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Calcium Channel $\alpha_2\delta$ Subunits—Structure and Gabapentin Binding

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ABSTRACT

High-voltage activated calcium channels are modulated by a series of auxiliary proteins, including those of the $\alpha_2\delta$ family. Until recently, only a single $\alpha_2\delta$ subunit was known, but two further members, $\alpha_2\delta$-2 and -3, have since been identified. In this study, the structure of these two novel subunits has been characterized and binding of the antiepileptic drug gabapentin investigated. Using antibodies directed against the amino terminal portion of the proteins, the gross structure of the subunits could be analyzed by Western blotting. Similar to $\alpha_2\delta$-1, both $\alpha_2\delta$-2 and -3 subunits consist of two proteins—a larger α_2 and a smaller δ that can be separated by reduction. The subunits are also highly *N*-glycosylated with approximately 30 kDa of their mass consisting of oligosaccharides. $\alpha_2\delta$-1 was detected in all mouse tissues studied, whereas $\alpha_2\delta$-2 was found at high levels in brain and heart. The $\alpha_2\delta$-3 subunit was observed only in brain. $\alpha_2\delta$-1 and $\alpha_2\delta$-2, but not $\alpha_2\delta$-3, were found to bind gabapentin. The K_d value of gabapentin binding to $\alpha_2\delta$-2 was 153 nM compared with the higher affinity binding to $\alpha_2\delta$-1 (K_d = 59 nM).

Voltage gated calcium channels are multisubunit complexes that permit the influx of calcium ions into cells after changes in the plasma membrane potential. A number of voltage gated calcium channels exist, which can be broadly grouped into high-voltage (HVA) and low-voltage activated (LVA) channels. The HVA channels can be further subgrouped as L-, R-, P/Q-, and N-type, depending on their biophysical characteristics. The channels consist minimally of an α_1 pore protein that conducts current, contains the voltage sensor, and is the target of several drugs. Seven genes have been identified for the α_1 subunits of HVA channels and three for LVA channels (for reviews, see Hofmann et al., 1999; Lacinová et al., 2000). Although it is known that HVA channels are modulated by accessory subunits, it remains to be unequivocally established whether the LVA channels are associated with other proteins. The HVA auxiliary subunits are β (four genes), $\alpha_2\delta$ (three), and γ (six). Most of these subunits have splice variants, giving rise to an even larger number of possible channel combinations and behaviors. Mutations of several channel proteins have been shown to be a causative factor in neurological disorders, making the calcium channel subunits target for therapeutic interventions (Burgess and Noebels, 1999).

The $\alpha_2\delta$ family consists of three genes. The first subunit identified was $\alpha_2\delta$-1 in rabbit skeletal muscle (Ellis et al., 1988). Five tissue-specific splice variants exist (Angelotti and Hofmann, 1996), but a functional significance of the splicing has not been established. Two new $\alpha_2\delta$ family members were subsequently identified in human and mouse, and were named $\alpha_2\delta$-2 and -3 (Klugbauer et al., 1999). The novel $\alpha_2\delta$ subunits are 56 and 30% homologous to $\alpha_2\delta$-1 at the amino acid level and share a number of structural motifs. The subunits have similar hydrophobicity profiles and all contain several potential *N*-glycosylation sites. Northern analysis has shown that $\alpha_2\delta$-1 is ubiquitously expressed, $\alpha_2\delta$-2 is found in several tissues including brain and heart, and $\alpha_2\delta$-3 is brain-specific (Klugbauer et al., 1999). An association of $\alpha_2\delta$-2 with tumors has been suggested, and the mouse homolog is a candidate for the *ducky* epileptic phenotype (Gao et al., 2000).

$\alpha_2\delta$-1 consists of two proteins—a highly glycosylated α_2 that is believed to be extracellularly located, and a smaller δ protein that anchors the α_2 to the cell membrane (Brickley et al., 1995; Wiser et al., 1996). These proteins are coded for by a single gene, the product of which is translated as a precursor polypeptide that is post-translationally cleaved (De Jongh et al., 1990). The α_2 and δ associate by disulfide bridges that form between the numerous cysteine residues found in both proteins.

The effects of $\alpha_2\delta$-1on the biophysical properties of α_1 subunits depend on the expression system and subunits used. The subunit invariably increases the current density of cal-

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ABBREVIATIONS: HVA, high voltage activated; LVA, low voltage activated; GBP, gabapentin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

cium channels by increasing the amount of functional channel at the cell surface, and has been reported to allosterically alter the activation and inactivation of several α_1 subunits (Singer et al., 1991; Bangalore et al., 1996, Felix et al., 1997, Klugbauer et al., 1999). $\alpha_2\delta$-1 is known to enhance dihydropyridine binding to L-type channels and ω-conotoxin GVIA to N-type channels (Brust et al., 1993; Felix et al., 1997). $\alpha_2\delta$-2 and -3 significantly enhance and modulate the current through a number of HVA and LVA channels (Klugbauer et al., 1999; Gao et al.; 2000, Hobom et al., 2000). The action of these subunits depends on the α_1 subunit expressed and $\alpha_2\delta$-2 and -3 may preferentially interact with $Ca_v2.3$ (α_{1E}), $Ca_v2.1$ (α_{1A}), or even $Ca_v3.1$ (α_{1G}).

Gabapentin (GBP) is an antiepileptic drug that has also found application in pain and anxiolytic disorders (Welty et al., 1993; Beydoun et al., 1995). GBP binds to rat brain (Hill et al., 1993) and skeletal muscle homogenates (Gee et al., 1996), with lower binding seen in heart, lung, and pancreas (Suman-Chauhan et al., 1993). GBP was subsequently found to bind specifically to $\alpha_2\delta$-1 of voltage activated calcium channels (Gee et al., 1996). Because voltage activated channels are involved in controlling the electrical excitability of neurons, it has been postulated that this drug reduces calcium current by modulating α_1 indirectly through its association with $\alpha_2\delta$-1 (Gee et al., 1996).

