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Tissue-specific Expression and Gabapentin-Binding Properties of Calcium Channel α2δ Subunit Subtypes

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Abstract. We report here the tissue-specific expression and gabapentin-binding properties of calcium channel α2δ subunits. Northern blot analysis demonstrated that human α2δ-1, -2, and -3 mRNA all had high levels of expression in brain, heart and skeletal muscle. However, the highest expression of human α2δ-2 mRNA was found in lung. Human α2δ-1, -2, and -3 mRNAs were detected in all portions of brain tested. Western blotting revealed that α2δ-2 protein was predominantly expressed in cerebellar cortex (brain) and undetectable in lung. The dissociation between mRNA and protein levels of human α2δ-2 in lung suggests possible post-transcriptional regulation. Although mouse α2δ-1 proteins exhibited a similar tissue distribution profile as that of human, tissue distribution of mouse α2δ-2 and -3 mRNA revealed a different profile. Mouse α2δ-3 mRNA was restricted to brain and mouse α2δ-2 mRNA was not detectable in lung. Gel electrophoresis under a reduced condition resulted in a mobility shift of both α2δ-1 and α2δ-2 proteins, suggesting that α2 and δ of α2δ-2 protein are linked by disulfide bond as are α2 and δ of α2δ-1. Scatchard plots revealed a single population of gabapentin binding sites for human α2δ-2 with the K_D value twofold higher than that of porcine α2δ-1 (156 ± 25 nM vs. 72 ± 9 nM). Inhibition of gabapentin binding to α2δ-2 by selected amino acids and gabapentin analogs produced a binding profile similar, but not identical to that of α2δ-1.

Key words: α2δ — calcium channel subunits — gabapentin — anticonvulsant

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Introduction

Gabapentin [1-(aminomethyl) cyclohexaneacetic acid] is an anticonvulsant with initial application for treating seizures refractory to conventional drug therapies (Taylor et al., 1998). Even though gabapentin was synthesized as a mimetic of the inhibitory neurotransmitter γ-amino butyric acid (GABA), it readily penetrates blood-brain barrier through the system L transport system (Su et al., 1995). Recent studies have shown that gabapentin is effective in treating many other diseases such as neuropathic pain (Harden, 1999), anxiety (Singh et al., 1996), and psychiatric and behavioral disorders (Schaffer & Schaffer, 1997; Cora-Locatelli et al., 1998; Ryback & Ryback, 1995; Herrmann, Lancot & Myszak, 2000). Despite its wide scope of therapeutic utility, the mechanism of gabapentin action remains poorly understood (Taylor et al., 1998). Recently, gabapentin has been reported to act as an agonist at the gb1a-gb2 heterodimer of $GABA_B$ receptor, suggesting a GABA mechanism of gabapentin action (Ng et al., 2001). One of the intriguing features of gabapentin is that gabapentin exhibits high-affinity binding activity to the α2δ subunit of the calcium channel (Hill, Suman-Chauhan & Woodruff, 1993; Suman-Chauhan et al., 1993; Thurlow, Hill & Woodruff, 1996; Gee et al., 1996; Dissanayake et al., 1997; Brown et al., 1998; Wang et al., 1999; Taylor & Bonhaus, 2000).

Since the first α2δ subunit was cloned, it had been thought for a long time that there existed only one α2δ gene. Recently, two additional α2δ isoforms, α2δ-2 and α2δ-3, have been cloned from human and mouse tissue (Klugbauer et al., 1999; Gao et al., 2000). Two previous studies have reported tissue-specific expression and functional properties of α2δ-2 and α2δ-3 (Klugbauer et al., 1999; Gao et al., 2000). Since the α2δ-2 protein shows very high sequence homology to α2δ-1, it would be interesting to know if α2δ-2 is also a gabapentin-binding protein. All previous gabapentin-binding studies

were in fact based on mixed isoforms of α2δ from brain tissue (Hill et al., 1993; Suman-Chauhan et al., 1993; Thurlow et al., 1996; Gee et al., 1996; Dissanayake et al., 1997; Brown et al., 1998) or based on cloned α2δ-1 (Gee et al., 1996; Wang et al., 1999). On the other hand, studies of tissue-specific expression of α2δ-2 mRNA have produced inconsistent results (Klugbauer et al., 1999; Gao et al., 2000). One report shows that expression of human α2δ-2 mRNA is ubiquitous (Klugbauer et al., 1999), while another report suggests that the same α2δ-2 gene is highly expressed in lung and barely detectable in skeletal muscle, as opposed to α2δ-1 (Gao et al., 2000). Since tissue-specific expression of different subtypes and splice variants of α2δ proteins could be functionally relevant (Angelotti & Hofmann, 1996), we thus determined to further investigate the tissue-specific expression of α2δ subunits by a combination of Northern blotting and Western blotting approaches. In this study, we demonstrated differential tissue expression of three α2δ subtypes at both mRNA and protein levels and gabapentin binding properties. We showed that gabapentin bound to both α2δ-1 and α2δ-2 with different affinity but not to α2δ-3. The data reported here have been previously presented as a poster (Gong et al., 2000).

