The Novel Anticonvulsant Drug, Gabapentin (Neurontin), Binds to the $\alpha_2\delta$ Subunit of a Calcium Channel*

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Gabapentin (1-(aminomethyl)cyclohexane acetic acid; Neurontin) is a novel anticonvulsant drug, with a mechanism of action apparently dissimilar to that of other antiepileptic agents. We report here the isolation and characterization of a $[3H]$gabapentin-binding protein from pig cerebral cortex membranes. The detergent-solubilized binding protein was purified 1022-fold, in a six-step column-chromatographic procedure, with a yield of 3.9%. The purified protein had an apparent subunit M, of 130,000, and was heavily glycosylated. The partial N-terminal amino acid sequence of the M, 130,000 polypeptide, EPFPFSAVTIK, was identical to that reported for the $\alpha_2\delta$ subunit of the L-type Ca$^{2+}$ channel from rabbit skeletal muscle (Hamilton, S. L., Hawkes, M. J., Brush, K., Cook, R., Chang, R. J., and Smilowitz, H. M. (1989) Biochemistry 28, 7625–7626). High levels of $[3H]$gabapentin binding sites were found in membranes prepared from rat brain, heart and skeletal muscle. Binding of $[3H]$gabapentin to COS-7 cells transfected with $\alpha_2\delta$ cDNA was elevated >10-fold over controls, consistent with the expression of $\alpha_2\delta$ protein, as measured by Western blotting. Finally, purified L-type Ca$^{2+}$ channel complexes were fractionated, under dissociating conditions, on an ion-exchange column; $[3H]$gabapentin binding activity closely followed the elution of the $\alpha_2\delta$ subunit. $[3H]$Gabapentin is the first pharmacological agent described that interacts with an $\alpha_2\delta$ subunit of a voltage-dependent Ca$^{2+}$ channel.

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1The abbreviations used are: GABA, $\gamma$-aminobutyric acid; BCH, 2-amino-5-bicyclo[2.2.1]heptane-2-carboxylic acid; BigCHAP, N,N-bis(3-o-glucosaminopropyl)cholamide; MK-801, $[3H]$(5R,10S)-(+)-5-methyl-10,11-dihydro-SH-dibenzo[a,d]cyclohepten-5,10-imine (Dizocilpine); CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

GABAPENTIN ACTIVITY TOWARDS VARIOUS NEUROPEPTIDIC RECEPTORS AND TRANSPORTERS

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rabbit skeletal muscle α2βδ cDNA in COS-7 and HEK cells; and radioligand binding and immunoblotting studies, after column fractionation of purified L-type Ca\(^{2+}\) channel complexes under dissociating conditions.

**EXPERIMENTAL PROCEDURES**

Materials—Pig brains were obtained fresh from the local abattoir and transported to the laboratory on ice. All detergents were from Calbiochem Ltd., Nottingham, United Kingdom (UK). Hydroxyapatite was obtained from J ones Chromatography, Hengoed, Mid Glamorgan, UK. All other chromatography media were from Pharmacia Biotech Ltd, Milton Keynes, Bucks, UK. Minimal essential II precast gels, and reagents for gelation, were from Bio-Rad Laboratories, Hemel Hempstead, Herts, UK. Ultrafiltration cells and YM-10 membranes were from Amicon Ltd., Stonehouse, Gloucester, UK. GF/B filters were from Whatman International, Maidstone, Kent, UK. Glycyltate F was from Boehringer Mannheims, Lewes, East Sussex, UK. Lipofectamine and cell culture media were obtained from Life Technologies Ltd., Paisley, Renfrewshire, UK. Monodonal antibodies raised against rabbit skeletal muscle α2 (12) and β (13) subunits were from TCS Biologicals Ltd., Stevenage, Herts, UK. Unlabeled gabapentin and the enantiomers of 3-isobutyl GABA were obtained from Parke-Davis, Ann Arbor, MI. LIGAND version 3.0 (Elsevier-Biosoft).

Pig brains (up to 35 g) were processed as described above for the lentil lectin column. Active fractions (2 ml each) were pooled and subjected to two cycles of ultrafiltration and dilution with 1 ml NaCl (final salt concentration ~ 2 M).

Hydroxyapatite Chromatography—Active fractions from the lectin column were pooled, concentrated to 8.0 ml by ultrafiltration using a YM-10 membrane, and subjected to gel filtration chromatography. The sample was loaded at 1 ml/min on to a Sephacryl S-400 column (2.6 cm (internal diameter) × 92 cm) equilibrated in 0.08% Tween 20, 150 mM NaCl, 10 mM Hepes/KOH, pH 7.4. Active fractions (10 ml each) were pooled and subjected to two cycles of ultrafiltration and dilution with 1 ml NaCl (final salt concentration ~ 2 M).

