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Inhibition of neuronal Ca^{2+} influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices

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1 Cytosolic calcium ion concentrations ($[Ca^{2+}]_i$) were measured in rat neocortical synaptosomes using fura-2, and depolarization of synaptosomal membranes was induced by K^+ (30 mM). The release of the endogenous excitatory amino acids glutamate and aspartate was evoked by K^+ (50 mM) and determined by HPLC. The release of [^{3}H]-noradrenaline from rat neocortical synaptosomes or slices was evoked by K^+ (15 and 25 mM) and measured by liquid scintillation counting.

2 Gabapentin produced a concentration-dependent inhibition of the K^+-induced $[Ca^{2+}]_i$ increase in synaptosomes ($IC_{50} = 14$ μM; maximal inhibition by 36%). The inhibitory effect of gabapentin was abolished in the presence of the P/Q-type Ca^{2+} channel blocker ω-agatoxin IVA, but not by the N-type Ca^{2+} channel antagonist ω-conotoxin GVIA.

3 Gabapentin (100 μM) decreased the K^+-evoked release of endogenous aspartate and glutamate in neocortical slices by 16 and 18%, respectively.

4 Gabapentin reduced the K^+-evoked [^{3}H]-noradrenaline release in neocortical slices ($IC_{50} = 48$ μM; maximal inhibition of 46%) but not from synaptosomes.

5 In the presence of the AMPA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro[f]quinoxaline-7-sulphonamide (NBQX), gabapentin did not reduce [^{3}H]-noradrenaline release. Gabapentin did, however, cause inhibition in the presence of the NMDA receptor antagonist DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 37849).

6 Gabapentin is concluded to reduce the depolarization-induced $[Ca^{2+}]_i$ increase in excitatory amino acid nerve terminals by inhibiting P/Q-type Ca^{2+} channels; this decreased Ca^{2+} influx subsequently attenuates K^+-evoked excitatory amino acid release. The latter effect leads to a reduced activation of AMPA receptors which contribute to K^+-evoked noradrenaline release from noradrenergic varicosities, resulting in an indirect inhibition of noradrenaline release.
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Keywords: Neocortex; synaptosomes; noradrenaline; glutamate; voltage-gated Ca^{2+} channels; ω-agatoxin IVA; ω-conotoxin GVIA; NMDA receptor; AMPA receptor; rat

Abbreviations: CGP 37849, DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EAA, excitatory amino acids; GBP, gabapentin; HVA, high-voltage activated; NA, noradrenaline; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro[f]quinoxaline-7-sulphonamide; PSS, physiological salt solution; VGCC, voltage-gated Ca^{2+} channels

Introduction

Gabapentin (GBP) is a clinically effective anticonvulsant drug originally launched as add-on therapy but more recently used as monotherapy for medically refractory patients with partial or secondary generalized seizures (Leiderman, 1994; Feely, 1999). This drug has also been used for treatment of other CNS disorders such as neuropathic pain (Caraceni *et al*., 1999) and social phobia (Pande *et al*., 1999).

The mechanism of action of GBP, however, remains unclear. Previous *in vitro* studies from our group revealed that GBP decreases monoamine release from the rat neocortex but not acetylcholine release from the rabbit caudate nucleus (Schlicker *et al*., 1985). Although the inhibitory effect was robust, GBP did not appear to interact with presynaptic receptors or neurotransmitter transporters. Since intracellular Ca^{2+} accumulation is important for spreading of epileptic discharges (Siesjö & Bengtsson, 1989; Tymianski & Tator, 1996), an effect of GBP on voltage-gated Ca^{2+} channels (VGCC) is possible. GBP is known to bind to the $\alpha_2\delta$ subunit of VGCC (Gee *et al*., 1996), yet the functional significance of this interaction is controversial. One report indicated that GBP inhibited VGCC in dissociated adult rat neocortical pyramidal cells (Stefani *et al*., 1998), and another found that this drug reduced EPSPs and postsynaptic sensitivity to glutamate in rat striatal neurons (Calabresi *et al*., 1999). Other groups, however, have obtained negative results with GBP using different types of cultured rodent neurons (Rock *et al*., 1993) or acutely dissociated human hippocampal granule cells (Schumacher *et al*., 1998).