Materials and Methods

Materials

Porcine α2δ-1 (pα2δ-1) and mouse α2δ-3 (mα2δ-3) cDNA were generous gifts from J. Brown (Brown et al., 1998) and F. Hofmann (Angelotti & Hofmann, 1996), respectively. Monoclonal antibody against α2δ-1 was purchased from Affinity Bioreagents (Golden, CO), polyclonal antibodies against α2δ-2 and α2δ-3 were from S. Duffy (Pfizer, Cambridge UK). The peptide sequences to generate antibodies were HRWQDNIKEEDI for α2δ-2, VSEDYTQTGDFFGE for α2δ-3. The anti-α2δ-2 and -3 antibodies do not show cross-reaction among α2δ-1, -2 and -3, but they cross-react with human and mouse orthologs. Human multiple tissue blots and cDNA were purchased from Clontech. Human Western blots were purchased from Research Genetics, PCR reagents were from Invitrogen and Tag DNA polymerase was from Life Technologies. ECL Western blot kit was from Amersham. Lipofectamine, growth media, restriction enzymes were from Life Technologies. HEK293 and COS-7 cell lines were from ATCC. All other chemicals were from Sigma (St. Louis, MD).

Cloning of the Human α2δ-2 Subunit

Human α2δ-2 (hα2δ-2) cDNA was amplified by PCR from a human brain cDNA library. Four overlapped DNA fragments, which covered the whole open reading frame of hα2δ-2 cDNA (GenBank accession number AF042792) from nt −14 to 994 (fragment *H*), 845 to 1816 (fragment *F*), 1517 to 2791 (fragment *D*), and 2681 to 3790 (fragment *C*), were generated by PCR and then cloned into the expression vector pcDNA3.1 by the TA cloning kit. The PCR primer pairs were 5′-TCTTGAATGGAAACATGGCGGTGC-3′ and 5′-TATACCAGGGTCTCCTTCGGACAT-3′ (fragment *H*); 5′-ATGTGTTCATGGAAAACCGCAGAC-3′ and 5′-AGCCGTTCAGGTCAATGGCAAACA-3′ (fragment *F*); 5′-CCATCCGCATCAACACACAGGAAT-3′ and 5′-GTAAGTCCTCATTGTTAACCTCGC-3′ (fragment *D*); 5′-CTGAGAAGTTCAAGGTGCTAGCCA-3′ and 5′-GATGTGATTTGGGTGCCAAACACC-3′ (fragment *C*). The resulting fragments were cut at internal unique restriction enzyme sites at nt 791 (PflM I), 1395 (Xba I), and 2628 (Hpa I), and assembled into the pcDNA3.1 vector at Hind III/Xho I sites. The hα2δ-2 clone was confirmed by alignment with the previously reported sequence.

Northern Blot Analysis

Multiple Tissue Northern Blots (Clontech) were hybridized and washed according to the manufacturer's recommendation. Digoxigenin-labeled probes specific for α2δ subtypes were generated by PCR and hybridized at 50°C overnight in 10 ml EasyHyb buffer (Roche). The primer pairs employed to generate the probes for human α2δ −1, −2 and −3 were 5′-GACGCGGTGAATAATATCACAGCC-3′ and 5′-ACAAATCGTGCTTTCACTCCCTTG-3′ (nt 958 to 2165; GenBank accession number M76559); 5′-CTGAGAAGTTCAAGGTGCTAGCCA-3′ and 5′-GATGTGATTTGGGTGCCAAACACC-3′ (nt 2534 to 3643; GenBank accession number AF042792); and 5′-CGTGTCCTTGGCAGATGAATGGTC-3′ and 5′-CATCTCAGTCAGTGTCACCTTGAG-3′ (nt 1920 to 3272; GenBank accession number AJ272213), respectively. The primers for mouse α2δ −1, −2 and −3 probes were 5′-AACAGATCTAAAGCCCTGGTGCGCC-3′ and 5′-ACCCATGGAGAAGCTGGATAATATCG-3′ for mouse α2δ-1 (nt 504 to 901; GenBank accession number U73483); 5′-ATTGACGGTGTGATGCGGATTTTTG-3′ and 5′-GACATCGTACAGGTCAATCTTCTTG-3′ for mouse α2δ-2 (nt 693 to 1280; GenBank accession number AF247139); 5′-GTGGGGAGATAAAATCCATCGCTG-3′ and 5′-GCTCTTTAACTGGGACATCTGTGC-3′ for mouse α2δ-3 (nt 149 to 1525; GenBank accession number NM_009785). The blots were washed twice, first in 2×SSC and 0.1% SDS at room temperature for 5 min, then in 0.1×SSC and 0.1% SDS at 68°C for 15 min. Detection of expression was in accordance with the manufacturer's instructions (Roche).