Lentil Lectin Chromatography—The sample from the hydroxyapatite column was loaded at 4 ml/min onto a wheat germ lectin-agarose column (1 cm (internal diameter) × 5 cm) equilibrated with 0.08% Tween 20, 450 mM NaCl, 10 mM Hepes/KOH, pH 7.4. The column was washed with 6 ml of equilibration buffer and eluted with 0.35 M ω-N-acetyl-α-glucosamine in equilibration buffer using the two-step procedure outlined above for the lentil lectin column. Active fractions (2 ml each) were pooled and diluted (1:5) with 0.08% Tween 20, 10 mM Hepes, pH 7.4 (at 22°C), before application to a high resolution ion exchange column.

Mono Q Chromatography—After filtration through a 0.2 μm filter, the sample was loaded by means of a 50-ml Superloop on to Mono Q HR 5/5 column equilibrated with 0.08% Tween 20, 10 mM Hepes/KOH, pH 7.4, at 22°C. Bound proteins were eluted with a linear NaCl gradient (0–750 mM) in a total volume of 50 ml of buffer A. Fractions (1 ml each) were collected and aliquots analyzed in the radioligand binding assay and by SDS-polyacrylamide gel electrophoresis.

Purification of Skeletal Muscle Ca\(^{2+}\) Channels—Membranes were prepared from fresh muscle tissue following the method of Nakayama et al. (14). Detergent solubilization was performed as described for brain membranes (see above) except that digitonin (1% final concentration) was used. Ca\(^{2+}\) channel complexes were purified by sequential chromatography on wheat germ lectin-agarose and Mono Q. Column buffers were identical to those described above, except that digitonin (0.1%) replaced Tween 20.

Deglycosylation with Glycopeptidase F—Aliquots (50 μl) of purified [\(^{3}H\)]gabapentin-binding protein were mixed with 25 μl of either 0.5% SDS, 0.1 M 2-mercaptoethanol or water and heated for 5 min at 100°C. After this, the following additions were made: 25 μl of 0.5 M Tris/HCl, pH 8.0, 10 μl of 0.1 mM EDTA/NaOH, pH 8.0, 10 μl of 10% Tween 20, 10 mM Hepes/KOH, pH 7.4, and either 0.5 μl or 2.5 μl of glycopeptidase F (200 units/ml for water controls. Deglycosylation was carried out for 18 h at 37°C, after which samples were analyzed by SDS-polyacrylamide gel electrophoresis.

**Protein Sequencing**—The purification procedure was modified to allow the production of sufficient material for N-terminal protein sequencing. Briefly, the hydroxyapatite and wheat germ lectin columns were treated as described above except that a Waters 2996 UV detector was used instead of the UV-absorbance detector. Full details of this modified procedure will appear elsewhere.2 The final sample of purified [\(^{3}H\)]gabapentin-binding protein (5 μg) was electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto an Immobilon P membrane. The blot was stained with Coomassie Blue and the 130,000 band excised and sequenced on an Applied Biosystems 477A sequencer.

Stable Expression of cDNA in HEK 293 Cells—HEK 293 cells were transfected using Lipofectamine-mediated transfection with two pcDNA3 derivatives, one containing rabbit skeletal muscle α2βδ cDNA and the other containing human neuronal β subunit cDNA. Stable cell lines were selected using G418 at a concentration of 600 μg/ml medium.

2. P. Brown, V. U. K. Dissanayake, A. Briggs, and N. S. Gee, manuscript in preparation.
These cells were originally developed for studies on recombinant Ca\(^{2+}\) channels with different \(\alpha\) subunits, hence the co-expression of \(\alpha_2\delta\) and \(\beta\) subunits. The cell line designated 2L contained high levels of \(\alpha_2\delta\) and \(\beta\) mRNA and protein as measured by Northern and Western blotting, respectively. Membranes were prepared from 2L and HEK 293 cells by hypotonic lysis and homogenization. Extracts were centrifuged at 3000 \(g\), and the resulting supernatant was centrifuged at 50,000 \(g\) to pellet membranes. Cell membranes were assayed for \([3H]\)gabapentin binding activity as described above.

**Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was performed in 4–20% gradient gels using the Laemmli (15) buffer system. Gels were stained using the Bio-Rad silver-staining kit according to the manufacturer's instructions.

