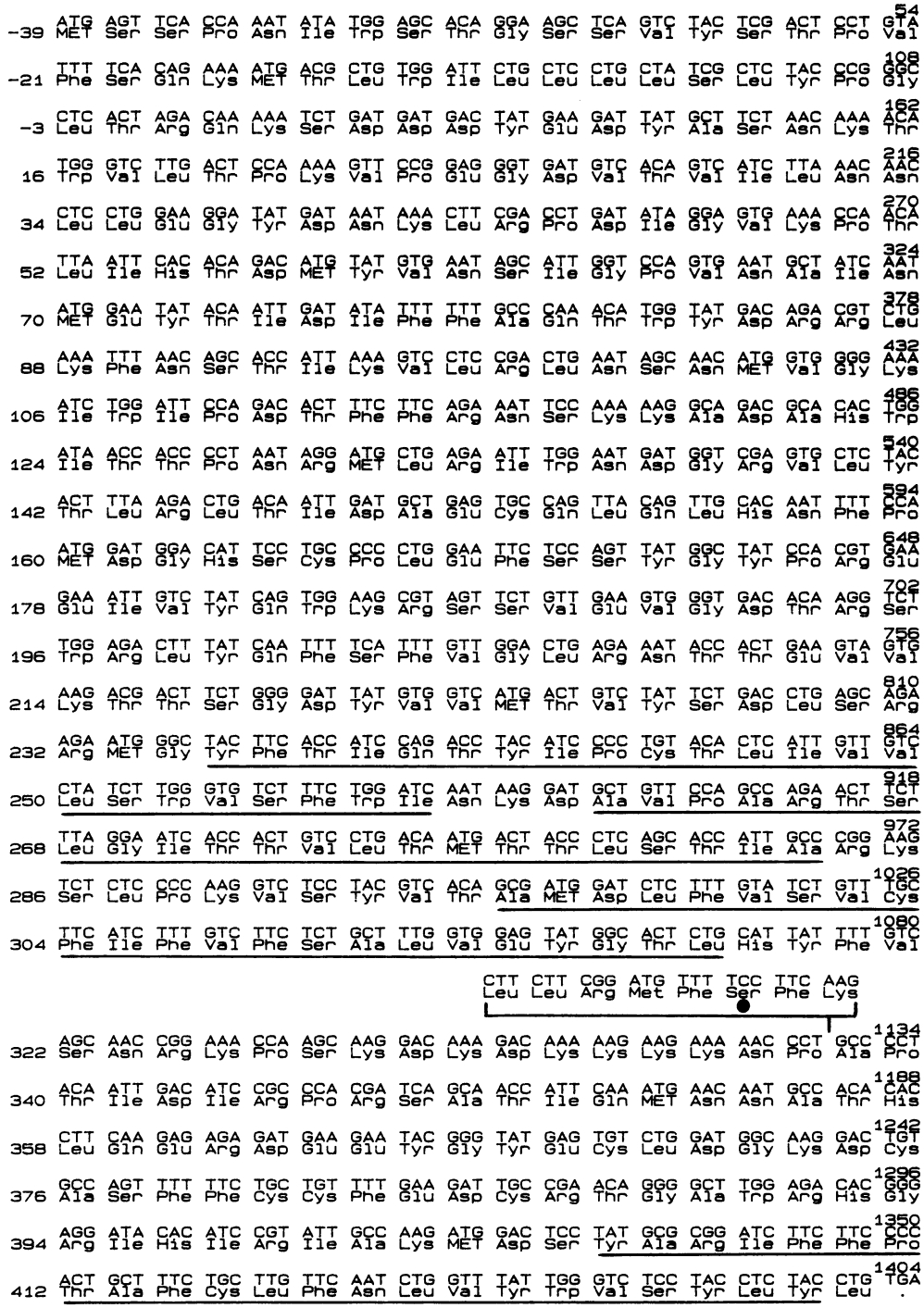

FIG. 1. Nucleotide and deduced amino acid sequence of bovine $GABA_A$ receptor γ2 cDNA clone. The nucleotide sequence is numbered at top right of each line, and the amino acid sequence, using the numbering for the human γ2 sequence (7), is numbered to the left of each line. Putative transmembrane regions are underlined. The 24-bp/8-amino acid insert of γ2L is included as a separate line. The consensus PKC phosphorylation site is indicated (●).

oligonucleotide probe that would have detected both γ2S and γ2L demonstrated that the γ2 subunit is relatively ubiquitous in its distribution in the brain (7, 8). Here we have confirmed expression of γ2 in the cortex, cerebellum, striatum, and hippocampus. Further, it appears that γ2S and γ2L may vary considerably in their relative abundance in different brain regions, suggesting that their expression is differentially regulated.