Cell Culture and Transfection

COS-7 and HEK293 cells were cultured in DMEM and RPMI 1640 media, respectively. The media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS), in a humidified incubator with 95% air and 5% CO_2 at 37°C. For transient transfection into COS-7 cells, 20 μg of plasmid DNA (vector or the same vector with α2δ insert) was incubated with 30 μl of lipofectamine. The mixture was overlaid onto the cells in 15 ml serum-free medium and incubated for 5 hr. Then FBS was added to the dishes to bring the final concentration to 10%. The medium was changed next morning. Forty-eight hours after the transfection, the cells were harvested for membrane preparation. For stable transfection of pα2δ-1 and hα2δ-2 into HEK 293 cells, the same procedure was applied as for transient transfection except that 800 μg/ml G418 was added to the cells 48 hr after the transfection. Two clones, GKS02 and GKS07, showed highest expression of pα2δ-1 and hα2δ-2, respectively, and were selected for further binding studies.

Membrane Preparation

Membranes were prepared from tissues or cultured cells. The cells were washed twice with cold PBS, then scraped off with cold buffer containing (in mM) 5 Tris (pH 7.4), 5 EDTA, 0.1 PMSF, 0.02 leupeptin, and 0.02 pepstatin. The cells were incubated on ice for 30 min, followed by sonication for 30–40 sec. For membrane preparations from

tissues, the tissues were sliced into small pieces and sonicated at intervals of 10 sec four times. The resulting homogenates from tissues or cultured cells were centrifuged for 10 min at 750–1000 × g, then the supernatants were centrifuged at 50,000 × g for 30 min. The resulting pellets were resuspended in the same buffer as described above.

Western Blot Analysis

The cell membranes (0.5 μg for GKS07 cells, 5 μg for GKS02 cells, 5 or 10 μg for transiently transfected cells or tissues) were resolved by 4–20% SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry transferring unit. The membranes were incubated with either mouse anti-α2δ-1 or rabbit anti-α2δ-2 and -3 antibodies for 1 hr at room temperature, followed by washing with 1 × PBS. The blots were incubated with anti-rabbit IgG for 1 hr and developed with ECL reaction according to the procedure recommended by manufacturer.

Binding Assays

The radioligand-binding assay was done using membrane proteins incubated in the presence of 20 nM [^{3}H] gabapentin. The membrane proteins (100 μg for transient transfected cells, 20 μg for GKS02 cells and 10 μg for GKS07 cells) were incubated in 10 mM Hepes (pH 7.4) for 40–50 min at room temperature, then filtered onto pre-wetted GF/C membranes and quickly washed five times with 3 ml of icecold 50 mM Tris buffer (pH7.4). The filters were dried and counted in a liquid scintillation counter. To determine nonspecific binding, the binding assays were performed in the presence of 10 μM (S+)-3-isobutylGABA (pregabalin; Gee et al., 1996). The specific binding was obtained by subtracting nonspecific binding from the total binding. Unless specified, all experiments were carried out in triplicate and values were expressed as mean ± SD.

Results

Tissue Distribution of α2δ Transcripts

Northern blots demonstrated that all three human α2δ genes were expressed in brain, heart and skeletal muscle (Fig. 1). The highest expression of human α2δ-1 (hα2δ-1) was detected in skeletal muscle, while the most abundant hα2δ-2 transcript was found in lung. In the present study we also detected a small amount of hα2δ-1 and hα2δ-3 mRNAs in kidney.

Human α2δ-1, -2 and -3 transcripts were detected in every portion of brain tissue tested, including cerebellum, cerebral cortex, medulla, occipital pole, frontal lobe, temporal lobe, and putamen. The level of hα2δ-2 transcript in cerebellum was higher than that of cerebral cortex, while the reverse was true for hα2δ-3. For hα2δ-1, its mRNA was approximately equally expressed in these two brain regions. These results were in accordance with previous in situ hybridization results (Klugbauer et al., 1999; Hobom et al., 2000). In addition, all three subtypes of human α2δ mRNA were found in spinal cord, but at a lower level than in the brain.

Northern blotting of mouse multiple tissue panels showed that mouse α2δ-1 (mα2δ-1) mRNA was expressed in brain, heart, and skeletal muscle (Fig. 2). Expression of mouse α2δ-2 (mα2δ-2) mRNA was predominantly found in brain, to much less extent in heart, and barely detectable in skeletal muscle. In contrast, mα2δ-3 mRNA was restricted to the brain. The brain-specific expression of mα2δ-3 was consistent with a previous report (Klugbauer et al., 1999).

Tissue Distribution of α2δ Proteins

Although the protein expression level is a function of the steady-state level of mRNA, the relative abundance of mRNA and protein of a specific gene is not always proportional, which may reflect post-transcriptional regulation (Jackson et al., 1997). To examine the relative levels of human α2δ subunits across tissues, we used antibodies raised against specific subtypes of α2δ protein for Western analysis. Equal amounts of proteins were loaded on SDS polyacrylamide gels. Western blotting showed that hα2δ-1 protein was widely distributed among various tissues except lung and jejunum. hα2δ-3 protein was only detected in brain, not in lung, testis, aorta, spleen, jejunum, ventricular muscle and kidney (Fig. 3). In contrast to high expression of hα2δ-2 transcript in lung (*see* Fig. 1), hα2δ-2 protein was undetectable in lung and predominantly found in brain (Fig. 3). In addition to brain, low levels of hα2δ-2 protein were also found in aorta, testis and ventricular muscle. There seemed to be two immunoreactive bands in testis with one equivalent to the predicted molecular weight of hα2δ-2 (175 kDa) and the other having slightly lower molecular weight. This low-molecular weight protein was similar in size to the predominant band in ventricular muscle. This lower band might represent the α2 subunit (~140 kDa) dissociated from the α2δ-2 protein, an isoform of α2δ-2, or a different level of glycosylation (Brown et al., 1998; Wang et al., 1999). Like hα2δ-1, mα2δ-1 protein was also expressed ubiquitously in almost every tissue tested (Fig. 3). Mouse α2δ-2 was highly expressed in brain and to less extent in heart, pituitary, and kidney. In addition, much weaker expression of mα2δ-2 was found in liver, lung, stomach, and pancreas. Differing from mα2δ-1 and -2, mα2δ-3 protein, like its mRNA (Fig. 2), was only detected in brain.