**Western Blotting**—Proteins were electrophoretically transferred to nitrocellulose sheets at 25 mA for 72 h according to the method of Towbin et al. (16). Blots were incubated with blocking buffer (2% milk powder in 50 mM Tris, 150 mM NaCl, pH 7.5) for 30 min, before incubation with primary antibody for 18 h at 4 °C. Blots were washed five times with blocking buffer, over 15 min, before addition of horse-radish peroxidase-conjugated second antibody. After 3 h, blots were washed as above, followed by three rinses in buffer lacking milk powder. Peroxidase activity was detected either using 3-amino-9-ethylcarbazole substrate, as described by Graham et al. (17), or by chemiluminescence using a kit supplied by Amersham International.

**Protein Determinations**—Protein concentration was determined by the method of Bradford (18) using bovine serum albumin as a standard.

**RESULTS**

**Solubilization of the \([3H]\)Gabapentin-binding Protein**—Although most studies on the \([3H]\)gabapentin-binding protein have been carried out in the rodent (7, 8, 19), a species such as the pig has obvious advantages as a tissue source for protein purification work. Binding of \([3H]\)gabapentin to membranes prepared from various pig brain regions was therefore examined. Specific activity values for occipital cortex, parietal cortex, frontal cortex, hippocampus, striatum, thalamus, cerebellum, and brainstem were 835, 691, 509, 399, 323, 278, 179, and 123 dpm/mg, respectively. Membranes prepared from whole cerebral cortex were used in all subsequent experiments.

**Purification of the \([3H]\)Gabapentin-binding Protein**—In the purification procedure the chromatographic columns were ordered to maximize resolution and minimize the number of intermediate conditioning steps. Solubilization of brain membranes in a low ionic strength buffer with Tween 20 (a non-ionic detergent) allowed direct application of the crude 100,000 \(g\) supernatant on to an anion-exchange column. Two batches of extract, each prepared from 250 g of pig cerebral cortex, were processed on Q-Sepharose FF. Fig. 1A shows a typical gradient...
elution profile obtained with this column. The peak of 

\[ \text{[H]}gabapentin binding activity emerged at around } 400 \text{ mM NaCl, although considerable trailing was usually observed. Re-chromatography of the later eluting fractions also yielded a peak of activity at } 400 \text{ mM NaCl, suggesting that the trailing phenomenon was not due to protein heterogeneity. Further chromatography was performed on lentil lectin-Sepharose, after addition of } Ca^{2+} \text{ and } Mn^{2+} \text{ ions (each } 1 \text{ mM) to the pooled Q-Sepharose extract. These ions caused little or no interference in the } [H]gabapentin binding assay. Equilibration of the lentil lectin column with sugar-containing buffer for } 2 \text{ h before elution resulted in a sharper peak of binding activity, and thus facilitated chromatography by gel filtration (Fig. 1B). The active material from the Sephacryl S-400 column was subjected to two cycles of ultrafiltration and dilution, to reduce the ionic strength of the sample, before application to the hydroxyapatite column. This method of salt removal was much faster than dialysis and gave a higher recovery of } [H]gabapentin-binding protein. While fractionation on hydroxyapatite (Fig. 1C) was straightforward, provided the elution buffer was prevented from crystallizing, the eluted material could not be frozen without a substantial loss of } [H]gabapentin binding activity. This phenomenon was not investigated systematically, but the degree of inactivation appeared to be related to the rate of sample cooling. As a precaution, we avoided freezing samples in phosphate buffer and applied the pooled active material directly on to the wheat germ lectin-agarose column. Further chromatography on Mono Q (Fig. 1D) allowed the removal of the sugar-containing buffer from the previous step and most of the remaining protein impurities. The progress of one purification is summarized in Table I. The specific activity value for the peak Mono Q fractions was 1584 pmol/mg, which corresponds to a 1022-fold purification over the starting membranes. A lower value of 1153 pmol/mg was calculated for the relatively dilute total Mono Q pool. Stepwise recoveries were in the range 45–

\[ 72\% \text{, and the overall yield was } 3.9\% \text{ or } 0.24 \text{ pmol/mg of gabapentin-binding protein}.

