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Spermine modulation of specific [^{3}H]-gabapentin binding to the detergent-solubilized porcine cerebral cortex $\alpha_2\delta$ calcium channel subunit

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1 Recent studies have identified the [^{3}H]-gabapentin-binding protein, purified from porcine cerebral cortical membranes, as the $\alpha_2\delta$ subunit of voltage-sensitive calcium channels (Gee *et al*., 1996). The present study investigates the influence of the polyamine spermine on specific [^{3}H]-gabapentin binding to detergent-solubilized porcine cerebral cortical membranes.

2 Spermine, spermidine, 1,10 diaminodecane, Mg^{2+} and Zn^{2+}, all divalent cations, displaced [^{3}H]-gabapentin binding to detergent-solubilized membranes in a concentration-dependent manner with a maximal inhibition of 65–75%. Radioligand binding studies showed that spermine did not directly interact with the [^{3}H]-gabapentin-binding site. Spermine inhibited [^{3}H]-gabapentin binding by interacting with a polyamine-sensitive allosteric site on the membrane protein. The steep concentration-dependence of spermine inhibition of [^{3}H]-gabapentin binding may suggest multi-site co-operativity.

3 Prolonged dialysis of cerebral cortical membranes and Tween 20-solubilized membranes resulted in a >2.0 fold increase in [^{3}H]-gabapentin binding. The increase in binding was due to the removal of a heat stable, low molecular weight (<12,000Da) endogenous molecule which influences [^{3}H]-gabapentin binding competitively.

4 Dialysis of detergent-solubilized cerebral cortical membranes also resulted in a decrease in the maximum inhibition of [^{3}H]-gabapentin binding by spermine. Since the rates of the increase in [^{3}H]-gabapentin binding and the loss of the ability of spermine to inhibit [^{3}H]-gabapentin binding on dialysis were different it was inferred that a second endogenous ligand was removed during dialysis.

5 During initial steps of purification of the [^{3}H]-gabapentin-binding protein there was a decrease in the maximum inhibition of [^{3}H]-gabapentin binding by spermine. The loss of the second endogenous molecule during initial purification would reasonably explain the reduction in inhibition of binding by spermine. However, spermine stimulation of [^{3}H]-gabapentin binding to material that eluted from the gel-filtration column later in the purification scheme does not appear to be due to removal of a dialysable endogenous factor or to the dissociation of other calcium channel subunit(s).

6 Adding back dialysate, before or after boiling, to detergent solubilized membranes resulted in a dose-dependent restoration of the inhibition of [^{3}H]-gabapentin binding and of the maximal inhibition [^{3}H]-gabapentin binding by spermine. This result is consistent with the re-addition of two endogenous heat stable ligands.

7 The finding that [^{3}H]-gabapentin binding to the pure $\alpha_2\delta$ subunit was stimulated by spermine indicates that the $\alpha_2\delta$ subunit of voltage-sensitive calcium channels bears a modulatory spermine site. Such a spermine site has not been identified before. Spermine stimulation of [^{3}H]-gabapentin binding to the purified protein was reversed to inhibition after adding back dialysate. Thus the inhibitory spermine effect in membranes is also probably due to one or more modulatory sites on the $\alpha_2\delta$ subunit.

Keywords: Gabapentin; neurontin; calcium channels; $\alpha_2\delta$ subunit; anticonvulsant; polyamines; spermine; spermine modulation; N-methyl-D-aspartate (NMDA); endogenous ligand

Introduction

Spermine, a naturally occurring polyamine, has been found to modulate the ligand binding properties of several neurotransmitter receptors. Notable among these is the N-methyl-D-aspartate (NMDA) receptor complex, where spermine interacts with a specific polyamine binding site (Ransom & Stec, 1988; Reynolds & Miller, 1989; Williams *et al*., 1989). Polyamines have been shown to modulate ligand binding to γ-aminobutyric$_A$ (GABA$_A$)-benzodiazepine receptors (Costa & Guidotti, 1979; Gilad *et al*., 1992), A_1 adenosine receptors (Wasserkort *et al*., 1991) and σ receptors (Paul *et al*., 1990). More recently polyamines have been shown to affect radioligand binding to the α_1 subunit of voltage-sensitive L- and N-type calcium channels (Pullan *et al*., 1990; Schoemaker, 1992). Spermine has also been shown to affect [^{3}H]-gabapentin binding to rat cortical membranes (Suman Chauhan *et al*., 1993).

Gabapentin, 1-(aminomethyl)cyclohexane acetic acid (neurontin), is a novel anticonvulsant drug (Rogawski & Porter, 1990; McLean, 1995) in clinical use in the U.K. and U.S.A. Its precise mechanism of action is unclear. Although initially designed as a lipophilic γ-aminobutyric acid (GABA) analogue, gabapentin has shown little activity at GABA$_A$ or GABA$_B$ receptors (Dooley *et al*., 1986; Taylor, 1993; Rock *et al*., 1993). Gabapentin does not influence GABA uptake or GABA transaminase activity (Taylor, 1993) although there is some evidence to suggest that gabapentin may act by increasing the accumulation/release of GABA (Loscher *et al*., 1991; Gotz *et al*., 1993). In addition, behavioural data have suggested a possible association between gabapentin and the glycine modulatory site of the NMDA receptor complex (Oles *et al*.,

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1990; Singh *et al*., 1996). However, binding studies have not shown any interaction between gabapentin and NMDA receptors (Suman Chauhan *et al*., 1993).

In vitro radioligand binding studies have revealed a high-affinity, novel binding site for [^{3}H]-gabapentin in rat and porcine cerebral cortical membranes (Suman Chauhan *et al*., 1993; Thurlow *et al*., 1993). Out of a diverse group of pharmacologically active compounds tested, gabapentin and 3-isobutyl GABA were the most active at this binding site. **S**(+)-3-isobutyl GABA was more potent than the **R**(−) enantiomer, mirroring their anticonvulsant potency. Autoradiographic studies have shown that the distribution of specific [^{3}H]-gabapentin binding sites is high in regions associated with seizure activity e.g. cerebral cortex and hippocampus (Hill *et al*., 1993). Taken together, these observations suggest that the [^{3}H]-gabapentin binding site is likely to be involved in the antiepileptic action of the drug.