The 8-Amino Acid Insert of γ2L Contains a PKC Phosphorylation Site. Analysis of the inserted 8-amino acid sequence indicated that it contained a PKC consensus phosphorylation site (Ser-Xaa-Lys) (21). Additionally, the presence of an

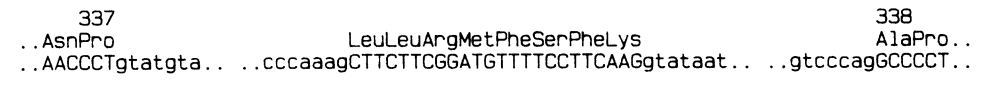

FIG. 2. Intron/exon arrangement of the human γ2 gene around the 24-bp insertion sequence. The exon base sequence is shown in uppercase, and the intron base sequence in lowercase.

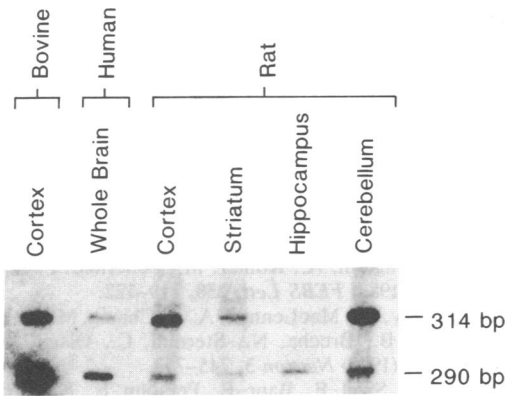

FIG. 3. Isoforms of $GABA_A$ receptor $\gamma 2$ subunit are expressed in nervous tissue. Electrophoresis and autoradiography were used to detect ^{32}P-labeled PCR products from RNA, synthesized using oligonucleotide primers flanking the putative large cytoplasmic loop of the $\gamma 2$ subunit. The 314-bp product corresponds to $\gamma 2L$, and the 290-bp product corresponds to $\gamma 2S$.

arginine three residues N-terminal to the serine would probably serve to enhance the V_{max} and K_m of phosphorylation of the serine residue (21). The only other $GABA_A$ receptor subunits reported to contain a PKC consensus phosphorylation site are $\alpha 5$ and $\alpha 6$ (12, 13). The location of the 8-amino acid insert in the large putative cytoplasmic loop domain, where phosphorylation sites are known to exist in other members of the ligand-gated ion-channel gene superfamily (22), suggested that this sequence may be a bona fide PKC phosphorylation site. To test this possibility, we investigated whether PKC could phosphorylate (*i*) the synthetic peptide Pro-Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys-Ala, (*ii*) the bacterially expressed fragment Leu^{317}–Met^{402} of $\gamma 2S$, and (*iii*) the bacterially expressed fragment Leu^{317}–Met^{402} of $\gamma 2L$. Fig. 4*A* shows SDS/PAGE and protein stain of the bacterially expressed fragments. The large putative cytoplasmic loop proteins of $\gamma 2L$ and $\gamma 2S$ are the predominant polypeptides in the insoluble material prepared from bacterial lysates. The predicted molecular weights of 12,850 and 11,980 are in good agreement with the apparent molecular weights of 15,500 and 14,500 ($\gamma 2L$ and $\gamma 2S$, respectively). The recombinant proteins were also further purified to homogeneity by preparative SDS/PAGE and electroelution (data not shown). Fig. 4*B* shows that PKC phosphorylates, in a phospholipid-dependent manner, Leu^{317}–Met^{402} of $\gamma 2L$, but not Leu^{317}–Met^{402} of $\gamma 2S$, both as "crude" insoluble material and as protein purified by preparative electrophoresis. The synthetic peptide was also phosphorylated by PKC in a phospholipid-dependent manner. Thus the 8-amino acid insert of $\gamma 2L$ contains a PKC phosphorylation site.