### Table I

| Fraction                  | Total activity | Total protein | Specific activity | Yield | Purification |
|---------------------------|----------------|---------------|------------------|-------|--------------|
|                           | pmol           | mg            | pmol/mg          | %     | -fold        |
| Membranes                 | 3504           | 2258          | 1.55             | 100   | 1            |
| Solubilized membranes     | 2476           | 449           | 5.51             | 70.7  | 3.55         |
| Q-Sepharose               | 1115           | 92.1          | 12.1             | 31.8  | 7.81         |
| Lentil lectin             | 599            | 10.4          | 57.6             | 17.1  | 37.2         |
| Sephacryl S-400           | 376            | 2.32          | 162              | 10.7  | 105          |
| Hydroxyapatite            | ND             | 0.42          | ND               | ND    | ND           |
| Wheat germ lectin         | 191            | 0.223         | 857              | 5.45  | 553          |
| Mono-Q (Fractions 18–25)  | 136            | 0.118         | 1153             | 3.88  | 744          |
| Mono-Q (Fractions 20/21)  | 49.1           | 0.031         | 1584             | 1.40  | 1022         |

Biochemical and Molecular Properties of the Purified [H]Gabapentin-binding Protein—As shown in Fig. 2, the predominant species in the Mono Q eluent was a polypeptide with an apparent subunit \( M_r \) of 130,000. An additional, faint, diffuse band \( M_2 \), 25,000), whose intensity paralleled that of the \( M_r \), 130,000 band could be visualized if gels were either overloaded or stained twice (data not shown). Under non-reducing conditions, neither of these bands was observed; instead, the purified protein migrated as a single species with an apparent \( M_r \) of 170,000 (data not shown). The [H]Gabapentin-binding protein is therefore composed of non-identical subunits covalently linked by at least one disulfide bridge. Gel filtration of the purified protein on a Superose 6 column yielded a molecular size for the Tween 20-[H]Gabapentin-binding protein complex of 260 kDa (n = 3). This is broadly consistent with a species of 170-kDa binding to a micelle of detergent. However, chromatography of Tween 20-solubilized membrane proteins yielded a molecular size for the [H]Gabapentin-binding protein of 430 kDa \( n = 4 \). Preliminary experiments suggest that the alteration in molecular size occurs during Q-Sepharose chromatography (data not shown).

The retention of the [H]Gabapentin-binding protein by lectin columns suggested that the protein was probably glycosylated. Incubation of the reduced SDS-denatured protein with high concentrations of glycopeptidase F led to a decrease in the apparent subunit \( M_r \) from 130,000 to 105,000 (Fig. 3). Purified [H]Gabapentin-binding protein that had been subjected to heat denaturation without SDS and 2-mercaptoethanol was resistant to the enzyme. Thus the [H]Gabapentin protein is heavily glycosylated, with N-linked oligosaccharide chains that are accessible to glycopeptidase F only under fully denaturing conditions.

Pharmacological Properties of the Purified Protein—Several compounds were evaluated in competition assays with [H]Gabapentin using the purified [H]Gabapentin-binding protein (Fig. 4). (S+) 3-Isobutyl GABA potently inhibited [H]Gabapentin binding with an IC\(_{50}\) value of 40 nM (cf. gabapentin, IC\(_{50} = 50\) nM), while the (R)-enantiomer was nearly an order of magnitude less effective (IC\(_{50} = 370\) nM). A stereoselective requirement at the [H]Gabapentin binding site was even more pronounced for the L- and D-enantiomers of leucine (IC\(_{50}\) values of 80 and 10,000 nM, respectively). The paradigm L-system substrate, BCh, displaced [H]Gabapentin binding with an IC\(_{50}\) of 691 nM. Similar results to these were obtained both with brain membranes and with Tween 20-solubilized preparations. Thus, the pharmacological characteristics of the
[\(\text{H}\)]gabapentin-binding protein appears to have been preserved following detergent solubilization and subsequent isolation of the protein.

**N-terminal Sequencing and Identification of the [\(\text{H}\)]Gaba-pentin-binding Protein**—The availability of microgram quantities of highly purified pig brain [\(\text{H}\)]gabapentin-binding protein allowed us to obtain a partial N-terminal sequence for the Mr 130,000 polypeptide. Two preparations of protein were analyzed, and, in each case, 10 cycles of readable sequence were obtained. Both samples gave the same sequence: EPFPSAVTIK. A homology search showed that the N-terminal sequence was identical to that reported for the \(\alpha_2\)d subunit of the rabbit skeletal muscle voltage-dependent L-type Ca\(^{2+}\) channel (20). The same stretch of amino acids is also found in the sequence deduced by cDNA cloning for the human brain \(\alpha_2\)d Ca\(^{2+}\) channel subunit (21). The corresponding sequence for the rat brain \(\alpha_2\)d sequence contains a prolyl instead of an alanyl residue at position 6 (22).