Recently, the [^{3}H]-gabapentin binding protein was purified from porcine cerebral cortical membranes and identified as an $\alpha_2\delta$ subunit of voltage-sensitive calcium channels (VSCCs) (Gee *et al*., 1996). In the present study, we have investigated the effect of spermine on specific [^{3}H]-gabapentin binding to the detergent-solubilized porcine cerebral cortex calcium channel $\alpha_2\delta$ subunit. We also showed that endogenous ligands may influence [^{3}H]-gabapentin binding and spermine modulation of [^{3}H]-gabapentin binding to the $\alpha_2\delta$ subunit.

Methods

Membrane preparation and detergent-solubilization

Porcine brains were obtained from the local abattoir and transported to the laboratory on ice. All steps described below were carried out at 0–4°C. The cerebral cortex (35g) was homogenized in 10 vol. of buffer A (0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES/KOH, pH 7.4 at 4°C) with a glass/teflon homogeniser. Following centrifugation at 1000 g_{max} for 10 min the pellet was discarded and the supernatant centrifuged at 30,000 g_{max} for 20 min. The resulting pellet was resuspended in 10 vol. of buffer B (buffer A lacking sucrose), stirred continuously for 30 min and centrifuged as above. In some experiments the last step was repeated to furnish the required number of washes. The final pellet was resuspended in 3 vol of 1.25 × buffer C (1.25 mM EDTA, 1.25 mM EGTA, 25% glycerol, 12.5 mM HEPES/KOH, pH 7.4 at 4°C) at a protein concentration of 3 mg ml^{-1}. This material was then used in solubilization experiments. Membranes (100 ml) were combined with 25 ml of 2% Tween 20 solution and mixed in an end-over-end fashion for 1 h. The solubilized membranes were then centrifuged at 100,000 g_{max} for 1 h and the soluble fraction frozen in small aliquots at −70°C until required. Typically, Tween 20 solubilized 60–70% of the [^{3}H]-gabapentin-binding sites in membranes.

Radioligand binding studies

Binding of [^{3}H]-gabapentin to solubilized membranes was carried out for 40 min at 22°C in 10 mM HEPES/KOH pH 7.4 (assay buffer) in a final assay volume of 250 μl, as described by Gee *et al*. (1996). Non-specific binding was defined as that remaining in the presence of 10 μM S(+)-3-isobutyl GABA. Non-specific binding was between 10–15% of total [^{3}H]-gabapentin binding. The final Tween 20 concentration in all assays was 0.017% unless otherwise stated. Separation of bound from free ligand was effected by filtration through 0.3% polyethylenimine-impregnated GF/B filters with 3×4 ml of 50 mM Tris-HCl, pH 7.4 at 4°C. Radioactivity on filters was determined by scintillation counting. [^{3}H]-gabapentin saturation studies were carried out in the presence of 12 concentrations of the radioligand, ranging from 0.5 to 250 nM. Displacement studies were carried out in the presence of 20 nM [^{3}H]-gabapentin unless otherwise stated, with 10 concentrations of the competing ligand.

Column chromatography

The [^{3}H]-gabapentin-binding protein was purified to homogeneity from porcine cerebral cortical membranes as described previously (Gee *et al*., 1996). Briefly, detergent-solubilized protein was purified by sequential chromatography on Q-Sepharose, lentil lectin, Sephacryl S-400, hydroxyapatite, wheatgerm lectin and Mono-Q. Active fractions from each stage of the scheme were pooled and frozen at −70°C. Within three days of the last step of the scheme, the influence of spermine (10^{-4}M to 3 × 10^{-9}M) on specific [^{3}H]-gabapentin binding to samples from each step were determined simultaneously, in binding assays described above.

Dialysis

Dialysis experiments were carried out in seamless dialysis tubing (prepared according to manufacturer's instructions) which retain proteins of a molecular weight of approximately 12,000 and above. Membranes or Tween 20-solubilized membranes (approximately 4.0 ml) were placed in dialysis bags and dialysed at 4°C against 100 volumes of dialysis buffer: buffer C (for membranes) or buffer C containing 0.4% Tween 20 (for solubilized membranes). At each time point (t) of 0, 0.08 (2 h), 1, 2, 3, 4, 6 and 7 days, a dialysis bag was removed and the contents frozen at −70°C. The dialysis buffer of the remaining samples was replaced with fresh buffer at each time point. Within three days of the last time point, all dialysed samples were simultaneously assayed to determine specific [^{3}H]-gabapentin binding and the influence of spermine (10^{-4}M to 3 × 10^{-9}M) on specific [^{3}H]-gabapentin binding. In order to obtain a concentrated solution of dialysate for the add-back experiments, ∼3.0 ml of solubilized membranes were dialysed against 3 volumes of dialysis buffer for 4 days.

Add-back experiments

The appropriate volume of dialysate i.e. 10, 25 or 75 μl was added to 25 μl of dialysed soluble material or purified gabapentin binding protein, and assay buffer to a final volume of 200 μl. The tubes were incubated on ice for 30 min when the remaining components of the binding assay i.e. radioligand ± drug were added to a final volume of 250 μl. The binding assay was then carried out as described above. Parallel controls were run for each set of experimental tubes where a corresponding volume of dialysis buffer replaced dialysate. Differences in results within each group of control and experimental tubes, due to factors such as buffer components was therefore eliminated. The final Tween 20 concentration in the presence of volumes 10, 25 and 75 μl of dialysate/dialysis buffer was 0.03, 0.06 and 0.14%, respectively. There was no appreciable difference in specific [^{3}H]-gabapentin binding between controls containing this range of detergent concentration.

Molecular size determination

After filtration through a 0.2 μm filter, samples of Tween 20-solubilised protein (200 μl) were chromatographed at 0.5 ml min^{-1} on an HR 10/30 Superose 6 gel-filtration fast protein liquid chromatography (f.p.l.c.) column equilibrated in 0.08% Tween 20, 450 mM NaCl, 10 mM Tris/HCl, pH 7.5 at 22°C. Fractions were collected and assayed for [^{3}H]-gabapentin binding. Molecular sizes were determined by reference to a calibration curve constructed with the following standards: ovalbumin, M_r 45,000; aldolase, M_r 158,000; catalase, M_r 232,000; ferritin, M_r 440,000; thyroglobulin, M_r 669,000. Elution volume values were (ml): 16.6, 15.1, 14.9, 13.7 and 11.7 respectively; the void volume was 7.5 ml.