In contrast to the above investigations, the effects of GBP on presynaptic VGCC have never been studied. The influx of Ca^{2+} through presynaptic VGCC is an important prerequisite to trigger neurotransmitter release from axon terminal varicosities (e.g., Nachshen, 1985; Suszkiw *et al*., 1989; Tareilus *et al*., 1993). Since axon terminal preparations such as synaptosomes are too small for an electrophysiological

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analysis, the synaptosomal cytosolic Ca^{2+} concentrations can only be monitored by using fluorescent ion specific probes such as fura-2. This approach allows a pharmacological characterization of presynaptic VGCC in mammalian neocortical synaptosomes (e.g. Blaustein & Goldring, 1975; Tareilus *et al*., 1993; Meder *et al*., 1997; 1999). The modulation of presynaptic Ca^{2+} influx alters exocytotic neurotransmitter release resulting postsynaptically in increased or decreased neuronal excitability. The purpose of the present study was to provide functional evidence for a modulation of presynaptic Ca^{2+} influx by GBP and subsequent changes in excitatory amino acid (EAA) and catecholamine release. Preliminary reports of this work have been presented previously (Fink *et al*., 1998; Dooley *et al*., 1999).

Methods

Synaptosomal $[Ca^{2+}]_i$ measurements

Neocortical synaptosomes were prepared as described previously from the neocortex of male Wistar rats (200–300 g) (Meder *et al*., 1997). Briefly, the neocortex was taken after sacrifice and maintained until further preparation for up to 60 min in cold (4°C) Krebs-Henseleit buffer (composition mM): NaCl 118, KCl 4.8, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 1.3, $MgSO_4$ 1.2, glucose 11, ascorbic acid 0.06, disodium EDTA 0.03; equilibrated with 95% O_2 and 5% CO_2. The neocortex was homogenized with a Potter-Elvehjem glass homogenizer (800 r.p.m., 6 strokes/2 min) in 40 vol (w v^{-1}) of 0.32 M sucrose. The homogenate was centrifuged (10 min, 1000 $\times g$ at 4°C) to remove nuclei and debris, and the supernatant was then centrifuged at 12,000 $\times g$ for 10 min. The buffy layer of pelleted synaptosomes was resuspended by gentle agitation in physiological but Ca^{2+}-free salt solution (PSS) composition (mM): NaCl 133, KCl 4.8, HEPES 10, Na_2HPO_4 1.2, $MgSO_4$ 1.2, glucose 10; pH adjusted to 7.4 with NaOH. The synaptosomal suspension (2 ml; ~3 mg of protein ml^{-1}) was incubated with fura-2/AM (5 μM) for 40 min at 37°C with gentle shaking. The fura-2-loaded synaptosomes were centrifuged at 1300 $\times g$ and the pellet was washed once with Ca^{2+}-free PSS and centrifuged again. The pellet was resuspended and stored on ice until use. Aliquots (200 μl) of the washed synaptosomal suspension were diluted with 1.8 ml PSS (final protein concentration 125 ± 4 $\mu g\ ml^{-1}$), containing 1.3 mM $CaCl_2$, placed in a quartz curvette at 37°C and preincubated for 6 min. The synaptosomes were kept in suspension with a magnetic stirrer. K^+ 30 mM 20 μl was added from the 360th second of incubation onward. Substances under investigation were present in the buffer from the beginning of the incubation until the end of the experiment. Fluorescence ($\lambda_{ex} = 340/380$ nm; $\lambda_{em} = 510$ nm) was measured with a spectrofluorometer (Perkin Elmer LS 50 B) and cytosolic calcium concentrations ($[Ca^{2+}]_i$) calculated according to Grynkiewicz *et al*. (1985).