The purpose of this study was to characterize the gross structure and properties of the novel $\alpha_2\delta$-2 and -3 subunits. It was found that $\alpha_2\delta$-1, -2, and -3 are similarly processed and post-translationally modified. $\alpha_2\delta$-1 and -2 bound GBP with differing affinities, but the more distantly related $\alpha_2\delta$-3 did not.

Materials and Methods

Transfection of COS-7 Cells. Plasmids containing the $\alpha_2\delta$ subunits, or pcDNA3 (Invitrogen, Groningen, The Netherlands) as control, were transiently transfected in COS-7 cells, using FuGene 6 (Roche, Mannheim, Germany). $\alpha_2\delta$-1 and -3 had been previously cloned into the expression vector pcDNA3, and $\alpha_2\delta$-2 into pcDNA3.1/V5/His-TOPO (Klugbauer et al., 1999). The cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Karlsruhe, Germany) containing 10% fetal calf serum, 100 U of penicillin, and 100 μg/ml streptomycin at 37°C, 6% CO_2; the cells were seeded at 1.5×10^6 cells per 100-mm culture plate. After 24 h, the cells were transfected in antibiotic-free medium with 6 μg of DNA using 18 μl of FuGene reagent per plate. The medium was replaced with complete medium after 16 h and harvested 60 to 72 h after the start of transfection.

Membrane Preparations. Tissues from BALB/c mice were crushed under liquid nitrogen using a mortar and pestle. The powder was homogenized in buffer A (20 mM MOPS, pH 7.4, 300 mM sucrose, 2 mM EDTA, 1 mM iodoacetamide, 1 mM orthophenanthroline, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml antipain) by two 20-s bursts with a Polytron homogenizer (Kinematica AG, Switzerland) and 8 strokes in a power-driven Potter pestle (Braun Biotech, Germany). Cell debris was pelleted by centrifugation at 5000 g for 10 min at 4°C. Low-speed pellets were re-extracted as above and the supernatants combined. The supernatants were centrifuged at 70,000 g for 30 min at 4°C. The pellets were resuspended in buffer A and stored in aliquots at −80°C. Membranes from transfected COS-7 cells were prepared similarly, with the following modifications: cells were harvested by scraping into 1× phosphate-buffered saline, pelleted by low speed centrifugation, resuspended in 5 mM Tris-Cl, 5 mM EDTA, pH 7.4, containing protease inhibitors (as above) and incubated on ice for 15 min before homogenization. The pellets resulting from ultracentrifugation were resuspended in 50 mM MOPS, pH 7.4, plus protease inhibitors. The protein content was determined by the Bradford method using BSA as a standard.

Anti-Peptide Antibodies. Antibodies were raised in rabbits by Gramsch Laboratories (Schwabhausen, Germany) against peptides of the human $\alpha_2\delta$-2 (amino acids 98–115) and murine $\alpha_2\delta$-3 (amino acids 59–76) subunits (Klugbauer et al., 1999). These sequences were selected because they have low homology to each other and to $\alpha_2\delta$-1, are found after the putative signal peptide sequence, and have no potential *N*-glycosylation sites. The antibodies were affinity purified using a SulfoLink coupling gel (Pierce, Rockford, IL) with bound antigen. Antibodies were eluted with 4.5 M $MgCl_2$, and concentrated using Centricon YM-50 centrifugal filter devices (Millipore Corporation, Bedford, MA). The antibody was made to 1 mg/ml BSA in phosphate-buffered saline and stored at −20°C in aliquots.

Western Blotting. Proteins were separated by discontinuous SDS-PAGE in 5 or 7.5% resolving gels. The membrane preparations were denatured in Laemmli sample buffer (with or without 0.1 M dithiothreitol) by boiling for 3 min. Typically, 50 to 100 μg of tissue or 3 μg of COS-7 microsomal membrane preparation was loaded per lane. After electroblotting and blocking in 3% BSA, the nitrocellulose membranes were incubated for 2 h at room temperature with primary antibodies used at 1/750 for $\alpha_2\delta$-2 and 1/500 for $\alpha_2\delta$-3. A commercially available anti-$\alpha_2\delta$-1 monoclonal antibody (ABR, Golden, CO) was used at a concentration of 1/500. Incubation with the secondary goat anti-rabbit- or goat anti-mouse-horseradish peroxidase antibodies (Dianova, Germany) proceeded for 1 h at room temperature at a dilution of 1/7000. Antibody binding was detected using the ECL system (Amersham Pharmacia Biotech, Freiburg, Germany).

Deglycosylation Assay. Membrane protein (70 μg) was denatured by boiling in 0.1 M β-mercaptoethanol, 0.5% SDS for 5 min. The reaction was brought to 15 mM Tris-Cl, pH 8.0, 20 mM orthophenanthroline, and 1% Triton X-100. Two units of *N*-glycosidase F (Roche, Mannheim, Germany) were added and the reaction allowed to proceed for 5 h at 37°C. The protein was separated by SDS-PAGE gel and analyzed by Western blotting as described above.