DISCUSSION

Pharmacological studies suggested the existence of three main classes of $GABA_A$ receptor (2, 3, 23), BZ1, BZ2, and a third class of receptor that is insensitive to most "classical" benzodiazepines but has high affinity for the "alcohol antagonist" Ro15-4513. cDNA cloning of $GABA_A$ receptor subunits has demonstrated the existence of a gene family of even greater diversity and heterogeneity (4–13). Here we demonstrate a further level of $GABA_A$ receptor diversity: alternative forms of the $\gamma 2$ subunit created by RNA splicing.

Alternative RNA splicing as a mechanism for creating diversity has been reported for other receptor systems. Recent studies have demonstrated that two forms of D_2 dopamine receptor exist as a result of alternative RNA splicing from a single gene (24–27). The physiological role of the two forms, which differ by a 29-amino acid insertion in the third cytoplasmic loop, has yet to be determined. Similarly, Beeson *et al.* (28) have reported that RNA splicing results in

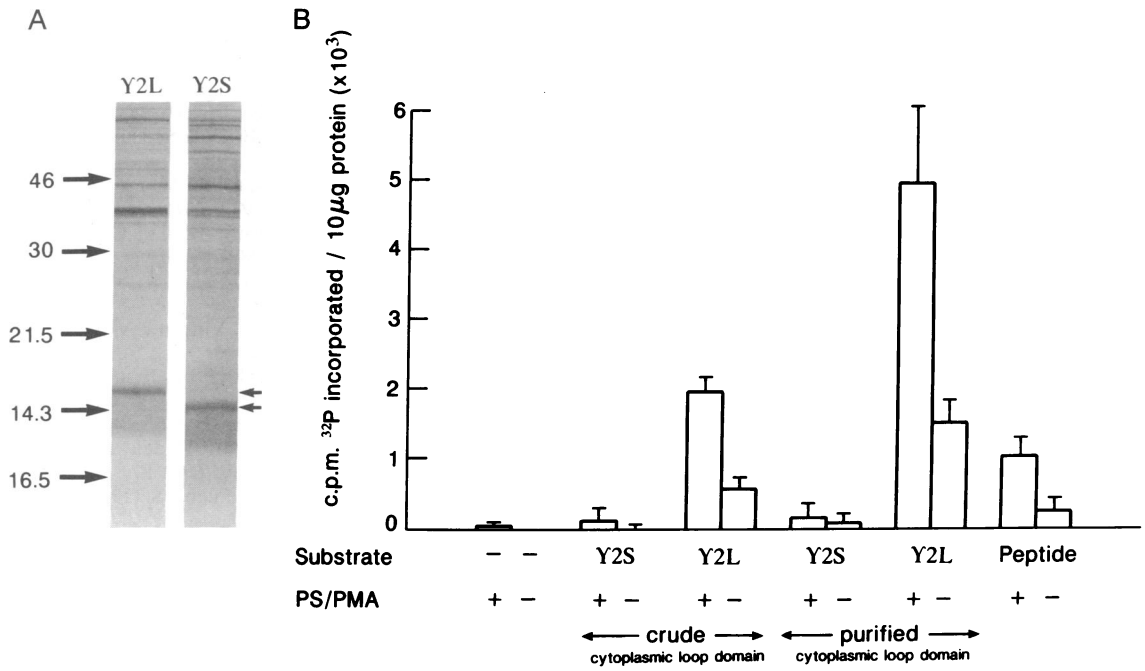

FIG. 4. Phosphorylation by PKC of the putative large cytoplasmic loop domain of $\gamma 2L$ but not $\gamma 2S$. (*A*) SDS/PAGE and protein stain of the insoluble material prepared from bacteria expressing the putative cytoplasmic loop domains of $\gamma 2S$ and $\gamma 2L$. Arrows at left indicate the apparent molecular weights ($M_r \times 10^{-3}$) of marker proteins resolved in the same gel. Arrows to the right of the lanes indicate the protein bands of $\gamma 2L$ (upper arrow) and $\gamma 2S$ (lower arrow) cytoplasmic loop proteins. (*B*) Phosphorylation by PKC of the bacterially expressed putative cytoplasmic domains (both as a crude bacterial cell pellet and as protein purified by preparative SDS/PAGE) and the synthetic peptide (Pro-Leu-Leu-Arg-Met-Phe-Ser-Phe-Lys-Ala) encompassing the 8-amino acid insert of $\gamma 2L$. Incorporation of ^{32}P is expressed as cpm/10 μg of protein and is shown as the mean ± SD of triplicate incubations. In the absence of PKC, there was no incorporation of ^{32}P (data not shown). PS, phosphatidylserine; PMA; phorbol 12-myristate 13-acetate.