**Tissue Distribution of [\(\text{H}\)]Gabapentin-binding Sites in the Rat**—The binding of [\(\text{H}\)]gabapentin to membranes prepared from some 14 rat tissues was examined. As shown in Fig. 6A, the highest level of [\(\text{H}\)]gabapentin binding sites was observed in skeletal muscle. Significant levels were found in cerebral cortex, cerebellum, forebrain, and heart. Trace amounts of [\(\text{H}\)]gabapentin binding sites were found in lung, spleen, liver, and kidney, but pancreas and intestine were devoid of activity. In rat muscle membranes [\(\text{H}\)]gabapentin bound to a single population of sites with high affinity (\(K_D = 29\) nM), similar to that reported for rat brain (8). Competition experiments with (\(S\) -) and (\(R\) -) 3-isobutyl-GABA, D- and L-leucine, BCH, and gabapentin gave a rank order of potency that was identical to that obtained with the purified pig [\(\text{H}\)]gabapentin-binding protein (see Fig. 4). The distribution of dihydropyridine-sensitive L-type Ca\(^{2+}\) channels as defined by [\(\text{H}\)]nitrendipine (Fig. 5B) was similar, although not identical, to that for \(\alpha_2\)d subunits labeled by [\(\text{H}\)]gabapentin.

**Heterologous Expression of \(\alpha_2\)d cDNA**—Binding of [\(\text{H}\)]gabapentin to 2L cells, which express the rabbit skeletal muscle \(\alpha_2\)d protein and a human neuronal \(\beta\) subunit, and to parental HEK 293 cells, was examined. As shown in Fig. 6A, specific [\(\text{H}\)]gabapentin binding to 2L cell membranes was approximately 85-fold higher than to HEK 293 cell membranes. The trace levels of specific [\(\text{H}\)]gabapentin binding to HEK 293 cells (and to kidney membranes; Fig. 5A) is probably explained by low level expression of endogenous Ca\(^{2+}\) channel subunits. Indeed, splice variants of \(\alpha_2\)d have been detected in HEK 293 cells by polymerase chain reaction methods (23). COS cells transfected with pcDNA3/\(\alpha_2\)d cDNA expressed 10-fold higher levels of [\(\text{H}\)]gabapentin binding sites than COS cells transfected with vector alone (Fig. 6A). This was consistent with increased expression of \(\alpha_2\)d protein as measured by Western blotting (Fig. 6B). The electrophoretic properties of the ex-
pressed \(\alpha_2\beta\) protein were consistent with those reported for the skeletal muscle protein (24). In COS-7 cells transfected with \(\alpha_2\beta\) cDNA, \(\[^{3}H\]gabapentin\) bound to a single population of sites, with high affinity (\(K_D = 13\) nM), with a maximum binding capacity of 17.8 pmol/mg of protein (Fig. 5C). Similar results to these were obtained in a second transfection experiment (\(K_D = 19.8\) nM; \(B_{\text{max}} = 25.9\) pmol/mg).

Fractionation of Partially Dissociated Ca\(^{2+}\) Channel Complexes—Ca\(^{2+}\) channel complexes were purified from digitonin-solubilized skeletal muscle membranes by sequential chromatography on wheat germ lectin-agarose and Mono Q. The system was used. Fig. 8 shows that the material applied to the Mono Q column contained \(\alpha_2\), \(\alpha_3\), \(\alpha_3\), and \(\beta\) subunits. Like the \(\alpha_3\) subunit, the \(\beta\) subunit was found only in the second peak of \(\[^{3}H\]gabapentin\) binding activity. The amount of \(\[^{3}H\]gabapentin\) binding activity in the three samples was broadly consistent with the amount of \(\alpha_\beta\) polypeptide as measured by immunoblotting.