Materials

[^{3}H]-gabapentin (104 Ci mmol^{-1}) (custom synthesized by Cambridge Research Biochemicals, Cambridge, U.K.), [^{3}H]-MK-801 (22 Ci mmol^{-1}; Du Pont Ltd., Stevenage, U.K.), **S**(+)- and **R**(−)-3-isobutyl GABA and gabapentin (Parke Davis & Co., Ann-Arbor, MI, U.S.A.). 1,10-Diaminodecane (DA-10) (Research Biochemicals Incorporated, Natick, MA, U.S.A.). All other chemicals were obtained from Sigma Chemicals Ltd. (Poole, U.K.).

Data analysis and statistical analysis

Saturation data were analysed by use of LIGAND (Munson & Rodbard, 1980). The inhibition data were analysed by ALLFIT (DeLean *et al*., 1978). Curves fitted without constraining any parameters. Differences between means were tested for statistical significance by Student's paired *t* tests (two-sided). $P<0.05$ was taken to be statistically significant.

Results

Influence of spermine, divalent cations and selected compounds on specific [^{3}H]-gabapentin binding to Tween 20-solubilised porcine cerebral cortical membranes

The potencies (IC_{50} values) of selected compounds at the [^{3}H]-gabapentin-binding site in Tween 20-solubilized membranes are shown in Table 1. Spermine, spermidine, DA-10, Mg^{2+} and Zn^{2+} inhibited [^{3}H]-gabapentin binding maximally by 65–75%. All other compounds at appropriate concentrations displaced specific [^{3}H]-gabapentin binding completely.

The influence of spermine on [^{3}H]-gabapentin binding to solubilised membranes was examined and compared with that of **S**(+)-3-isobutyl GABA. Displacement studies were performed with four different [^{3}H]-gabapentin concentrations. Increasing concentrations of radioligand resulted in a rightward shift of **S**(+)-3-isobutyl GABA dose-response curves (Figure 1a). At 6.4, 22, 55 and 108 nM [^{3}H]-gabapentin, IC_{50} values were 87.1, 109, 139 and 246 nM, respectively. Linear regression analysis of **S**(+)-3-isobutyl GABA IC_{50} values vs [^{3}H]-gabapentin concentration showed a close correlation ($r^2=0.97$, $P=0.01$). Similar observations were made in a second experiment ($r^2=0.96$, $P=0.02$). When [^{3}H]-gabapentin was displaced with spermine, such a correlation ($r^2=0.05$, 0.07 and $P=0.78$, 0.92 respectively, $n=2$) was not apparent. In a representative experiment, IC_{50} values were 2.29, 2.38, 2.29 and 2.35 μM, respectively when measured against 6.4, 22, 55 and 108 nM [^{3}H]-gabapentin (Figure 1b). The corresponding maximum percentage inhibition of [^{3}H]-gabapentin binding by spermine was 65, 62, 60 and 57%, respectively.

Table 1 Potencies of selected compounds at the [^{3}H]-gabapentin-binding site in porcine cerebral cortex preparations

	IC_{50} (μM)	
Compound	*Solubilized membranes*	*Purified protein*
Gabapentin	0.07±0.01 (−1.04)	0.05±0.02 (−1.06)
S(+)-3-isobutyl GABA	0.08±0.17 (−0.91)	0.04±0.01 (−1.04)
R(−)-3-isobutyl GABA	0.50±0.91 (−1.06)	0.37±0.05 (−0.76)
L-Leucine	0.10±0.02 (−1.20)	0.08±0.02 (−0.78)
Mg^{2+}	70.2±32.3 (−1.50)	146±8.00 (+1.23)*
Zn^{2+}	80.6±3.10 (−2.70)	6.06±2.63 (−0.85)
Spermine	2.56±0.26 (−1.85)	0.94±0.28 (+1.06)*
Spermidine	48.0±4.40 (−0.86)	ND
DA-10	54.8±6.95 (−1.08)	ND

[^{3}H]-gabapentin (20 nM) was displaced with 10 concentrations (performed in duplicate) of each compound in binding assays described under Methods. The IC_{50} and slope values were determined by ALLFIT analysis. Each IC_{50} value represents the mean±s.e. from 3–10 experiments. Mean slope values are given in parentheses. ND, not determined. *Mg^{2+} and spermine stimulated binding to the purified protein, therefore these two values represent EC_{50} values.

Effect of dialysis on specific [^{3}H]-gabapentin binding to Tween 20-solubilized membranes

Increase in [^{3}H]-gabapentin binding When Tween 20-solubilized membranes were dialysed, [^{3}H]-gabapentin binding to the dialysed material was observed to increase progressively with time reaching a maximum after the third day (Figure 2). When solubilized membranes were frozen at −70°C for different lengths of time (up to 3 months), there was no change in [^{3}H]-gabapentin binding (data not shown). Therefore, it was established that the increase observed in binding after dialysis was not due to a freezing artifact.

When porcine cerebral cortical membranes were washed repeatedly, a comparable rise in [^{3}H]-gabapentin binding was observed. [^{3}H]-gabapentin (20 nM) binding to membranes after each of three washes were 5618 ± 55, 9402 ± 228 and 10488 ± 188 d.p.m. without further change after each of the next two washes ($n=3$). Furthermore, when membranes were dialysed and assayed at t=0, 0.08, 1, 2, 3, 4 and 6 days, [^{3}H]-gabapentin (20 nM) binding increased gradually from 6898 ± 368 (t=0) to 14589 ± 742 d.p.m. (t=2 days) there being no subsequent change ($n=3$).