Gabapentin Binding Assay. COS-7 membrane preparations (10 to 30 μg) were incubated in 200 μl volumes with various concentrations of [^{3}H]gabapentin (143 Ci/mmol, custom synthesized by Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in 10 mM HEPES/KOH, pH 7.4, for 30 min at room temperature. The protein was precipitated using 8 ml of ice-cold precipitation buffer (22.5 mM HEPES, pH 7.4; 11.25% polyethylene glycol 6000; 11.25 mM $CaCl_2$) and filtered over GF/B filters soaked in the same buffer. Two further 8-ml volumes of precipitation buffer were used as washes. The activity of the filters was counted in a scintillation counter. Concentrations greater than 20 nM were achieved by adding nonradioactive gabapentin to the required concentration. The corrected binding was calculated using the equation, Total GBP bound = (specific dpm) × [1 + (concentration nonradioactive gabapentin/concentration [^{3}H]gabapentin)].

Nonspecific binding was determined in the presence of 10 μM unlabeled gabapentin. The background binding was less than 20% of the counts without addition of unlabeled gabapentin. To assess the effect of the carbohydrates on GBP binding, 30 μg of native mouse brain membranes were incubated with 2 U of *N*-glycosidase F in 15 mM Tris-Cl containing protease inhibitors (as used for preparation of the membranes), for 18 h at 37°C without prior denaturing.

Results

Analysis of $\alpha_2\delta$ Protein Structure. The specificity of the peptide antibodies was tested using membrane preparations of COS-7 cells transfected with the various $\alpha_2\delta$ proteins. The commercial $\alpha_2\delta$-1 antibody did not recognize $\alpha_2\delta$-2 and -3

preparations. Similarly, the peptide antibodies only recognized the $\alpha_2\delta$ against which they were raised (Fig. 1). The antibodies were also preincubated with an excess of peptide antigen before incubation with the protein blots to test for cross-reactivity. The bands corresponding to the $\alpha_2\delta$ proteins were not detected, indicating that the antibodies are specific (results not shown).

Using antibodies against all three $\alpha_2\delta$ proteins, various murine tissues were analyzed by Western blotting. In our study, $\alpha_2\delta$-1 in brain had a mass of 200 kDa under nonreducing conditions and 140 kDa in a reducing environment (Fig. 2), which is in accord with the values reported previously (Jay et al., 1991). $\alpha_2\delta$-2 in mouse brain had an apparent molecular mass of 190 kDa under nonreducing conditions, which shifted to 138 kDa when DTT was added. $\alpha_2\delta$-3 in brain had the lowest mobility of the $\alpha_2\delta$ proteins, with a mass of 166 kDa under nonreducing and 131 kDa under reducing conditions (Fig. 2A). Our results indicate that, similar to $\alpha_2\delta$-1, $\alpha_2\delta$-2, and $\alpha_2\delta$-3 consist of separate, disulfide linked α_2 and δ proteins. Because the antibodies recognize the amino-terminal domains of the $\alpha_2\delta$-2 and -3 subunits, which correspond to the putative α_2, the shift in migration is due to the loss of the δ protein caused by reduction of the disulfide bonds. The mass estimated by subtraction for δ of $\alpha_2\delta$-1 is 53 kDa, 57 kDa for $\alpha_2\delta$-2, and 35 kDa for $\alpha_2\delta$-3. A shift in electrophoretic mobility of both $\alpha_2\delta$-2 and -3 upon reduction was also observed in COS-7 membranes expressing the proteins (Fig. 4, insert). The multiple bands detected are most probably caused by the glycosylation and processing of the overexpressed proteins by the COS-7 cells.

To test whether the novel subunits are *N*-glycosylated, mouse brain membrane preparations were incubated with *N*-glycosidase F and studied by Western blotting. Deglycosylation resulted in a loss of approximately 30 kDa for all $\alpha_2\delta$ proteins (Fig. 2B).

The tissue distribution of the three $\alpha_2\delta$ subunits in mouse was also analyzed by immunoblotting. As can be seen in Fig. 3, $\alpha_2\delta$-1 is expressed in all tissues studied, namely brain, heart, skeletal muscle, liver, and lung. The highest expression was seen in skeletal muscle and brain; in the latter case only 40 μg of protein was used while 100 μg of the other preparations were loaded. The heart subunit had a larger apparent mass than that of the brain subunit −150 kDa as compared with 140 kDa (reducing conditions). The $\alpha_2\delta$-1b splice variant is exclusively found in brain, whereas the predominant forms in heart are $\alpha_2\delta$-1c and -d (Angelotti and Hofmann, 1996). Because the $\alpha_2\delta$-1c and -d heart splice variants have 5 and 12 fewer amino acids than that of the brain isoform, respectively, the difference in mass is believed to be caused by glycosylation.

$\alpha_2\delta$-2 was found to be expressed at high levels in brain and, to a lesser extent, in heart (Fig. 3). Weaker reactive bands of a similar size were seen in lung, liver, and skeletal muscle, in addition to bands at approximately 120 kDa in brain, heart, and lung. The apparent mass of the primary reactive band in heart was 153 kDa under reducing conditions, which is larger than that seen for $\alpha_2\delta$-2 in brain (138 kDa). This may also be caused by differential glycosylation and/or splice variation, because four splice variants have been described so far for $\alpha_2\delta$-2 (Gao et al., 2000; Hobom et al., 2000). To determine whether the protein detected in the tissues is in fact $\alpha_2\delta$-2, the antibody was preincubated with an excess of antigen. The bands at approximately 160 and 120 kDa disappeared, indicating a specific reaction (data not shown). Because the antibody recognized both bands, the protein detected at lower masses is caused by the presence of more than one splice form, partial degradation, or incompletely processed forms of the highly glycosylated protein. The latter option is possible as the membrane preparations also contain endoplasmic reticulum with associated protein. $\alpha_2\delta$-3 was only found to be expressed in brain, and the smears below and above the size expected for $\alpha_2\delta$-3 in skeletal muscle are believed to be caused by spurious reactions with contractile elements (Fig. 3).