Endogenous aspartate and glutamate release from neocortical slices

Neocortical slices (0.4 mm thick, 5 mm diameter) were prepared and incubated for 30 min at 22°C in Krebs-Henseleit buffer routinely containing the glutamate uptake inhibitor L-*anti-endo*-3,4-methano-pyrrolidinedicarboxylic acid (100 μM). Within one experiment slices of the same origin were used for control or GBP exposure. The slices were washed, and two slices (24 mg wet weight) were placed in each well of a sterile 24-well plate along with 1 ml of prewarmed buffer. The plate was placed in a 22°C shaking water bath with a continuous O_2 (100%) supply. After 15 min, the buffer was removed and 500 μl of buffer containing GBP (100 μM) was added to half of the slices and buffer without GBP was added to the control slices. The plate was shaken for 7.5 min after which all buffer was removed and replaced with 500 μl of fresh buffer with or without GBP. After an additional 7.5 min of shaking, 200 μl of buffer was removed (i.e., basal fraction) and placed into microcentrifuge tubes; 200 μl of stimulation buffer (final concentration of 50 mM K^+) with or without GBP was added and the plate shaken for a final 7.5 min. A 200 μl aliquot was removed from each well (i.e., stimulated fraction) and placed into tubes. Slices were then removed and weighed. Samples (viz., basal and stimulated fractions) and aspartate and glutamate standards were prepared for HPLC analysis by *o*-phthaldialdehyde derivatization. The results were converted to concentrations (pmol aspartate and glutamate mg^{-1} slice wet weight), and the values for net aspartate and glutamate release (stimulated minus basal release) were determined for both control and GBP treatment.

[^{3}H]-Noradrenaline release from neocortical slices and synaptosomes

Neocortical slices (0.3 mm thick, 3 mm diameter) were prepared and incubated for 30 min with 50 nM [^{3}H]-noradrenaline (specific activity 42.0, 46.8 or 54.3 Ci $mmol^{-1}$). Neocortical synaptosomes were prepared by homogenizing the tissue in 0.32 M sucrose, centrifuging at 1000 $\times g$ for 10 min, and incubating 9 ml of the supernatant with 6 ml of Krebs-Henseleit buffer for 7 min at 37°C. After addition of [^{3}H]-noradrenaline, the incubation was continued for another 7 min. The labelled synaptosomes were pelleted at 600 $\times g$ for 10 min and resuspended in 2.25 ml ice-cold Krebs buffer (final protein content: 4656 ± 155 $\mu g\ ml^{-1}$). Slices or synaptosomal suspension aliquots were layered on Whatman GF/C or GF/B filters in chambers and superfused at 0.6 ml min^{-1} with Krebs-Henseleit buffer. Tritium overflow was evoked by a K^+ stimulus (15 or 25 mM; all K^+ concentrations applied in this study caused submaximal stimulation). The superfusate was continuously collected in 4 min (synaptosomes) or 5 min (slices) fractions and the tritium content determined by liquid scintillation counting. Tritium efflux was calculated as the fraction of tritium content in the slice or synaptosomes at the beginning of the respective collection period. Basal tritium efflux was assumed to decline linearly during fraction collection. Stimulation-evoked tritium overflow was calculated by subtracting basal efflux from total overflow.

Analysis of data

IC_{50} values and 95% confidence intervals were calculated from a sigmoidal logistic equation fitted to the concentration-response data by iterative nonlinear regression analysis using Prism 2.01 for PC (GraphPad Software Inc., San Diego, U.S.A.). Results are given as means ± s.e.mean of *n* experiments. For comparison of mean values, Student's *t*-test (unpaired or, it specified, paired) was used. In case of multiple comparisons, 1-way or 2-way ANOVA was applied, followed by Dunnett's *post-hoc* test. The minimal level of significance was $P \leqslant 0.05$ (2-tail criterion).