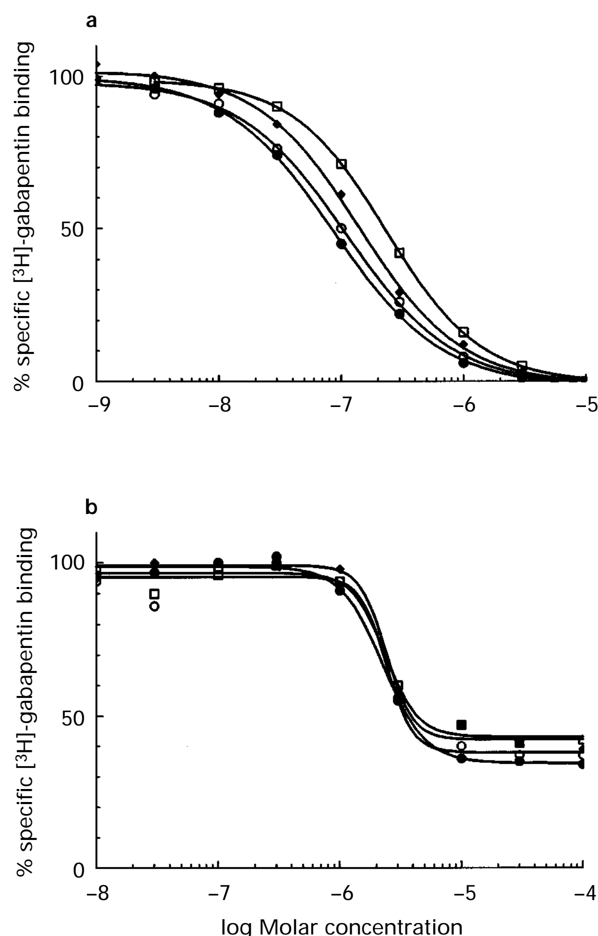

Figure 1 Displacement of [^{3}H]-gabapentin binding to Tween 20-solubilized pig cerebral cortical membranes by (a) **S**(+)-3-isobutyl GABA and (b) spermine with various concentrations of [^{3}H]-gabapentin (● 6.4 nM, ○ 22 nM, ◆ 55 nM, □ 108 nM). Points shown are from a single experiment performed in duplicate. Similar results were obtained in a further experiment.

The nature of the increase in [3H]-gabapentin binding to dialysed solubilized membranes was investigated by saturation analysis (Figure 3). Before dialysis, the K_D and B_{max} of [3H]-gabapentin (n=3) were 67.6 $\pm$ 9.57 nM and 6.97 $\pm$ 0.16 pmol mg^{-1} protein (Hill coefficient 0.97 $\pm$ 0.01), respectively, and following dialysis (t=4 days) the corresponding values were 20.1 $\pm$ 5.06 nM and 6.92 $\pm$ 0.61 pmol mg^{-1} protein (Hill coefficient 1.13 $\pm$ 0.03). The 3.4 fold shift in K_D was significant (P=0.01, Student's paired two-tailed t test).

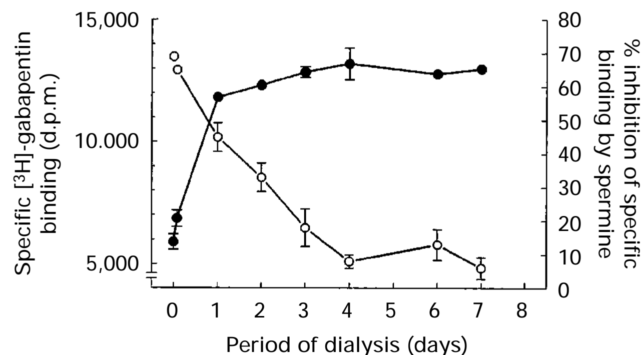

Figure 2 Effect of dialysis on [3H]-gabapentin (20 nM) binding to Tween 20-solubilized membranes. Solubilized membranes were dialysed as described under Methods. Samples were taken at each time point for estimation of [3H]-gabapentin binding (●) and spermine inhibition of [3H]-gabapentin binding as described under Methods. % maximum spermine (10^{-4}M) inhibition of binding at each time point is represented by (○). Results are mean values of 3 separate experiments performed in duplicate, vertical lines show s.e.mean.

Reduction in maximal inhibition of specific [3H]-gabapentin binding by spermine Spermine displaced [3H]-gabapentin binding to dialysed Tween 20-solubilised membranes in a dose-dependent manner. Maximum inhibition of [3H]-gabapentin binding by spermine (10^{-4}M) after different time points of dialysis is shown in Figure 2. Dialysis attenuated spermine inhibition of [3H]-gabapentin binding. Dialysis of solubilized membranes did not result in spermine stimulation of [3H]-gabapentin binding.

Effect of adding back dialysate to dialysed Tween 20-solubilized membranes and to purified [3H]-gabapentin-binding protein

To ascertain if the effects of dialysis could be reversed, dialysate was added back to dialysed solubilised membranes. The influence of dialysate on specific [3H]-gabapentin binding was compared to that of controls (+ buffer) as described in Methods section (Figure 4a). At each volume (10, 25 and 75 μl) of dialysate tested, [3H]-gabapentin binding (compare

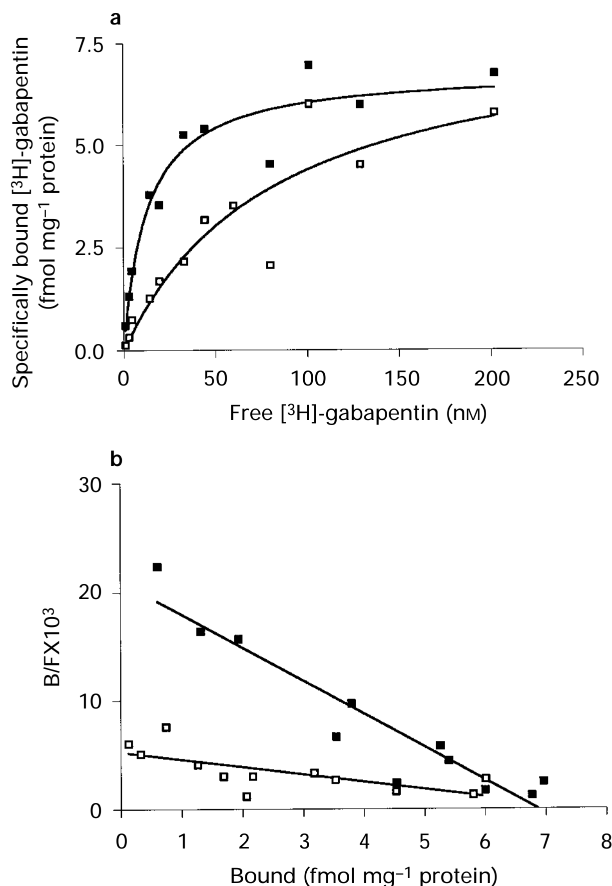

Figure 3 (a) Saturation curves for [3H]-gabapentin binding to Tween 20-solubilized membranes (□) and solubilized membranes dialysed for 4 days (■). (b) Scatchard transformation of the corresponding binding data. Non-specific binding of [3H]-gabapentin was linear over the selected concentration range. Points shown are from a single representative experiment performed in triplicate. Similar results were obtained in two additional experiments. B, bound; F, free.