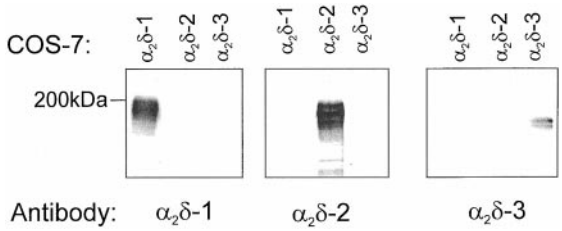

Fig. 1. The anti-$\alpha_2\delta$-1, -2, and -3 antibodies are specific. The specificity of the antibodies was tested using membrane preparations of COS-7 cells transiently transfected with $\alpha_2\delta$ constructs. Three micrograms of each membrane preparation was denatured in the presence of DTT and analyzed by Western blotting. The subunit transfected is indicated above the lanes. The antibodies applied, anti-$\alpha_2\delta$-1, $\alpha_2\delta$-2, and $\alpha_2\delta$-3, are shown below the blots.

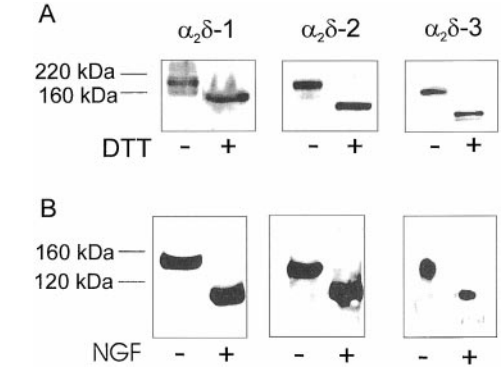

Fig. 2. The $\alpha_2\delta$ proteins are highly glycosylated and consist of two proteins. The sizes and structures of $\alpha_2\delta$-1, -2 and -3 were assessed by Western blotting. Changes in the apparent molecular mass of $\alpha_2\delta$-1, -2, and -3 were observed upon reduction (A). Brain membrane preparations were separated by SDS-PAGE with (+) and without (−) DTT, and analyzed by immunoblotting. Deglycosylation of denatured, reduced brain preparations using *N*-glycosidase F (NGF +) led to a shift in the protein mobility. For all three proteins this corresponded to a loss of approximately 30 kDa relative to untreated (−) controls (B). The antibodies used are indicated above the blots.

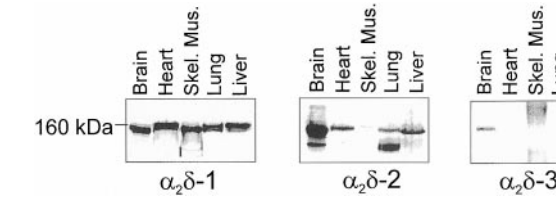

Fig. 3. Western blot showing the tissue distribution of $\alpha_2\delta$-1, 2, and -3 in mouse tissues. Membrane preparations of the indicated tissues were probed with $\alpha_2\delta$-1, $\alpha_2\delta$-2, and $\alpha_2\delta$-3 antibodies. Forty micrograms of brain was used for $\alpha_2\delta$-1 and -2 and 100 μg for the $\alpha_2\delta$-3 blots. One hundred micrograms of the other tissues was loaded. Both $\alpha_2\delta$-1 and -2 gels were electrophoresed under reducing conditions, whereas that for $\alpha_2\delta$-3 was nonreducing.

Gabapentin Binding Assays. Binding of GBP to the $\alpha_2\delta$ subunits was assessed using COS-7 cells that overexpressed the proteins. Membrane preparations were incubated with [^{3}H]gabapentin for 30 min. The protein was subsequently precipitated, captured on filters, and measured by scintillation counting. $\alpha_2\delta$-1 and $\alpha_2\delta$-2 were found to specifically bind GBP, whereas $\alpha_2\delta$-3 did not show any binding (Fig. 4). Cells transfected with pcDNA3 alone as a control did not interact with GBP. $\alpha_2\delta$-1 bound GBP with a relatively high affinity, and a dissociation constant (K_d) of 59 ± 6 nM (S.E.M.) was calculated by Scatchard analysis of data from three experiments (Fig. 5). $\alpha_2\delta$-2 was found to bind GBP with a K_d of 153 ±14 nM (S.E.M.) in six independent experiments. In both cases, GBP seemed to bind to a single binding site, as judged by the Scatchard plots. The contribution of $\alpha_2\delta$ carbohydrate side chains to GBP binding was also investigated. The binding of [^{3}H]GBP to native mouse brain membranes following deglycosylation for 18 h with *N*-glycosidase F was not significantly altered relative to controls incubated without enzyme (not shown).

Discussion

This study was undertaken to clarify the gross protein structure and characteristics of the novel $\alpha_2\delta$-2 and -3 subunits. It was of particular interest to determine whether the characteristic traits of $\alpha_2\delta$-1, namely its composition of two proteins derived from a single precursor protein, and its high level of glycosylation, are conserved. Because GBP, a drug increasingly used for neurological disorders, binds to $\alpha_2\delta$-1, the novel subunits were also tested for drug binding.