Disulfide Linkage of α2 and δ Proteins

It has been shown that the α2 and δ subunits of α2δ-1 are linked by disulfide (Wang et al., 1999). Since the amino acid sequence in the δ region is less conserved between α2δ-1 and α2δ-2, it is of interest to know if α2δ-2 protein is also post-translationally cleaved into two subunits. To examine such a possibility, cell membranes from

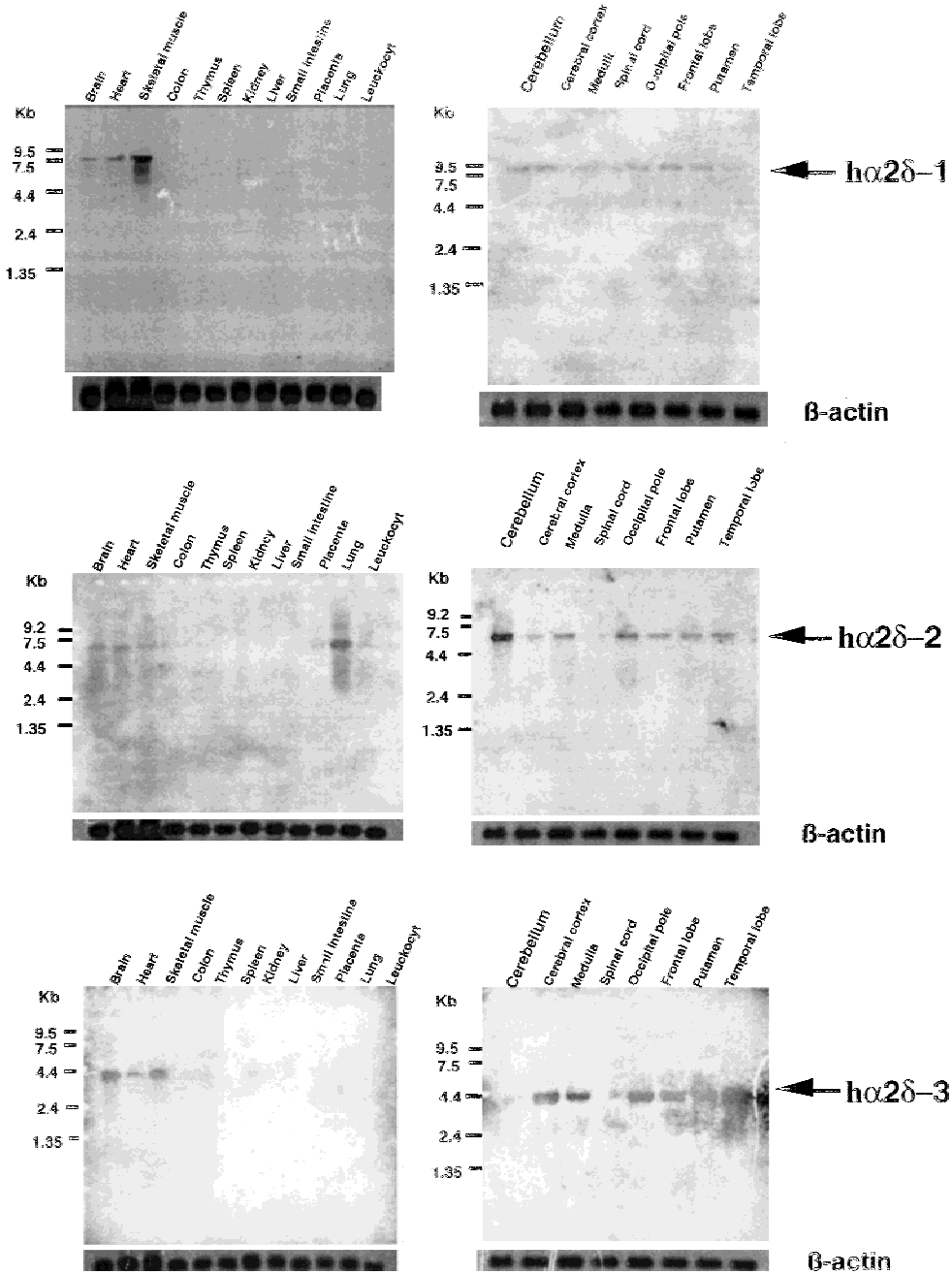


Fig. 1. Northern blot analysis of human α2δ tissue distribution. Northern blotting was carried out as described in Materials and Methods. Human multiple tissue blots (Clontech) were hybridized with Digoxigenin-labeled cDNA synthesized from nucleotide 958 to 2165 (hα2δ-1), 2534 to 3643 (hα2δ-2) and 1920 to 3272 (hα2δ-3). The positions of marker RNA are indicated to the left.