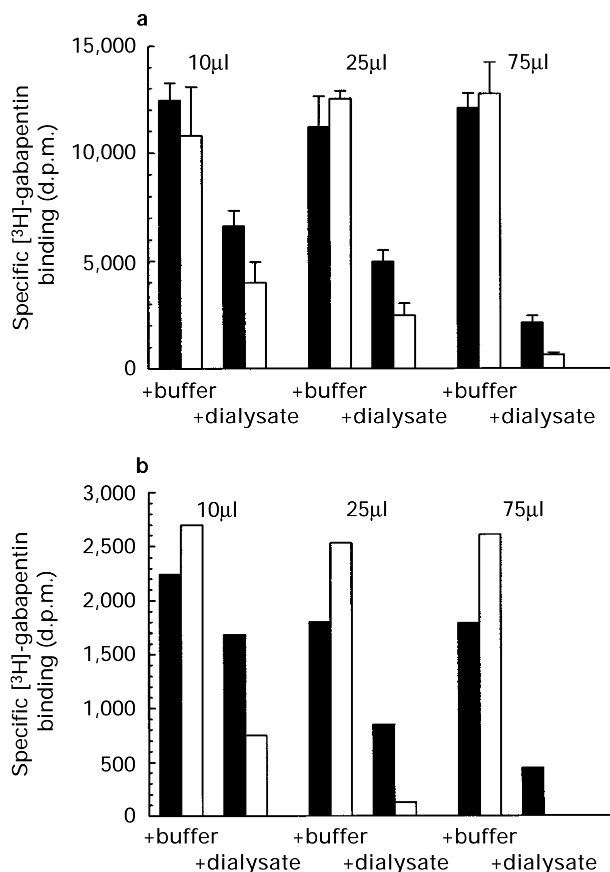

Figure 4 Changes in [3H]-gabapentin (20 nM) binding after adding back dialysate to dialysed Tween 20-solubilized membranes (a) and to purified [3H]-gabapentin-binding protein (b). Add back experiments were carried out as described in the Methods section. Effect of 10, 25 or 75 μl dialysate on specific [3H]-gabapentin binding (solid columns, + dialysate), where control samples had an equivalent volume of dialysis buffer added to them (solid columns, + buffer). Open columns reflect [3H]-gabapentin binding in the presence of spermine (10^{-4}M). Results in (a) are mean values$\pm$s.e.mean of 3 separate experiments while those in (b) represent mean values from 2 separate experiments.