Using antibodies directed against the putative α_2 of the $\alpha_2\delta$-2 and -3 subunits, it could be assessed whether precursor proteins are cleaved and form complexes via disulfide bridges. Both $\alpha_2\delta$-2 and -3 in native tissues were found to be composed of an α_2 and δ, which could be separated by reduction. The difference in mass of the nonreduced complex relative to the reduced protein roughly corresponds to that of δ alone and was approximately 50 kDa for each of the $\alpha_2\delta$ proteins. The cleavage site of $\alpha_2\delta$-1 is between A^{934} and A^{935}, when the signal sequence is not taken as part of the protein (De Jongh et al., 1990; Jay et al., 1991). The alanine at position 934 in $\alpha_2\delta$-1 is conserved in $\alpha_2\delta$-2 and -3, although the rest of the sequence in the region diverges. Although the $\alpha_2\delta$-1 cleavage site cannot be assumed to be the same as for the novel proteins, the approximate sizes of the δ proteins and the conservation of the alanine suggest that this could be the case. If the alanine is assumed to be the first residue of δ for both $\alpha_2\delta$-2 and -3, then the mass calculated for δ of $\alpha_2\delta$-2 based on its amino acid content is 17.2 kDa, and for $\alpha_2\delta$-3, 15.1 kDa. The mass estimated indirectly is larger than that calculated using the amino acid sequence. This difference is likely to be due to the difficulty associated with inferring sizes based on shifts in SDS-PAGE migration of reduced and nonreduced proteins, as well as post-translational modifications such as *N*-glycosylation (both δ proteins contain potential *N*-glycosylation sites). A similar pattern of modification has been observed for the δ of $\alpha_2\delta$-1, which has a calculated mass of 19 kDa, and a migration of 24 and 27 kDa using antibodies against the δ (De Jongh et al., 1990).

All three $\alpha_2\delta$ proteins were found to be highly glycosylated, with 30 kDa of the mass of the proteins consisting of oligosaccharides. Because glycosylation is essential for current stimulation by $\alpha_2\delta$-1 of $Ca_v2.1$ (α_{1A}) (Gurnett et al., 1996), this is likely to be the case for the novel subunits as well.

The distribution of $\alpha_2\delta$ protein in mouse tissue was compared with that of the mRNA expression pattern (Klugbauer et al., 1999). $\alpha_2\delta$-3 was detected only in brain, which corresponds with the results of the Northern analysis. $\alpha_2\delta$-2 mRNA was originally described to be ubiquitously expressed, with the highest levels in brain, heart, pancreas, and skeletal muscle (Klugbauer et al., 1999). Lower levels of the mRNA were seen in other tissues after longer exposures. In this study, $\alpha_2\delta$-2 protein was detected at very low levels in skeletal muscle, although the Northern analysis had produced a

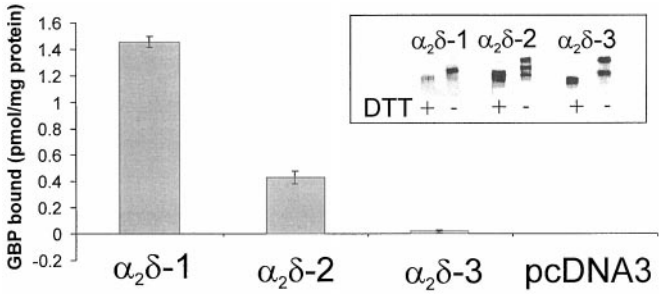

Fig. 4. Gabapentin binds to $\alpha_2\delta$-1 and -2, but not to $\alpha_2\delta$-3. The binding of [^{3}H]gabapentin to membranes of COS-7 cells transiently transfected with $\alpha_2\delta$-1, -2, and -3 was measured. Thirty micrograms of protein was incubated with 20 nM [^{3}H]gabapentin. No binding was observed with membrane preparations of cells transfected with pcDNA3 alone. The data are from three separate experiments done in duplicate. The error bars show the standard error of the mean. The inset shows Western blots showing the expression and cleavage of the proteins in the preparations used.

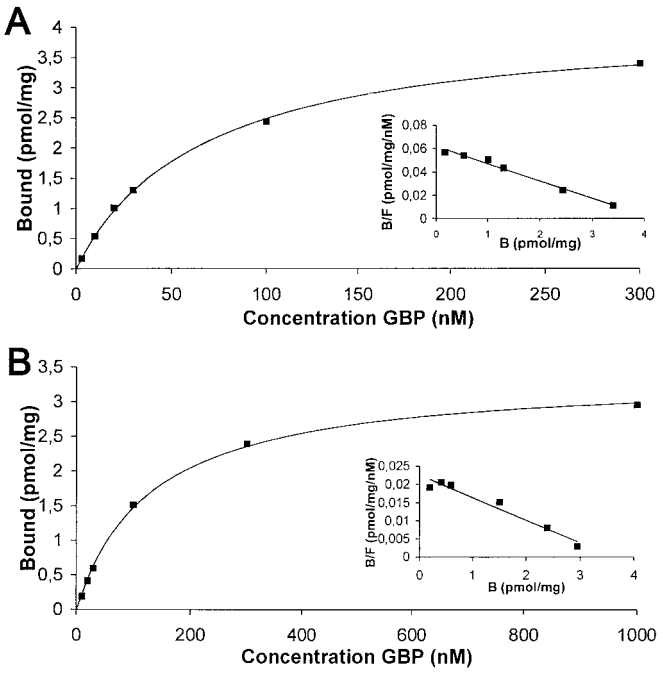

Fig. 5. Gabapentin binds to $\alpha_2\delta$-1 and -2 with different affinities. Ten micrograms of protein was incubated with the indicated concentrations of gabapentin. A representative saturation curve for each subunit is shown, with the corresponding Scatchard plot. K_d values of 59 ± 6 nM (S.E.M.) were calculated for $\alpha_2\delta$-1 (A) and 153 ± 14 nM (S.E.M.) for $\alpha_2\delta$-2 (B). Three independent experiments for $\alpha_2\delta$-1 and six for $\alpha_2\delta$-2 were performed, with each assay done in duplicate. Specific binding was determined in the presence of 10 μM unlabeled gabapentin.

signal comparable with brain. Gao et al. (2000) reported high levels of $\alpha_2\delta$-2 mRNA in human lung and cloned the subunit from a lung library. Although $\alpha_2\delta$-2 protein was observed at low levels in mouse lung in this study, it is possible that certain lung cell types express high levels. Whereas the Gao group showed overexpression of $\alpha_2\delta$-2 protein in various tumor cell lines, no data was presented on the endogenous levels in healthy lung tissue.