expression of two forms of muscle nicotinic acetylcholine receptor α subunit that differ by the presence or absence of a 25-amino acid insertion at amino acid 58 of the polypeptide. Again, the function of this insertion remains unknown.

Our data suggest that isoforms of $GABA_A$ receptors exist that differ in the presence or absence of a PKC phosphorylation site on the $\gamma 2$ subunit. Further studies are needed to determine which other subunits are associated with $\gamma 2S/\gamma 2L$ *in vivo* to form a native receptor macromolecule, whether the PKC site of $\gamma 2L$ can be phosphorylated in the native receptor, and what effect this has upon receptor function/pharmacology. It is known that $GABA_A$ receptors can be phosphorylated by PKC and that activators of PKC significantly decrease $GABA_A$ receptor function (29–31). Additionally, there is a precedent from other receptor systems for this possibility. For instance, nicotinic acetylcholine receptors of *Torpedo* electric organ, which are structurally homologous to $GABA_A$ receptors (32), can be phosphorylated by PKC at sites located in the large putative cytoplasmic loop domain of the δ subunit. This PKC phosphorylation site is conserved in the δ subunit of muscle nicotinic acetylcholine receptor (33), and activation of PKC in cultured muscle cells results in a decreased sensitivity to acetylcholine and increased rate of receptor desensitization (34). Activation of receptor systems (e.g., some muscarinic, domaminergic, and serotininergic systems) coupled through the phosphatidylinositol second-messenger system leads to the generation of 1,2-diacylglycerol, which activates PKC. Thus it is possible to speculate that PKC-mediated phosphorylation of $GABA_A$ receptors, and subsequent modulation of their function, may allow interaction and cross-talk between neurotransmitter pathways. Interestingly, the different subspecies of PKC have a discrete cellular distribution in the rat cerebral neocortex; 80% of the $\beta 1$ type has been shown to be localized adjacent to the plasma membrane of GABAergic neurons (35). It will be important to map the distribution of the $\gamma 2L$ subunit. Additionally, since the $\gamma 2$ subunit is required for binding of benzodiazepine drugs to $GABA_A$ receptors (7), it may be important to determine whether PKC phosphorylation of $\gamma 2L$-containing $GABA_A$ receptors (resulting perhaps in changes in receptor function) has any relationship to the mechanisms underlying the tolerance and dependence that develop in response to long-term administration of benzodiazepine anxiolytic drugs.