**DISCUSSION**

The purification of the detergent-solubilized \(\[^{3}H\]gabapentin\)-binding protein from pig brain was achieved by sequential chromatography on Q-Sepharose, lentil lectin, Sephacryl S-400, hydroxypatite, wheat germ lectin, and Mono Q. The protein in the Mono Q eluent was analyzed on a 4–20% gradi-
sent SDS-polyacrylamide gel, which covers a broad range of molecular weights. The amount of radioligand binding paralleled the intensity of the M\(_1\), 130,000 band, although other faint co-eluting bands were observed. The possibility that \[^{3}H\]gabapentin might bind to one of these quantitatively minor components was excluded in other experiments (see below). The final specific activity, based on the peak Mono Q fractions, was 1584 pmol/mg. This value is approximately 5-fold less than that expected, given a starting specific activity value of 1.55 pmol/mg for brain membranes. However, the low concentration of protein in the Mono Q eluent, and the presence of Tween 20, which interfered with the protein assay, precluded an accurate determination of specific activity. The susceptibility of the protein to inactivation, which was particularly evident in phosphate buffers, may also have been a contributory factor. In all, we have prepared five batches of \[^{3}H\]gabapentin-binding protein using this, or a very similar, procedure. Preparations of higher purity, though with reduced microgram yields, could be obtained either by reducing the amount of starting material, or by judicious selection of fractions to be pooled. However, the purification scheme described here offers a reasonable compromise between yield and purity.

The partial N-terminal sequencing of the M\(_1\), 130,000 polypeptide was a crucial step in the identification of the \[^{3}H\]gabapentin-binding protein as an \(\alpha_2\delta\) subunit of a Ca\(^{2+}\) channel. The sequence homology with the \(\alpha_2\delta\) subunit predicted a more widespread tissue distribution for the \[^{3}H\]gabapentin-binding protein than has previously been acknowledged (7). Indeed, radioligand binding assays revealed high concentrations of \[^{3}H\]gabapentin binding sites not only in brain, but also in skeletal muscle and heart. The distribution of \[^{3}H\]trendipine sites was broadly similar to that for \[^{3}H\]gabapentin, although detailed differences in the relative levels of the binding sites were apparent. However as \[^{3}H\]gabapentin probably labels both L- and non-L-type voltage-dependent Ca\(^{2+}\) channels, these differences are not unexpected. To further confirm the identity of the \[^{3}H\]gabapentin-binding protein, we performed radioligand binding assays on cell lines expressing the \(\alpha_2\delta\) subunit from rabbit skeletal muscle. High levels of specific \[^{3}H\]gabapentin binding in the 2L cell line, which expresses both \(\alpha_2\delta\) and \(\beta\) calcium channel subunits, were observed, whereas the parental HEK 293 line was almost devoid of activity. COS-7 cells transfected with \(\alpha_2\delta\) cDNA alone, expressed >10-fold higher levels of \[^{3}H\]gabapentin binding sites than control cells. These data were consistent with expression of the \(\alpha_2\delta\) protein, as measured by Western blotting using a polyclonal antibody raised against the \(\alpha_2\) polypeptide. The expressed \(\alpha_2\) protein bound \[^{3}H\]gabapentin with high affinity (\(K_D = 16 \text{ nm; } n = 2\)), broadly similar to that determined for rat muscle membranes. These studies argue strongly that \[^{3}H\]gabapentin binds to the \(\alpha_2\delta\) subunit in our purified preparation and not to a minor contaminant.