The specific binding of gabapentin to $\alpha_2\delta$-1 was the first described interaction between a regulatory subunit of voltage activated calcium channels and a pharmaceutical agent. The K_d of porcine brain $\alpha_2\delta$-1 was reported as 9.4 nM (Brown et al., 1998) but as 37.5 nM for porcine $\alpha_2\delta$-1 expressed in COS-7 cells (Brown and Gee, 1998) and 16 nM for rabbit $\alpha_2\delta$-1 in COS-7 cells (Gee et al., 1996). In this study, K_d values of 59 nM for $\alpha_2\delta$-1 and 153 nM for $\alpha_2\delta$-2 were determined. In a preliminary report by Su et al. (2000), $\alpha_2\delta$-2 is described as having two binding sites (K_d values of 147 and 25 nM). The reason for variations in K_d values is not clear, but may be attributable to species differences and assay methods. Because GBP is used for a variety of neurological disorders, it is interesting to note that it binds to two auxiliary calcium channel subunits that have been found to exert differing modulatory effects on α_1 pore subunits in heterologous expression systems.

The effect of GBP on the physiological activity of calcium channels is not clearly understood. In patch-clamp studies with hippocampal granule cells, no effect of GBP was reported (Schumacher et al., 1998). However, in other studies, modest to dramatic changes in calcium current were noted. A reduction in the calcium current in isolated neurons (Stefani et al., 1998) and in rat neocortical slices (Fink et al., 2000) upon application of GBP has been described. Calabresi et al. (2000) found GBP to reduce most excitatory properties of striatal spiny neurons, which could account for the anticonvulsant effect of the drug. Dooley et al. (2000) have also shown that GBP and a related compound, pregabalin, reduce the release of norepinephrine when stimulated by potassium and electrical pulses. The calcium channels affected are not known, but candidates are L-type (Stefani et al., 1998) and P/Q (Fink et al., 2000; Meder and Dooley, 2000). No consistent effect of GBP on $Ca_v1.2$ (α_{1C}), $Ca_v2.1$ (α_{1A}), and $Ca_v3.2$ (α_{1G}) currents in the heterologous human embryonic kidney 293 expression system were observed in this study. The complexity of the interaction between GBP and $\alpha_2\delta$ is further illustrated by a temperature dependent influence of ruthenium red, $MgCl_2$, and spermine on GBP binding (Taylor and Bonhaus, 2000). Initial studies on GBP binding using rat tissue homogenates showed strong binding in skeletal muscle and brain, where $\alpha_2\delta$-1 is most highly expressed (Gee et al., 1996). A much lower binding of the drug was seen in liver and kidney, which express considerable levels of the protein, as judged by Western blotting. A possible explanation for these conflicting results is that the binding of GBP to $\alpha_2\delta$ is modulated by other subunits (e.g., the α_1 pore protein). It is possible that the effects of GBP depend on the composition and environment of the channel. The lack of clinical side effects of the drug on skeletal muscle and other $\alpha_2\delta$-1 expressing tissues supports this view (Beydoun et al., 1995). GBP has also been shown to be an agonist of certain γ-aminobutyric $acid_B$ receptors, and this has also been postulated to be involved in the clinical action of the drug (Ng et al., 2001).

Gabapentin binding is dependent on the presence of both α and δ subunits, which do not have to be translated as a single precursor protein (Wang et al., 1999). Their interaction is important, however, because neither α nor δ bind the drug when expressed alone (Wang et al., 1999). Cleavage of the precursor protein is also not required for binding (Brown and Gee, 1998). Mutation analysis of porcine $\alpha_2\delta$-1 by Brown and Gee (1998) led to the identification of a region (960–994) in δ-1, containing a zinc-like finger, that is important for gabapentin binding. Another study identified residues 206 to 222, 516 to 537, and 383 to 603 as essential for binding (Wang et

Amino acids 205-223

$\alpha_2\delta$-1	T	P	N	K	I	D	L	Y	*D*	V	*R*	**R**	*R*	P	*W*	*Y*	*I*	Q	G
$\alpha_2\delta$-2	A	P	K	K	I	D	L	Y	*D*	V	*R*	**R**	*R*	P	*W*	*Y*	*I*	Q	G
$\alpha_2\delta$-3	D	E	N	G	V	I	A	F	*D*	C	*R*	N	*R*	K	*W*	*Y*	*I*	Q	A

Amino acids 515-538

$\alpha_2\delta$-1	T	L	*D*	F	L	D	A	*E*	L	*E*	N	F	I	K	V	E	I	*R*	N	K	*M*	I	D	G
$\alpha_2\delta$-2	T	L	*D*	F	L	D	A	*E*	L	*E*	D	E	N	K	E	E	I	*R*	R	S	*M*	I	D	G
$\alpha_2\delta$-3	S	V	*D*	L	S	E	V	*E*	W	*E*	D	R		D	D	V	L	*R*	N	A	*M*	V	N	R

Amino acids 582-604

$\alpha_2\delta$-1	T	Y	S	F	Y	*Y*	I	K	A	K	I	E	E	T	I	T	Q	A	R	Y	S	*E*	T
$\alpha_2\delta$-2	P	Y	S	T	F	*Y*	L	Q	A	N	L	S	D	Q	I	L	Q	V	K	Y	F	*E*	F
$\alpha_2\delta$-3	R	G	H	G	K	*Y*	F	F	R					G			N	V	T	I	E	*E*	G