HEK 293 cell lines overproducing pα2δ-1 (GKS02) and hα2δ-2 (GKS07) proteins were treated or not treated with 100 mM DTT before gel electrophoresis. In the presence of DTT, both pα2δ-1 and hα2δ-2 proteins shifted to a position predicted for α2 (~140 kDa), suggesting that hα2δ-2 also consists of two subunits that are linked by disulfide bonds (Fig. 4).

[^{3}H] Gabapentin Binding

To determine the gabapentin binding properties of the cloned hα2δ-2, membranes were isolated from COS-7 cells that were transiently transfected with pα2δ-1, hα2δ-2, mα2δ-3, and vector pcDNA3.1. Expression of the corresponding α2δ proteins was examined by West-

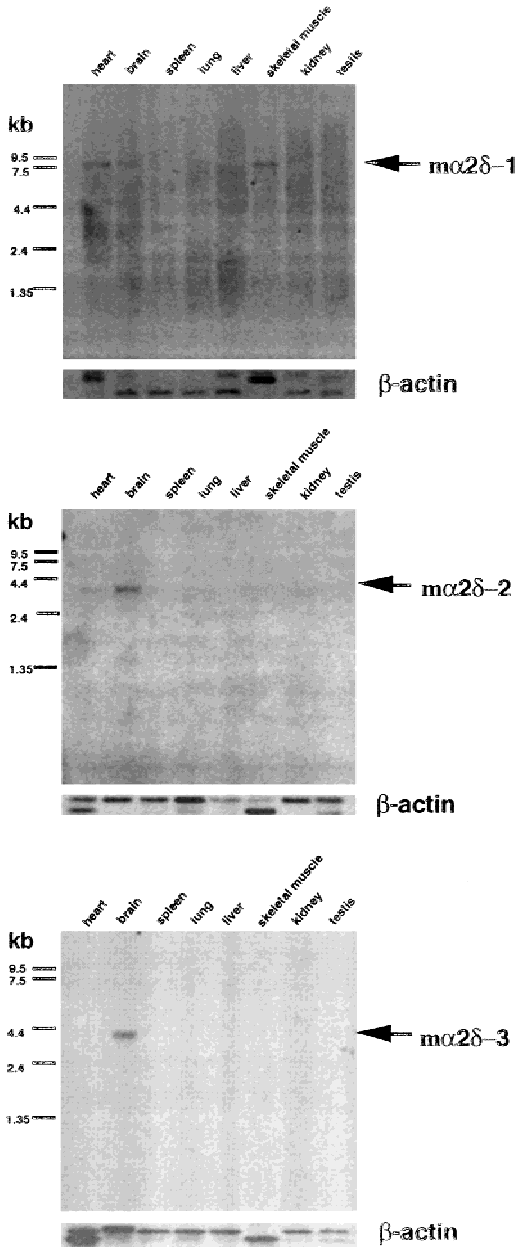


Fig. 2. Northern blot analysis of mouse α2δ tissue distribution. Northern blotting was carried out as described in Materials and Methods. Mouse multiple tissue blots (Clontech) were hybridized with Digoxigenin-labeled cDNA synthesized from nucleotide 504 to 901 (mα2δ-1), 693 to 1280 (mα2δ-2) and 149 to 1525 (mα2δ-3). The positions of marker RNA are indicated to the left.

ern blots. As shown in Fig. 5, transfection of the cells with pα2δ-1 resulted in a prominent increase in gabapentin binding. Similarly, the cells expressing hα2δ-2 exhibited an about fourfold increase in gabapentin-binding activity. In contrast to pα2δ-1 and hα2δ-2, we did not observe gabapentin binding activity with mα2δ-3. Although a slightly increased binding activity was observed in the cells transfected with pcDNA3.1 vector alone, statistic analysis did not show that this smaller change was significant.

Gabapentin-binding K_D and the binding properties of pα2δ-1 and hα2δ-2 were further determined in cell lines GSK02 (pα2δ-1) and GKS07 (hα2δ-2). In HEK293 cells stably expressing pα2δ-1, [^{3}H] gabapentin bound to a single population of sites, as demonstrated in a previous report (Gee et al., 1996), with a K_D value of 72 ± 9 nM (Fig. 6*A*). Similarly, a single population of binding sites was also observed in hα2δ-2-containing membranes (Fig. 6*B*), but the K_D value was higher than that of pα2δ-1 (156 ± 25 nM). We previously observed that gabapentin contained two binding sites on α2δ-2, but because of the big variation in that experiment, we could not confirm the high-affinity (25 nM) binding site (Gong et al., 2000). To determine pharmacological properties of hα2δ-2, several compounds were selected for competition with [^{3}H] gabapentin binding. A similar, but not identical profile of competition was seen in the two subtypes of α2δ protein (Table). For instance, binding to both subtypes of α2δ was stereo-selective because L-leucine was markedly more potent than its D-enantiomer. The affinities of BCH, a model substrate of system L transport (Su et al., 1995), and phenylalanine were weak for both subtype proteins. On the other hand, gabapentin was more selective for α2δ-1 (72 ± 8 nM vs. 190 ± 10 nM; $P < 0.001$), and pregabalin was slightly more potent for α2δ-2 (70 ± 0.5 nM vs. 106 ± 4 nM; $P < 0.001$).