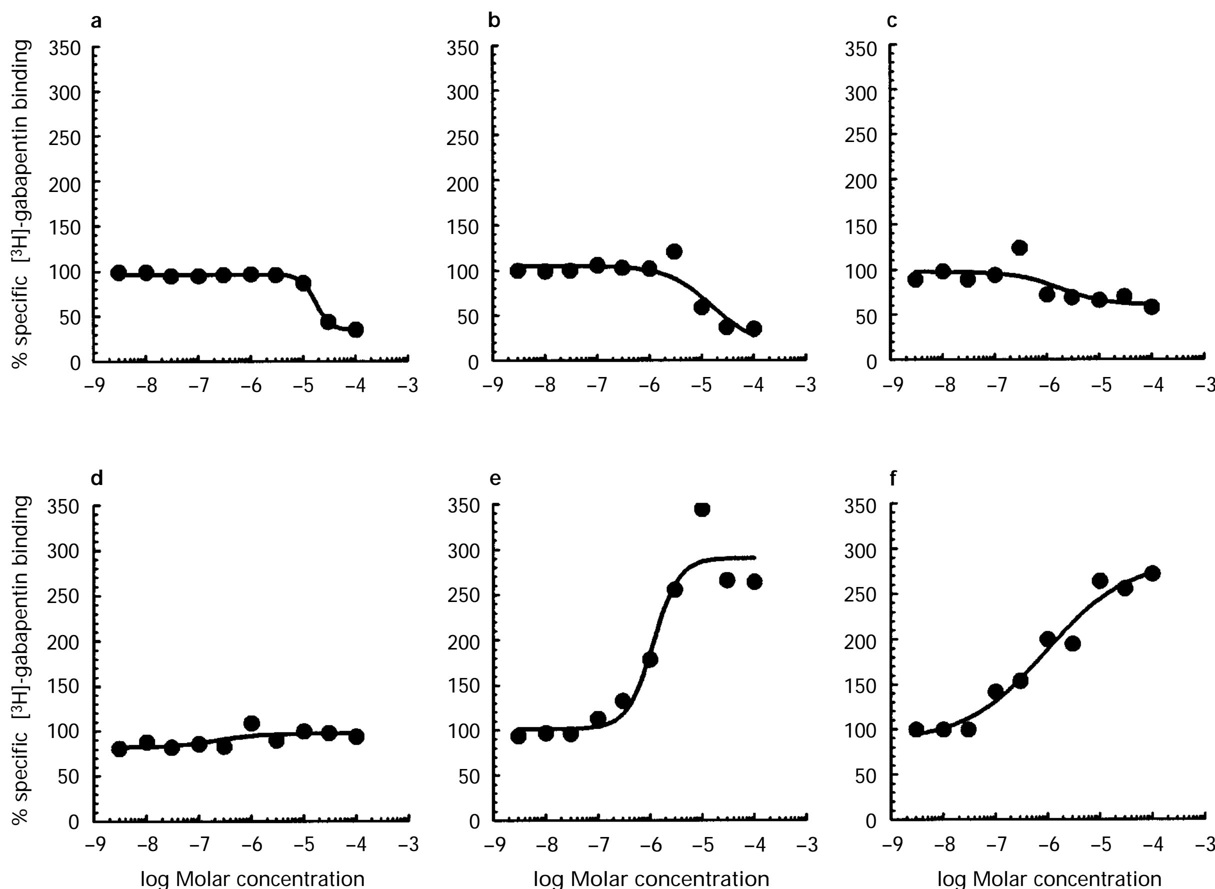

Figure 5 Spermine modulation of [^{3}H]-gabapentin (20 nM) binding to fractions from the [^{3}H]-gabapentin-binding protein purification scheme. (a) Membranes, (b) Tween 20-solubilized membranes, (c) Q-Sepharose, (d) lentil lectin, (e) Sephacryl S-400 and (f) hydroxyapatite columns. Data from wheat-germ lectin and Mono-Q fractions are not shown as they did not differ from (e) or (f). Material from each stage was tested in the binding assay as described in the Methods section. Binding studies on all fractions were performed simultaneously and results presented are from a single representative experiment, done in duplicate. Similar results were obtained in a further experiment.

solid columns) was significantly reduced from the corresponding control ($P=0.02$, 0.002 and 0.002, respectively, Student's paired two-tailed t test, $n=3$).

The influence of spermine (10^{-4}M) on specific [^{3}H]-gabapentin binding to dialysed membranes when dialysate was present was compared to specific binding in control samples (Figure 4a). In control samples, spermine did not affect [^{3}H]-gabapentin binding significantly (compare solid and open columns, $P=0.41$, 0.33 and 0.72, Student's paired two-tailed t test, $n=3$). However, in test samples with dialysate, spermine decreased [^{3}H]-gabapentin binding by 40% (10 μl dialysate), 51% (25 μl) and 70% (75 μl).

When the dialysate was heated to 100°C for 5 min, and then added back to dialysed solubilized membranes, similar results to that described above were obtained (data not shown). When dialysate was added to purified [^{3}H]-gabapentin-binding protein, again specific binding was reduced from the corresponding control (Figure 4b, $n=2$). In control samples, spermine potentiated [^{3}H]-gabapentin binding. However, in test samples with dialysate, spermine reduced [^{3}H]-gabapentin binding by 55% (10 μl dialysate), 85% (25 μl) and 100% (75 μl).

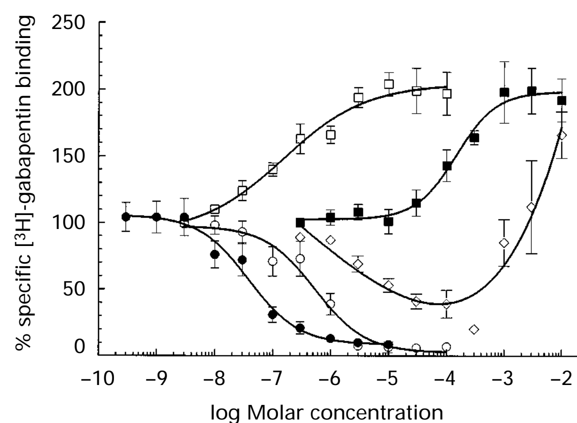

Figure 6 Displacement of [^{3}H]-gabapentin (20 nM) binding to the purified $\alpha_2\delta$ protein by **S**(+)-3-isobutyl GABA (●), **R**(−)-3-isobutyl GABA (○), spermine (□), Mg^{2+} (■) and Zn^{2+} (◇). Points shown represent mean of three separate experiments performed in duplicate; vertical lines show s.e.mean.

Effect of spermine on specific [^{3}H]-gabapentin binding to fractions during $\alpha_2\delta$ purification

Spermine displaced [^{3}H]-gabapentin binding to membranes, solubilized membranes and Q-Sepharose column-eluted material in a dose-dependent manner with a maximal inhibition of 65%, 65% and 33%, respectively (Figure 5). Spermine had a minimal influence on [^{3}H]-gabapentin binding to material eluted from the lentil lectin column (Figure 5). Profiles of spermine curves during early stages of purification resembled those during dialysis in that there was a gradual reduction in maximal inhibition of [^{3}H]-gabapentin binding. However, there was a sharp switch to a dose-dependent stimulation of [^{3}H]-gabapentin binding to material eluted from a Sephacryl S-400 gel-filtration column (Figure 5). Spermine also stimulated [^{3}H]-

gabapentin binding to material eluted from each of the last three columns i.e hydroxyapatite (Figure 5), wheat-germ lectin and Mono Q (data not shown) of the purification scheme. At the higher concentrations of the dose-response curve, spermine maximally stimulated [^{3}H]-gabapentin binding ~3 fold.

Potencies (IC_{50}s) of selected compounds at the [^{3}H]-gabapentin-binding site of the purified $\alpha_2\delta$ subunit are shown in Table 1. Like spermine, Mg^{2+} also stimulated [^{3}H]-gabapentin binding 2-3 fold (Figure 6). Zinc showed an initial inhibition followed by stimulation of binding at higher concentrations (Figure 6). The IC_{50} of the inhibitory phase is given in Table 1. All other compounds listed in the table fully inhibited specific binding. Glycine, glutamate and (+)-5-methyl-10,11-dihydro-5H-di-benzo[a,d]cyclohepten-5,10-imine maleate (MK-801) did not displace [^{3}H]-gabapentin binding up to concentrations of 100 μM (data not shown). Specific [^{3}H]-MK-801 (5.0 nM) binding to the purified gabapentin binding protein preparation could not be detected, under conditions described by Ransom and Stec (1988, data not shown).

Size exclusion chromatography to determine the molecular size/subunit composition of the [^{3}H]-gabapentin-binding membrane protein during purification

As described above, the magnitude and direction of spermine modulation of [^{3}H]-gabapentin binding changed during purification of the protein. We attempted to investigate if these changes were caused by the removal of an endogenous ligand from the binding protein (dialysis studies described before) and/or the removal of one or more subunits (α_1 and β) of the calcium channel complex from the $\alpha_2\delta$ subunit (size exclusion studies). The subunit profile of the [^{3}H]-gabapentin-binding membrane protein at each stage of purification could not be assessed by immunoprecipitation techniques due to the unavailability of antibodies to porcine VSCC subunits. We therefore chose to assess the subunit composition of the protein indirectly by size exclusion chromatography.