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1. Olsen, R. W. & Venter, J. C., eds. (1986) *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties* (Liss, NY).
2. Lippa, A. S., Garrett, K. M., Tabakoff, B., Beer, B., Wennogle, L. P. & Meyerson, L. R. (1985) *Brain Res. Bull.* **14,** 189–195.
3. Squires, R. F., Benson, D. I., Braestrup, C., Coupet, J., Klepner, C. A., Myres, V. & Beer, B. (1979) *Pharmacol. Biochem. Behav.* **10,** 825–830.
4. Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H. & Barnard, E. A. (1987) *Nature (London)* **328,** 221–227.
5. Levitan, E. S., Schofield, P. R., Burt, D. R., Rhee, L. M., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H. F., Stephenson, A., Darlison, M. G., Barnard, E. A. & Seeburg, P. H. (1988) *Nature (London)* **335,** 76–79.
6. Ymer, S., Schofield, P. R., Draguhn, A., Werner, P., Kohler, M. & Seeburg, P. H. (1989) *EMBO J.* **8,** 1665–1670.
7. Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Kettenmann, H., Schofield, P. R. & Seeburg, P. H. (1989) *Nature (London)* **338,** 582–585.
8. Shivers, B. D., Killisch, I., Sprengel, R., Sontheimer, H., Kohler, H., Schofield, P. R. & Seeburg, P. H. (1989) *Neuron* **3,** 327–337.
9. Ymer, S., Draguhn, A., Kohler, M., Schofield, P. R. & Seeburg, P. H. (1989) *FEBS Lett.* **258,** 119–122.
10. Krestchatisky, M., MacLennan, A. J., Chiang, M.-Y., Xu, W., Jackson, M. B., Brecha, N., Sternini, C., Olsen, R. W. & Tobin, A. J. (1989) *Neuron* **3,** 745–753.
11. Malherbe, P., Sigel, E., Baur, E., Persohn, E., Richards, J. G. & Mohler, J. (1990) *FEBS Lett.* **260,** 261–265.
12. Pritchett, D. B. & Seeburg, P. H. (1990) *J. Neurochem.* **54,** 1802–1804.
13. Luddens, H., Pritchett, D. B., Lohler, M., Killisch, I., Keinanen, K., Monyer, H., Sprengel, R. & Seeburg, P. H. (1990) *Nature (London)* **346,** 648–651.
14. Pritchett, D. B., Sontheimer, H., Gorman, C. M., Kettenmann, H. K., Seeburg, P. H. & Schofield, P. R. (1989) *Science* **242,** 1306–1308.
15. Pritchett, D. B., Luddens, H. & Seeburg, P. H. (1989) *Science* **245,** 1389–1391.
16. Berger, S. L. & Kimmel, A. R., eds. (1987) *Methods Enzymol.* **152,** 33–556.
17. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 437–489.
18. Diehl, R. E., Whiting, P., Potter, J., Gee, N., Ragan, C. I., Linemeyer, D., Schoepfer, R., Bennett, C. & Dixon, R. A. F. (1990) *J. Biol. Chem.* **265,** 5946–5949.
19. Rosenberg, A. H., Lack, B. N., Chui, D. S., Lin, S. W., Dunn, J. J. & Studier, F. W. (1987) *Gene* **56,** 125–135.
20. Shapiro, M. B. & Senopathy, P. (1987) *Nucleic Acids Res.* **15,** 7155–7174.
21. Woodgett, J. R., Gould, K. L. & Hunter, T. (1986) *Eur. J. Biochem.* **161,** 177–184.
22. Hemmings, H. C., Nairn, A. C., McGuiness, T. L., Huganir, R. L. & Greengard, P. (1989) *FASEB J.* **3,** 1583–1592.
23. Harris, C. M. & Lal, H. (1988) *Drug Dev. Res.* **13,** 187–203.
24. Toso, R. D., Sommer, B., Ewert, M., Herb, A., Pritchett, D. B., Bach, A., Shivers, B. D. & Seeburg, P. H. (1989) *EMBO J.* **8,** 4025–4034.
25. Giros, B., Sokoloff, P., Martres, M.-P., Riou, J.-F., Emorine, L. J. & Schwartz J.-C. (1989) *Nature (London)* **342,** 923–925.
26. Monsma, F. J., Jr., McVittie, L. D., Gerfen, C. R., Mahan, L. C. & Sibley, D. R. (1989) *Nature (London)* **342,** 926–928.
27. Chio, C. L., Hess, G. F., Graham, R. S. & Huff, R. M. (1990) *Nature (London)* **343,** 266–269.
28. Beeson, D. A., Morris, A., Vincent, A. & Newsom-Davis, J. (1990) *EMBO J.* **9,** 2101–2106.
29. Sigel, E. & Baur, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6192–6196.
30. Moran, O. & Dascal, N. (1989) *Mol. Brain Res.* **5,** 193–202.
31. Browning, M. D., Bureau, M., Dudek, E. M. & Olsen, R. W. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1315–1318.
32. Safran, A., Neumann, D. & Fuchs, S. (1987) *J. Biol. Chem.* **262,** 10506–10511.
33. Nef, P., Mauron, A., Stalder, R., Alliod, C. & Ballivet, M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7975–7979.
34. Eusebi, F., Molinaro, M. & Zani, B. M. (1985) *J. Cell Biol.* **100,** 1339–1342.
35. Tsujino, T., Kose, A., Saito, N. & Tanaka, C. (1990) *J. Neurosci.* **10,** 870–884.