Discussion

Voltage-gated calcium channels (VGCCs) play a pivotal role in intracellular Ca^{2+}-dependent processes (for review, *see* Moreno, 1999; Randall & Benham, 1999; Varadi et al., 1999). VGCCs are formed by heteromultimeric complexes consisting of a pore-forming protein α1 (170–240 kDa) and 2 or 3 auxiliary proteins including β (52 kDa) and α2δ (175 kDa), and sometimes a transmembrane protein γ (95 kDa). Molecular cloning has revealed that there are at least 10 α1, 4 β, 4 γ and 3 α2δ genes in mammalian cells (Randall & Benham, 1999). Many of these genes also encode multiple splice variants. These multiple isoforms and their splice variants exhibit tissue-, subcellular location- and development-dependent expression, and by combination form markedly diversified calcium channels. It is generally thought that the primary biophysical properties of VGCCs are determined by the α1 subunit, and all other subunits are promiscuously associated with the α1 subunit, fine-tuning the channel function (Walker & De Waard, 1998). When α2δ-1, α2δ-2 or α2δ-3 subunits are coexpressed with α1C/β2a and α1E/β3, all three α2δ subunits increase the current density and shift the steady-state inactivation to more negative potentials (Hobom et al., 2000). However,

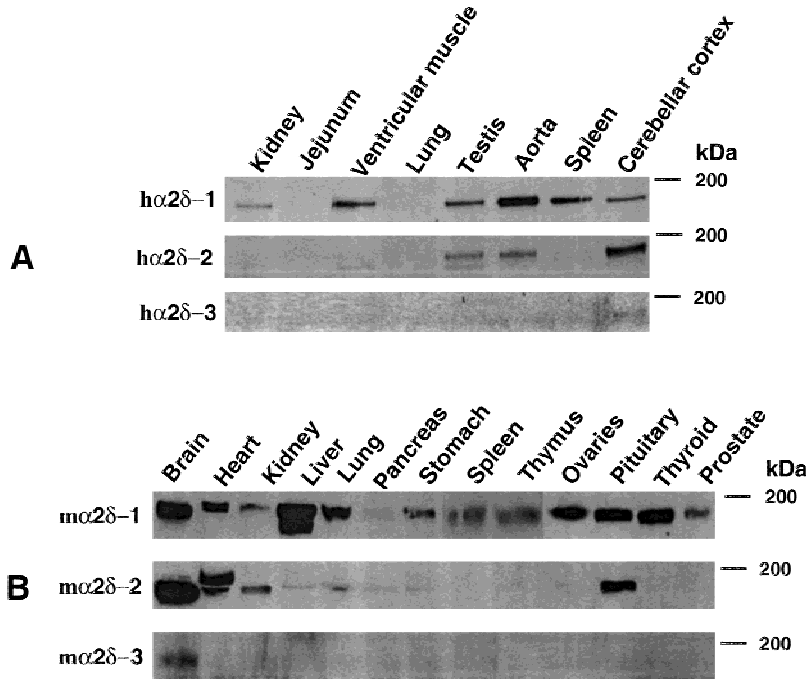


Fig. 3. Western blot analysis of human (*A*) and mouse (*B*) α2δ tissue distribution. Membrane proteins from different human (5μg) and mouse (10 μg) tissues were loaded on 4–20% SDS-PAGE (NOVEX) and subjected to Western blot analysis (*see* Materials and Methods). The blots were probed with anti-α2δ-1 monoclonal antibody or polyclonal antibodies against α2δ-2 and α2δ-3.

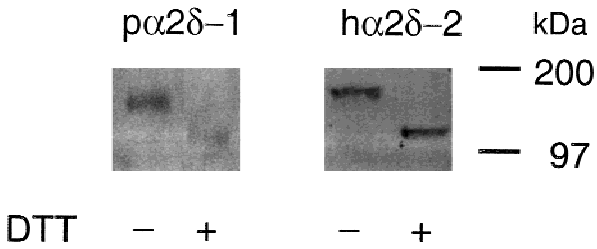


Fig. 4. Disruption of disulfide-linkage between α2 and δ subunits. An equal amount of membrane proteins from stable cell lines (0.5 μg for GKS02-pα2δ-1 and 5 μg for GKS07-hα2δ-2) was incubated in the presence or absence of 100 mM DTT for 10 min and resolved on a nonreducing SDS-PAGE and transferred to a PVDF membrane. The blots were probed with either an anti-α2δ-1 antibody (*left*) or an anti-α2δ-2 antibody (*right*). The positions of marker proteins are indicated to the right.

only α2δ-2 and α2δ-3 shift the voltage dependence of activation of α1E/β3, suggesting that the neuronal α1E calcium channel is differentially modulated by these α2δ subunits.