Most studies on the structure and functional domains of Ca\(^{2+}\) channels have focused on the L-type channel from rabbit skeletal muscle. This channel is a heteromultimeric complex composed of an \(\alpha_2\) subunit, which forms the Ca\(^{2+}\) conducting pore, and three accessory subunits: \(\alpha_2\delta\), \(\beta\), and \(\gamma\). Although it is now well established that the \(\alpha_2\) subunit is the target for dihydropyridines, early preparations of the "dihydropyridine receptor" apparently contained only an \(\alpha_2\delta\) subunit (25). It is now known that these samples were "contaminated" with \(\alpha_2\) subunits that were not visible on SDS gels. In view of the heterologous expression studies described above, the possibility of \[^{3}H\]gabapentin's binding to \(\alpha_2\) subunits in our preparation of purified pig brain \(\alpha_2\delta\) was unlikely. However, \(\alpha_2\delta\) and \(\beta\) subunit cDNAs have been shown to enhance the cell surface expression of co-transfected \(\alpha_2\) subunits (21, 23, 26). We considered the possibility that transfection of \(\alpha_2\delta\) cDNA might enhance the expression of host-derived \(\alpha_2\) subunits. To address this, we isolated L-type Ca\(^{2+}\) channel complexes from rabbit skeletal muscle membranes. Digitonin was employed as the solubilizing agent, as it is reported to preserve the oligomeric structure of Ca\(^{2+}\) channels (20). Purification was achieved using a combination of lectin chromatography and ion-exchange chromatography, as used by others (20, 25, 27). To allow fractionation of individual subunits, we planned to disrupt the Ca\(^{2+}\) channel complexes by exchanging detergents. However, we found that this step was not necessary: partial dissociation occurred during chromatography on Mono Q, even in digitonin-containing buffers. Two peaks of \[^{3}H\]gabapentin binding activity, which closely followed the elution of the \(\alpha_2\delta\) polypeptide, were observed. The \(\alpha_2\) and \(\beta\) subunits were found only in the second peak, presumably as \(\alpha_2\beta\) and \(\alpha_2\beta_\gamma\delta\) complexes. The earlier elution, from ion-exchange columns, of dissociated \(\alpha_2\delta\) subunits is in agreement with another study (20). The profile of the \(\gamma\) subunit was not assessed by immunoblotting, although a faint band of the expected size (35 kDa) was seen in the peak \(\alpha_2\) fraction (Fig. 7A, lane 7). Data from heterologous expression studies and purification experiments show conclusively that the single high affinity \[^{3}H\]gabapentin binding site found in brain and muscle membranes is the \(\alpha_2\delta\) subunit. Moreover, it is clear that the binding of \[^{3}H\]gabapentin does not require the presence of the \(\alpha_1\) and \(\beta\) subunits. However, these subunits may modulate the binding of \[^{3}H\]gabapentin to the \(\alpha_2\delta\) subunit in hetero-oligomeric complexes. A slight enhancement of binding to the \(\alpha_2\delta\) subunit, in the presence of the \(\alpha_1\) and \(\beta\) subunits, is suggested from the data in Fig. 7 (compare lanes 5 and 7) and Fig. 8 (\(\alpha_2\) immunoblot; compare lane 2 with lanes 1 and 3). It is
interesting to note that the \( \alpha_2\delta \) subunit itself modulates the binding of \( \omega \)-conotoxin to the \( \alpha_1 \) subunit of the N-type \( \text{Ca}^{2+} \) channel (23).

The biochemical properties of the purified pig brain \([\text{H}]\)gabapentin-binding protein are strikingly similar to those of the \( \alpha_2\delta \) subunit from rabbit skeletal muscle; the muscle \( \alpha_2\delta \) subunit exhibits a characteristic mobility shift on SDS-polyacrylamide gels, in the presence and absence of reducing agents (24, 27); moreover, the removal of N-linked carbohydrate from the \( \alpha_2 \) subunit, by exhaustive digestion with glycopeptidase F, results in an apparent molecular mass of 105 kDa (28). The \( \alpha_2\delta \) subunit is thought to be anchored to the membrane by a hydrophobic segment located in the 25-kDa \( \delta \) peptide (24), although transmembrane segments in the larger 140-kDa \( \alpha_2 \) component have also been postulated (29). Although we have yet to confirm the identity of the 25-kDa component in our purified preparation by N-terminal sequencing, the poor staining of the band on SDS-polyacrylamide gels is certainly a characteristic feature of the \( \delta \) polypeptide (28, 30).

The alteration in the hydrodynamic properties of the pig brain \([\text{H}]\)gabapentin-binding protein, which occurs during purification, is almost certainly explained by the dissociation of the \( \alpha_2 \) and \( \beta \) subunits. Takahashi et al. (28) found that the skeletal muscle \( \alpha_2\delta \) subunit was associated with an \( \alpha_2\beta \) heterotrimeric complex in solutions containing either 0.5% digitonin or 0.1% CHAPS. However, in 0.5% Triton X-100, the \( \alpha_2\delta \) subunit was found to dissociate from the tripartite complex. Our hydrodynamic data suggest that the \([\text{H}]\)gabapentin-binding protein is associated with other proteins immediately following solubilization with 0.4% Tween 20, and that dissociation begins on the Q-Sepharose column. The presence of both free and complexed \( \alpha_2\delta \) subunits may explain trailing of the \([\text{H}]\)gabapentin binding activity profile for this column.

Mechanism of Action of Gabapentin—All anticonvulsant drugs must ultimately exert their actions by modulating the activity of the basic mediators of neuronal excitability: voltage- and neurotransmitter-gated ion channels. Our data suggest that the \( \alpha_2\delta \) \( \text{Ca}^{2+} \) channel subunit may be the critical target at which gabapentin exerts its antiepileptic action. This is supported by previous studies that have shown a correlation between the affinity of ligands at the \([\text{H}]\)gabapentin binding site and anticonvulsant activity (8). Other ligands acting at \( \text{Ca}^{2+} \) channels have been shown to possess anticonvulsant properties in animal seizure models. Flunarizine is effective against electroshock-induced seizures in rodents (31, 32); dihydropyridines prevent seizures elicited by pentyleneetrazol (31, 33), ethanol withdrawal (34), N-methyl-DL-aspartic acid, and the L-type \( \text{Ca}^{2+} \) channel agonist, BAY K 8644 (31). However, gabapentin is unique among \( \text{Ca}^{2+} \) channel ligands in that it acts at the \( \alpha_2\delta \) subunit rather than at the \( \alpha_2 \) subunit.