Gel-filtration of the purified protein on a Superose 6 column yielded a molecular size for the Tween 20-solubilized [^{3}H]-gabapentin-binding protein of 260 kDa (elution volume 14.8 ml, $n=3$). This is broadly consistent with a species of 170 kDa ($\alpha_2\delta$) binding to a micelle of detergent. However, chromatography of Tween 20-solubilized membrane protein yielded a molecular size of 430 kDa (elution volume 13.8 ml, $n=4$). Gel-filtration of material from each chromatography step showed that the alteration in molecular size from 430 to 260 kDa was not gradual but occurred during Q-Sepharose chromatography (elution volume 14.8 ml, $n=3$).

The purity of fractions, expressed as specific activity of [^{3}H]-gabapentin (20 nM) binding in pmol mg^{-1} and (fold purification), from each stage of a typical scheme were: membranes, 1.55 (1); solubilized membranes, 5.51 (3.6); Q-Sepharose, 12.1 (7.8); lentil lectin, 57.6 (37); Sephacryl S-400, 162 (105); hydroxylapatite, not determined; wheat-germ lectin, 857 (553); Mono-Q, 1584 (1022) (Gee *et al*., 1996).

Discussion

Spermine inhibits [^{3}H]-gabapentin binding to solubilized membranes by interacting with a polyamine-sensitive allosteric site

This study demonstrates that spermine allosterically modulates [^{3}H]-gabapentin binding to solubilized porcine cerebral cortical membranes. Thus, when displacement studies were carried out in the presence of increasing concentrations of [^{3}H]-gabapentin, spermine IC_{50} values were unaffected. However, the maximum inhibition of [^{3}H]-gabapentin binding by spermine was decreased. In contrast, under the same experimental conditions, S(+)-3-isobutyl GABA curves showed a parallel rightward shift. S(+)-3-isobutyl GABA IC_{50} values increased linearly with [^{3}H]-gabapentin concentration, indicating a competitive interaction between the two ligands. Polyamines such as spermidine and DA-10 also dose-dependently inhibited binding, suggesting that the [^{3}H]-gabapentin-binding site is modulated by a polyamine site. Other divalent cations, e.g. Mg^{2+} and Zn^{2+}, which displaced [^{3}H]-gabapentin binding by 65–75% may also act at the polyamine site. Inhibition of [^{3}H]-gabapentin binding by spermine, Mg^{2+} and Zn^{2+}, has a steep concentration-dependence, suggestive of multi-site co-operativity.

Recently the [^{3}H]-gabapentin-binding protein was identified as an $\alpha_2\delta$ subunit of VSCCs (Gee *et al*., 1996). Therefore, we could reason that the modulatory spermine site(s) is located on the [^{3}H]-gabapentin-binding $\alpha_2\delta$ subunit in porcine cortical membranes. Spermine is also known to modulate allosterically [^{3}H]-nitrendipine, [^{3}H]-verapamil and [^{3}H]-diltiazem binding to the α_1 subunit of L-type VSCCs (Schoemaker, 1992) and to affect [^{125}I]-ω-conotoxin binding and function at N-type VSCCs (Pullan *et al*., 1990). It is not yet known whether these allosteric spermine sites are located on the α_1 subunit of the calcium channel complex or whether they are located on another subunit (eg. $\alpha_2\delta$) and exert their influence indirectly on radiolabelled ligand binding to the α_1 subunit.

Dialysis removes an endogenous ligand which influences [^{3}H]-gabapentin binding competitively

Dialysis of Tween 20-solubilized membranes resulted in a 2.5 fold increase in specific [^{3}H]-gabapentin binding suggesting that a small inhibitory molecule, less than 12,000 Da, was removed during dialysis. When the dialysate was added back, there was a dose-related reduction in specific [^{3}H]-gabapentin binding to dialysed solubilized membranes. The results suggest therefore that [^{3}H]-gabapentin binding to the protein is modulated by an endogenous molecule. The endogenous component is resistant to boiling, therefore we speculate that it is a small peptide, amino acid, biogenic amine or ion.

Saturation studies demonstrating a 3.4 fold increase in the affinity of [^{3}H]-gabapentin following dialysis provides convincing evidence that the endogenous ligand acts competitively at the [^{3}H]-gabapentin-binding site. Large neutral amino acids have high affinity for the [^{3}H-gabapentin-binding site (Thurlow *et al*., 1993) and it is not inconceivable that one or several of these interact with the gabapentin binding site *in vivo*. The nature of this endogenous ligand is currently under investigation.

A greater than two fold increase in [^{3}H]-gabapentin binding was also observed after repeated washing or dialysis of membranes. The increase in binding is therefore most likely due to the removal of a molecule that is normally associated with the [^{3}H]-gabapentin-binding protein, and not due to the removal of a factor which artificially associates with the binding protein following Tween 20-solubilization. However, we cannot exclude the possibility that the endogenous component becomes associated with the [^{3}H]-gabapentin-binding protein when cells are disrupted during tissue homogenization. Detailed investigation of the effects of spermine on [^{3}H]-gabapentin binding was carried out with detergent-solubilized membranes because changes in the influence of spermine were observed during purification, after the binding protein had been solubilized from membranes.

Endogenous modulators isolated from brain tissue, although incompletely characterized, have been shown to inhibit [^{3}H]-nitrendipine binding to the α_1 subunit of voltage-sensitive L-type calcium channels (Ebersole *et al*., 1988; Janis *et al*., 1988; Ebersole & Molinoff, 1992) and to block L- and T- type Ca^{2+} currents in neuronal tissue (Callewaert *et al*., 1989). It is interesting therefore that binding of [^{3}H]-gabapentin to the $\alpha_2\delta$ subunit of VSCCs is also modulated by an endogenous molecule. It is probable that the function of VSCCs may be physiologically regulated by endogenous molecules that interact with one or more subunits of the calcium channel complex.

Dialysis also removes a second putative endogenous ligand which influences spermine modulation of [^{3}H]-gabapentin binding

Although [^{3}H]-gabapentin binding to solubilized membranes increased after dialysis, maximal inhibition by spermine decreased. However, the two changes did not parallel one another. For example, at t = 2 days, [^{3}H]-gabapentin binding had almost reached its peak, whereas maximal spermine inhibition was still decreasing (Figure 2). The two changes, therefore, are most likely independent of one another. As adding back dialysate reversed the effects of spermine following dialysis, we could speculate that there is an additional dialysable, heat-stable low molecular weight endogenous factor which influences spermine modulation of [^{3}H]-gabapentin binding.