In this study we demonstrated that α2δ-1, -2 and -3 subunits were differentially expressed among tissues. For example, hα2δ-1, -2, -3 and mα2δ-1 and -2 mRNAs were detected in brain, heart and skeletal muscle, whereas mα2δ-3 mRNA was only found in brain. The undetectable expression of mα2δ-3 mRNA by Northern blots in peripheral tissues agrees well with one previous report (Klugbauer et al., 1999). We further demonstrated that mα2δ-3 mRNA and mα2δ-3 protein were detected merely in brain. In contrast to the relatively tissue-specific expression of α2δ-2 and -3 proteins, expression of α2δ-1 appeared to be ubiquitous. Human α2δ-1 mRNA was expressed about equally well in cerebellum and cerebral cortex, hα2δ-2 mRNA in cerebellum was much higher than cerebral cortex, and the reverse was true for hα2δ-3. In contrast to the relative abundance of hα2δ-2 mRNA in lung, we found that the level of hα2δ-2 protein in lung was barely detectable. The highest amount of hα2δ-2 protein was found in brain, although in comparison with lung the hα2δ-2 mRNA in brain was much less. The striking differences between the relative abundance of mRNA and the amount of protein of the same gene in a given tissue have been reported for other genes (Jackson et al., 1997), suggesting possible post-transcriptional regulation. The tissue-specific expression pattern of hα2δ-2 mRNA was consistent with a recent study (Gao et al., 2000), but differed from an earlier report (Klugbauer et al., 1999). The high level of hα2δ-2 mRNA expression in lung was further supported by RT-PCR analysis of hα2δ-2 tissue distribution and by sequencing analysis of the corresponding PCR clone from lung (*data not shown*). The failure to detect highest expression of hα2δ-2 mRNA in lung in the latter report could be due to the primers being less specific for the gene (Gao et al., 2000).

In addition to the tissue-specific expression, α2δ expression may vary depending on species. Despite the ubiquitous expression of both human and mouse α2δ-1, the tissue distribution patterns of α2δ-2 and -3 mRNA in mouse differ from human. Mouse α2δ-3 is restricted in brain at both mRNA and protein levels, whereas human

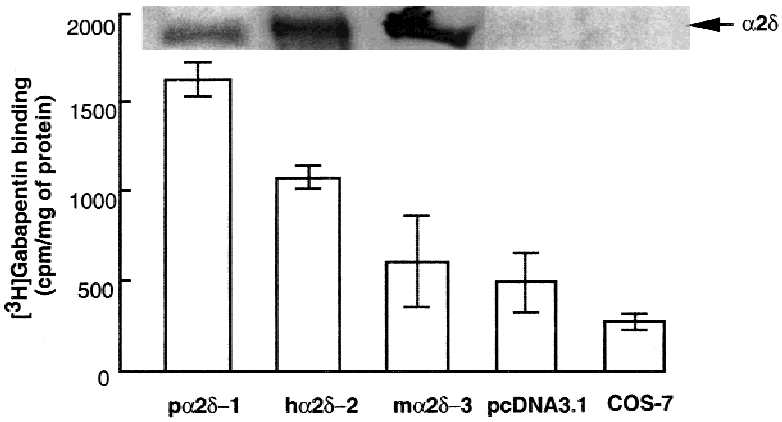
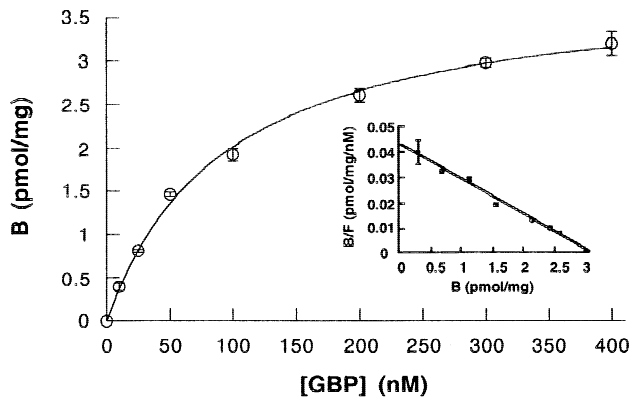


Fig. 5. Binding of [^{3}H] Gabapentin to membranes from COS-7 cells transfected with α2δ cDNA. COS-7 cells were transfected with 20 μg of pcDNA3.1 (pcDNA3.1), pcDNA3.1/porcine α2δ-1 construct (pα2δ-1), pcDNA3.1/mouse α2δ-3 (mα2δ-3), and pcDNA3.1/human α2δ-2 construct (hα2δ-2). The membranes were prepared for [^{3}H] gabapentin binding assays (*see* Materials and Methods). Data are an average of three independent assays with triplet in each determination. The same membranes (10 μg) were subjected to Western blot analysis with corresponding antibodies as described in Materials and Methods.