The physiological role of the \( \alpha_2\delta \) subunit is not well understood at present. Co-expression of \( \alpha_2\delta \) with the \( \alpha_2 \) and \( \beta \) subunits is known to be required for efficient assembly and functional expression of \( \text{Ca}^{2+} \) channel complexes (21, 23, 26). Since the \( \alpha_2\delta \) subunit appears to be common to all voltage-dependent \( \text{Ca}^{2+} \) channels (26, 35), it is conceivable that gabapentin modulates the activity of more than one type of neuronal \( \text{Ca}^{2+} \) channel. In mouse spinal cord neurons gabapentin blocked responses to BAY K 8644 (36), but in other studies gabapentin did not significantly affect L-, N-, or T-type voltage-dependent \( \text{Ca}^{2+} \) channels (37). However, given the structural diversity of \( \text{Ca}^{2+} \) channels, as revealed by molecular cloning studies (26, 35), data from a few electrophysiological studies should be interpreted with caution. At least six genes encoding \( \text{Ca}^{2+} \) channel \( \alpha_1 \) subunits have been identified. Classes C, D, and S are sensitive to dihydropyridines (L-type), class B is sensitive to \( \omega \)-conotoxin GVIA (N-type), and class A is sensitive to \( \omega \)-agatoxin IVA (P-type). Class E is resistant to the agents listed above (B- and T-type channels). At least four genes encode \( \beta \) subunits, one gene encodes the \( \alpha_2\delta \) subunit, and multiple splice variants of \( \alpha_2 \), \( \beta \) and \( \alpha_2\delta \) have been described (26, 35). The potential combinational heterogeneity of \( \text{Ca}^{2+} \) channels at the structural level is enormous. It is possible that gabapentin exerts functional effects only with particular combinations of subunits. Moreover, these effects may be observed only under conditions that mimic closely the excessive repetitive discharges that characterize clinical epilepsy. Further studies on the cellular electrophysiological actions of gabapentin, in a variety of systems, are required before the action of the drug at \( \text{Ca}^{2+} \) channels can be fully understood.

Finally, the data reported here show that the protein labeled by \([\text{H}]\)gabapentin in brain membranes is not the L-system transporter. However, the high affinity interaction of certain L-system substrates (e.g. L-leucine and L-methionine; Ref. 11) with the \( \alpha_2\delta \) subunit is intriguing. To our knowledge the effects of neutral amino acids on the functional activity of voltage-dependent \( \text{Ca}^{2+} \) channels have not been investigated. However, endogenous ligands such as these presumably compete with gabapentin in vivo for the binding site on the \( \alpha_2\delta \) subunit. This perhaps explains why the therapeutic concentration of gabapentin (2) is well above the \( K_0 \) of the drug at the \([\text{H}]\)gabapentin binding site. We cannot say from present data whether the gabapentin binding site is located on the \( \alpha_2 \) or the \( \delta \) component, or whether it is extracellularly or intracellularly disposed. However, the heavy glycosylation of \( \alpha_2\delta \) (24, 28) and weak labeling by hydrophobic photoaffinity probes (28) suggest that the bulk of the \( \alpha_2\delta \) subunit is found at the extracellular surface. Further studies on the topology of the \( \alpha_2\delta \) subunit and the precise location of the \([\text{H}]\)gabapentin binding site are required.

In summary, we have purified and characterized a high affinity \([\text{H}]\)gabapentin-binding protein from pig brain membranes. N-terminal sequencing has identified the protein as an \( \alpha_2\delta \) subunit of a voltage-dependent \( \text{Ca}^{2+} \) channel. This conclusion is supported by tissue distribution studies, by dynamic data, by heterologous expression of cloned \( \alpha_2\delta \) cDNA in COS-7 and HEK cells, and by radioligand binding and immuno blotting studies on fractionated \( \text{Ca}^{2+} \) channel subunits. \([\text{H}]\)Gaba pentin is the first ligand described that interacts with the \( \alpha_2\delta \) subunit. We suggest that modulation of voltage-dependent neuronal \( \text{Ca}^{2+} \) channels may be important to the antiepileptic action of gabapentin.

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