The attenuation of maximum inhibition of [^{3}H]-gabapentin binding by spermine during initial steps of purification is similar to that observed during dialysis. Therefore the loss of this second putative endogenous molecule may sufficiently explain the gradual loss of spermine inhibition of [^{3}H]-gabapentin binding during initial steps of purification. The gradual loss of VSCC subunits from [^{3}H]-gabapentin-binding $\alpha_2\delta$ during purification could not be responsible for a reduction in spermine inhibition of [^{3}H]-gabapentin binding, as the loss of associated proteins (perhaps α_1 and β subunits) occurred during a single step (see below).

Spermine stimulation of [^{3}H]-gabapentin binding during purification is not due to removal of other VSCC subunits from $\alpha_2\delta$

Spermine stimulation of [^{3}H]-gabapentin binding to Sephacryl S-400 purified material could not be explained by the removal of an endogenous modulator, as prolonged dialysis did not cause stimulation of binding. We attempted to investigate if spermine stimulation was caused by the removal of one or more calcium channel subunits from $\alpha_2\delta$. The molecular size (430 kDa) of the Tween 20-solubilized protein is broadly consistent with the existence a complex of α_1 (170 kDa), $\alpha_2\delta$ (170 kDa) and β (60 kDa) subunits and a Tween 20 micelle (70 kDa). The size of the purified [^{3}H]-gabapentin protein is 260 kDa which is consistent with there being an $\alpha_2\delta$ subunit and a Tween 20 micelle. It would appear therefore that column chromatography disrupted the calcium channel complex resulting in the loss of perhaps α_1 and β subunits. The removal of these subunits was not gradual but occurred sharply during Q-Sepharose chromatography, there being no size change after Sephacryl S-400 gel-filtration. Based on these data, it is unlikely that spermine stimulation of [^{3}H]-gabapentin binding is linked to the dissociation of a known subunit of the calcium channel complex from $\alpha_2\delta$. Furthermore, a marked change in purity was also not observed after gel-filtration purification. At present, therefore, we are unable to explain the change in the response of $\alpha_2\delta$ to spermine after gel-filtration chromatography. Conceivably the change in spermine effects is associated with the loss of a hitherto unknown small polypeptide or factor. The size change that occurred during Q-Sepharose chromatography does not appear to be accompanied by a significant change in the influence of spermine on [^{3}H]-gabapentin binding to the protein.

The $\alpha_2\delta$ subunit of VSCCs carries a distinct spermine modulatory site

Spermine stimulated [^{3}H]-gabapentin binding to the purified protein demonstrating that the $\alpha_2\delta$ subunit of VSCCs carries a distinct spermine modulatory site. Such a site has not been identified before. Are both spermine effects observed in this study i.e. inhibition of [^{3}H]-gabapentin binding to detergent-solubilized membranes and potentiation of [^{3}H]-gabapentin binding to the purified protein, due to a spermine site(s) on the $\alpha_2\delta$ subunit? The observation that spermine-induced stimulation of [^{3}H]-gabapentin binding to the purified protein was reversed after adding back dialysate would most likely mean that both effects are due to a spermine site(s) present on the $\alpha_2\delta$ subunit. We are unable to conclude from the present data whether the effects are due to separate sites on the subunit.

NMDA receptor ligands are ineffective at the [^{3}H]-gabapentin-binding site

In vivo behavioural studies have implied the possible involvement of the NMDA/glycine complex in the anticonvulsant action of gabapentin (Oles *et al.*, 1990; Singh *et al.*, 1996). The inactivity of glycine, glutamate and MK-801 at the [^{3}H]-gabapentin-binding site of the purified protein, and the absence of specific [^{3}H]-MK-801 binding to the purified protein demonstrates that the [^{3}H]-gabapentin-binding site is not a component of the NMDA complex. The identification of the [^{3}H]-gabapentin-binding protein as the $\alpha_2\delta$ subunit of a calcium channel further confirms this finding. The mechanism of D-serine reversal of the anticonvulsant action of gabapentin (Oles *et al.*, 1990; Singh *et al.*, 1996) is therefore as yet unexplained.

Conclusions

Spermine inhibited [^{3}H]-gabapentin binding to Tween 20-solubilized porcine cortical membranes by interacting with a polyamine-sensitive allosteric site. The steep slope of spermine inhibition curves suggests multi-site co-operativity. Dialysis of both membranes and detergent-solubilized membranes resulted in an increase in [^{3}H]-gabapentin binding. The increase in binding was due to the removal of a heat stable, low molecular weight endogenous molecule which influences [^{3}H]-gabapentin binding competitively. Dialysis also reduced maximal spermine inhibition of [^{3}H]-gabapentin binding. The rates of increase in [^{3}H]-gabapentin binding and reduction of spermine inhibition of [^{3}H]-gabapentin binding were different. We speculate therefore that a second endogenous molecule was removed after dialysis. The loss of this second putative molecule may explain the reduction in maximum inhibition of [^{3}H]-gabapentin binding by spermine during the initial purification of $\alpha_2\delta$.

Following gel-filtration purification of the [^{3}H]-gabapentin-binding protein, spermine stimulated rather than inhibited [^{3}H]-gabapentin binding. This phenomenon is not directly due to loss of a dialysable endogenous factor. There was no apparent change in molecular size of the [^{3}H]-gabapentin-binding protein after gel-filtration chromatography. Therefore the switch in the effects of spermine does not appear to be due to dissociation of one or more other known subunits of the VSCC complex from $\alpha_2\delta$. The fact that spermine stimulated [^{3}H]-gabapentin binding to the purified protein indicates that the $\alpha_2\delta$ subunit of VSCCs bears a distinct spermine modulatory site. Spermine-induced stimulation of [^{3}H]-gabapentin binding to the purified protein was reversed after adding back dialysate implying that both inhibition and stimulation by spermine are due to one or more spermine sites present on the $\alpha_2\delta$ subunit of VSCCs.

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