A



B

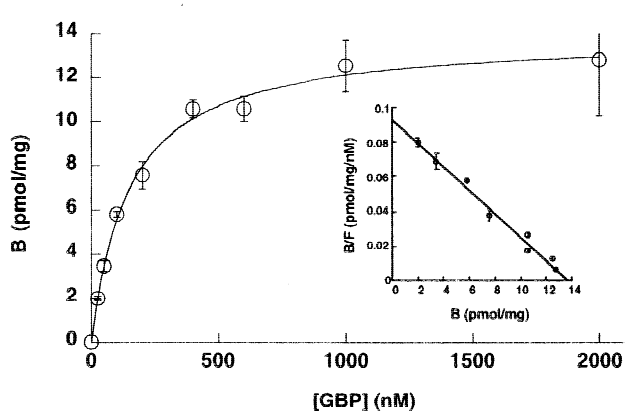


Fig. 6. Scatchard analysis of [^{3}H] gabapentin (GBP) binding to membranes from HEK293 cells overproducing porcine α2δ-1 (*A*) and human α2δ-2 (*B*). The cell membranes were prepared from GKS02, a stable cell line for porcine α2δ-1, and GKS07, a stable cell line for human α2δ-2. The specific [^{3}H] Gabapentin binding was carried out as described in Materials and Methods. The binding activity was expressed as pmole of gabapentin bound per mg of protein. Each binding reaction contained 20 μg of GKS02 membrane proteins or 10 μg of GKS07 membrane proteins. Data were averages of three assays.

α2δ-3 mRNA is also expressed in heart, skeletal muscle and kidney. The highest expression of α2δ-2 mRNA in lung can only be found in humans, not in mouse. It is unclear whether the high α2δ mRNA levels found in tissues other than brain in humans reflects the species differences in post-transcriptional regulation of α2δ genes. Further studies of the species-dependent expression of α2δ may facilitate better understanding of the functional role of α2δ across species.

The data presented here demonstrated that α2δ-1 and -2, but not -3 bind anticonvulsant gabapentin. Autoradiographical study of gabapentin binding in rat brain has shown that the highest level of binding sites is located in the outer layers of the cerebral cortex (Hill et al., 1993). Based on our present study, it is likely that the highest binding activity previously observed in cerebral cortex is contributed by at least two subtypes of α2δ subunit, α2δ-1 and -2. The link between gabapentin

Table. Inhibition of [^{3}H] gabapentin binding by selected amino acids to the membranes from porcine α2δ-1 (GKS02)- and human α2δ-2 (GKS07)-overproducing cell lines

Compounds	*Ki* (nM)		*P* value
	GKS02 (pα2δ-1)	GKS07(hα2δ-2)	
Gabapentin	72 ± 8	190 ± 10	<0.0008
Pregabalin	106 ± 4	70 ± 0.5	<0.0008
L-Leucine	93 ± 4	182 ± 4	<0.0001
L-Phenylalanine	642 ± 36	2625 ± 114	<0.0001
D-Leucine	15267 ± 2437	13741 ± 2223	
BCH	799 ± 110	669 ± 81	

Data are presented as mean ± SEM of 3 experiments.

binding and calcium channel function remains to be clarified. Several lines of evidence suggest an interesting correlation between gabapentin binding and its therapeutic actions. First, the rank order of affinity at this site correlates to the rank order of anticonvulsant activity in the animal models, suggesting that the gabapentin binding protein may modulate neuronal excitability (Taylor et al., 1993, 1998; Bryans & Wustrow, 1999). Second, the distribution pattern of gabapentin binding sites in the brain are co-localized to the areas where excitatory input is commonly associated with seizure activity (Hill et al., 1993). Third, modest inhibitory effects of gabapentin on neuronal Ca^{2+} currents have been observed in cultured neuronal cells (Alden & Garcia, 1999; Stefani, Spadoni & Bernardi, 1998). Fourth, gabapentin interacts with known calcium allosteric modulators such as spermine, Mg^{2+} (Dissanayake et al., 1997) and ruthenium red (Taylor & Bonhaus, 2000), suggesting that the principal endogenous binding site for gabapentin is a calcium channel subunit (Taylor & Bonhaus, 2000). Finally, genomic mapping reveals that an inherited mouse mutant, called ducky, develops epileptic phenotype possibly due to the defect in α2δ-2 expression (Meier, 1968; Barclay et al., 2001). All these results suggest that studies of the relevance of gabapentin binding to the function of defined subsets of calcium channels are clearly warranted (Taylor & Bonhaus, 2000).

Like studies of the α2δ role in modulation of calcium channel, studies of gabapentin binding effects on calcium channel function have produced controversial results (Alden & Garcia, 1999; Stefani et al., 1998; Rock, Kelly & Macdonald, 1993; Schumacher et al., 1998). One difficulty in evaluating the gabapentin effect through action on an ancillary subunit such as α2δ is that gabapentin may interact with a specific subpopulation of calcium channel (Taylor & Bonhaus, 2000). Previous and present studies have demonstrated that the functional roles and tissue distribution patterns of different isoforms of α2δ are distinct (Gao et al., 2000; Hobom et al., 2000). The modest differences observed in the binding profile between α2δ-1 and α2δ-2 predict the possibility to develop isoform-specific ligands. Given that gabapentin binding to certain isoforms of α2δ in a subpopulation of calcium channels at a specific cellular location is pharmacologically relevant, further studies of α2δ-2 binding under normal and diseased conditions using more specific ligands may bring more insight into the gabapentin mechanism of action.

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