Amino acids 960-988 (zinc-finger like motif)

$\alpha_2\delta$-1	C	G	N	C	S	R	I	*F*	H	V	E	K	L	M	N	T	*N*	*L*	I	F	I	M	V	E	S	K	G	T	C
$\alpha_2\delta$-2	C	G	N	C	S	R	L	*F*	H	A	Q	R	L	T	N	T	*N*	*L*	L	F	V	V	A	E	K	P	L	C	S
$\alpha_2\delta$-3	C	E	D	C	S	K	S	*F*	V	I	Q	Q	I	P	S	S	*N*	*L*	F	M	V	V	V	D	S	S	C	L	C

Fig. 6. Alignment of $\alpha_2\delta$ sequences important for GBP binding. $\alpha_2\delta$-1 is compared with $\alpha_2\delta$-2 and $\alpha_2\delta$-3 in the regions believed to be involved in GBP binding (Brown et al., 1998; Wang et al., 1999). Residues conserved in $\alpha_2\delta$-1, $\alpha_2\delta$-2 and $\alpha_2\delta$-3 are italicized. Residues only found in $\alpha_2\delta$-1 and -2 are underlined. A^{217} is shown in bold. The alignment of the sequences was done using Clustal V.

al., 1999). Because $\alpha_2\delta$-2 has in this study been shown to bind gabapentin, it is instructive to compare the sequences identified as putative binding sites in previous reports (Fig. 6). Mutations of charged amino acids in $\alpha_2\delta$-1 was performed by Wang et al. (1999), and Arg^{217} found to be the most important. This residue is found in $\alpha_2\delta$-2, but not in $\alpha_2\delta$-3, supporting its role in the binding of GBP. It is, however, not clear whether these sites are essential for the association of α_2 with δ, or whether they form a gabapentin-binding pocket.

Gabapentin analogs have been developed that bind to $\alpha_2\delta$-1 with a higher affinity than gabapentin and are effective in an animal model of epilepsy (Bryans et al., 1998). Because $\alpha_2\delta$ subunits may associate preferentially with α_1 subunits in the brain, these drugs could have differing therapeutic actions if more than one of $\alpha_2\delta$ family binds these drugs.

In summary, we present results indicating that $\alpha_2\delta$-2 and -3 consist of two proteins, derived from a single transcript, that are associated by disulfide bonds. Both novel subunits are highly glycosylated. $\alpha_2\delta$-2, but not $\alpha_2\delta$-3, is capable of binding the antiepileptic drug GBP, which has a higher affinity for $\alpha_2\delta$-1 than for $\alpha_2\delta$-2. Further investigation into the molecular action of GBP and its analogs on voltage gated calcium channels should enable the fine-tuning of treatment of neurological disorders.

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References

Angelotti T and Hofmann F (1996) Tissue-specific expression of splice variants of the mouse voltage-gated calcium channel $\alpha_2\delta$ subunit. *FEBS Lett* **397:**331–337.

Bangalore R, Mehrke G, Gingrich K, Hofmann F and Kass RS (1996) Influence of L-type Ca channel $\alpha_2\delta$-subunit on ionic and gating current in transiently transfected HEK 293 cells. *Am J Physiol* **270:**H1521–H1528.

Beydoun A, Uthman BM and Sackellares JC (1995) Gabapentin: Pharmacokinetics, Efficacy and Safety. *Clin Neuropharmacol* **18:**469–481.

Brickley K, Campbell V, Berrow N, Leach R, Norman RI, Wray D, Dolphin AC and Baldwin SA (1995) Use of site-directed antibodies to probe the topography of the α_2 subunit of voltage-gated Ca^{2+} channels. *FEBS Lett* **364:**129–133.

Brown JP, Dissanayake VU, Briggs AR, Milic MR and Gee NS (1998) Isolation of the [^{3}H]gabapentin-binding protein $\alpha_2\delta$ Ca^{2+} channel subunit from porcine brain: development of a radioligand binding assay for $\alpha_2\delta$ subunits using [^{3}H]leucine. *Anal Biochem* **255:**236–243.

Brown JP and Gee NS (1998) Cloning and deletion mutagenesis of the $\alpha_2\delta$ calcium channel subunit from porcine cerebral cortex. *J Biol Chem* **273:**25458–25465.

Brust PF, Simerson, McCue AF, Deal CR, Schoonmaker S, Williams ME, Velicelebi G, Johnson EC, Harpold MM and Ellis SB (1993) Human neuronal voltage-dependent calcium channels: studies on subunit structure and role in channel assembly. *Neuropharmacology* **32:**1089–1102.

Bryans JS, Davies N, Gee NS, Dissanayake VUK, Ratcliffe GS, Horwell DC, Kneen CO, Morrell AI, Oles RJ, O'Toole JC, et al. (1998) Identification of novel ligands for the gabapentin binding site on the $\alpha_2\delta$ subunit of a calcium channel and their evaluation as anticonvulsant agents. *J Med Chem* **41:**1838–1845.

Burgess DL and Noebels JL (1999) Single gene defects in mice: the role of voltage-dependent calcium channels in absence models. *Epilepsy Res* **36:**111–122.

Calabresi P, Centonze, Marfia GA, Pisani A and Bernardi G (1999) An *in vitro* electrophysiological study on the effects of phenytoin, lamotrigine and gabapentin on striatal neurons. *Br J Pharmacol* **126:**689–696.

De Jongh KS, Warner C and Catterall WA (1990) Subunits of purified calcium channels. *J Biol Chem* **265:**14738–14741.

Dooley DJ, Donovan CM and Pugsley TAJ (2000) Stimulus-dependent modulation of [^{3}H]Norepinephrine release from rat neocortical slices by gabapentin and pregabalin. *J Pharmacol Exp Ther* **295:**1086–1093.

Ellis SB, Williams ME, Ways NR, Brenner R, Sharp AH, Leung AT, Campbell KP, McKenna E, Koch WJ, Hui A, Schwartz A and Harpold MM (1988) Sequence and expression of mRNAs encoding the α_1 and α_2 subunits of a DHP sensitive calcium channel. *Science (Wash DC)* **241:**1661–1664.

Felix R, Gurnett CA, De Waard M and Campbell KP (1997) Dissection of functional domains of the voltage-dependent Ca^{2+} channel $\alpha_2\delta$ subunit. *J Neurosci* **17:**6884–6891.

Fink K, Meder W, Dooley DJ and Göthert M (2000) Inhibition of neuronal Ca^{2+} influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices. *Br J Pharmacol* **130:**900–906.

Gao B, Yoshitaka S, Maximov A, Saad M, Forgacs E, Latif F, Wei MH, Lerman M, Lee J-H, Perez-Reyes E, et al. (2000) Functional properties of a new voltage-dependent calcium channel $\alpha_2\delta$ auxiliary subunit gene (CACNA2D2). *J Biol Chem* **275:**12237–12242.

Gee NS, Brown JP, Dissanayake VUK, Offord J, Thurlow R and Woodruff GN (1996) The novel anticonvulsant drug, gabapentin (Neurontin) binds to the $\alpha_2\delta$ subunit of a calcium channel. *J Biol Chem* **271:**5768–5776.

Gurnett CA, de Waard M and Campbell KP (1996) Dual function of the voltage-dependent Ca^{2+} $\alpha_2\delta$ subunit in current stimulation and subunit interaction. *Neuron* **16:**431–440.

Hill DR, Suman-Chauhan N and Woodruff GN (1993) Localization of [^{3}H]gabapentin to a novel site in rat brain: autoradiographic studies. *Eur J Pharmacol* **244:**303–309.

Hobom M, Dai S, Marais E, Lacinová L, Hofmann F and Klugbauer N (2000) Neuronal distribution and functional characterization of the calcium channel $\alpha_2\delta$-2 subunit. *Eur J Neurosci* **12:**1217–1226.

Hofmann F, Lacinová L and Klugbauer N (1999) Voltage-dependent calcium channels: from structure to function. *Rev Physiol Biochem Pharmacol* **139:**33–87.

Jay SD, Sharp AH, Kahl SD, Vedvick TS, Harpold MM and Campbell KP (1991) Structural characterization of the dihydropyridine-sensitive calcium channel α_2-subunit and the associated δ peptides. *J Biol Chem* **266:**3287–3293.

Klugbauer N, Lacinová L, Marais E, Hobom M and Hofmann F (1999) Molecular diversity of the calcium channel $\alpha_2\delta$ subunit. *J Neurosci* **19:**684–691.

Lacinová L, Klugbauer N and Hofmann F (2000) Low voltage activated calcium channels: from genes to function. *Gen Physiol Biophys* **19:**121–136.

Meder WP and Dooley DJ (2000) Modulation of K^+-induced synaptosomal calcium influx by gabapentin. *Brain Res* **875:**157–159.

Ng GYK, Bertrand S, Sullivan R, Ethier N, Wang J, Yergey J, Belley M, Trimble L, Bateman K, Alder L, et al. (2001) γ-Aminobutyric acid type B receptors with specific heterodimer composition and postsynaptic actions in hippocampal neurons are targets of anticonvulsant gabapentin action. *Mol Pharmacol* **59:**144–152.

Singer D, Biel M, Lotan I, Flockerzi V, Hofmann F and Dascal N (1991) The roles of the subunits in the function of the calcium channel. *Science (Wash DC)* **253:**1553–1557.

Schumacher TB, Beck H, Steinhäuser C, Schramm J and Elger CE (1998) Effects of phenytoin, carbamazepine, and gabapentin on calcium channels in hippocampal granule cells from patients with temporal lobe epilepsy. *Epilepsia* **39:**355–363.

Stefani A, Spadoni F and Bernardi G (1998) Gabapentin inhibits calcium currents in isolated rat brain neurons. *Neuropharmacology* **37:**83–91.

Su T, Gong CH, Hang J, Kohler W and Dickerson M (2000) Human $\alpha_2\delta$-2 subunit of calcium channel: a novel gabapentin binding protein in brain. Society for Neuroscience 30th Annual Meeting, 2000 Nov 4–9, New Orleans, LA. Society for Neuroscience, Washington DC.

Suman-Chauhan N, Webdale L, Hill DR and Woodruff GN (1993) Characterisation of [^{3}H]gabapentin binding to a novel site in rat brain: homogenate binding studies. *Eur J Pharmacol* **244:**293–301.

Taylor MT and Bonhaus DW (2000) Allosteric modulation of [^{3}H]gabapentin binding by ruthenium red. *Neuropharmacology* **39:**1267–1273.

Wang M, Offord J, Oxender DL and Su T-Z (1999) Structural requirement of the calcium-channel subunit $\alpha_2\delta$ for gabapentin binding. *Biochem J* **342:**313–320.

Welty DF, Schielke GP, Vartanian MG and Taylor CP (1993) Gabapentin anticonvulsant action in rats: disequilibrium with peak drug concentrations in plasma and brain microdialysate. *Epilepsy Res* **16:**175–181.

Wiser O, Trus M, Tobi D, Halevi S, Giladi E and Atlas D (1996) The $\alpha_2\delta$ subunit of voltage sensitive Ca^{2+} channels is a single transmembrane extracellular protein which is involved in regulated secretion. *FEBS Lett* **379:**15–20.

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