Materials

Substances and drugs included fura-2/AM (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy) ethane-N,N,N′,N′-tetraacetic acid pentaacetoxy methyl ester), veratridine free base (Sigma, Deisenhofen, Germany); ω-agatoxin IVA (Research Biochemicals; Natick, MA, U.S.A.); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), L-*anti-endo*-3,4-methano-pyrrolidinedicarboxylic acid (Tocris Cookson; Bristol, U.K.); DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 37849) (Novartis; Basel, Switzerland); 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro[f]quinoxaline-7-sulphonamide (NBQX), gabapentin (Parke-Davis, Ann Arbor, MI, U.S.A.); and (−)-[ring-2,5,6-^{3}H]-noradrenaline (NEN; Dreieich, Germany). Stock solutions of compounds were prepared with deionized water with the exceptions of L-*anti-endo*-3,4-methano-pyrrolidinedicarboxylic acid (solubilized in 1 N NaOH), and fura-2/AM, CNQX, and NBQX (dissolved in DMSO). The final DMSO concentration did not exceed 0.1%, and was without effect in our experiments.

Results

Effect of GBP on basal $[Ca^{2+}]_i$ and K^+-induced $[Ca^{2+}]_i$ increase in neocortical synaptosomes

The basal $[Ca^{2+}]_i$ in neocortical synaptosomes, under control conditions as measured after 360 s, was 297±19 nM ($n=14$). Basal $[Ca^{2+}]_i$ was not affected by the presence of GBP at the concentrations investigated in this study (results not shown). GBP caused a concentration-dependent inhibition of the K^+-induced $[Ca^{2+}]_i$ increase ($IC_{50}=14$ μM, $CI_{95}=0.4-434$ μM; maximal calculated inhibition 36%; Figure 1).

Effect of GBP in combination with ω-conotoxin GVIA and ω-agatoxin IVA on K^+-induced $[Ca^{2+}]_i$ increase in neocortical synaptosomes

In the presence of the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (0.1 μM; Kerr & Yoshikami, 1982), which by itself inhibited K^+-induced $[Ca^{2+}]_i$ increase by 26% ($P<0.001$; data not shown), GBP still inhibited this variable by further 17% ($P<0.05$; Figure 2). However, in the presence of the selective P/Q type Ca^{2+} channel blocker ω-agatoxin IVA (0.2 μM; Mintz *et al*., 1992), which by itself inhibited K^+-induced $[Ca^{2+}]_i$ increase by 45% ($P<0.0001$; data not shown), no additional inhibition by GBP (300 μM) was observed (Figure 2).

Effect of GBP on K^+-evoked endogenous aspartate and glutamate release from neocortical slices

GBP did not alter basal aspartate or glutamate release (data not shown). GBP (100 μM) significantly decreased K^+ (50 mM)-evoked aspartate and glutamate release by 20% each (Figure 3). The control values (pmol mg^{-1} wet weight) for glutamate were 10±1 (basal release) and 111±21 (stimulated

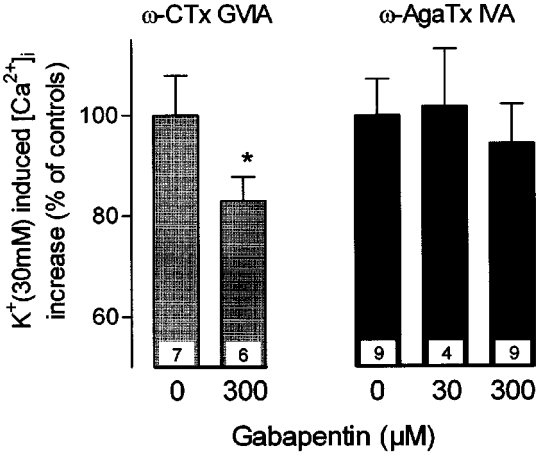

Figure 2 Effect of GBP in combination with ω-conotoxin GVIA (ω-CTx GVIA; 0.1 μM) and ω-agatoxin IVA (ω-AgaTx IVA; 0.2 μM) on K^+(30 mM)-induced $[Ca^{2+}]_i$ increase in fura-2-loaded rat neocortical synaptosomes. For depolarization K^+ concentration in the PSS was elevated by 30 mM. The effects on the K^+-induced $[Ca^{2+}]_i$ increase are presented as percentage of control in the presence of ω-CTx GVIA or ω-AgaTx IVA but not gabapentin. Means±s.e.mean of n experiments in duplicate; n is indicated at the bottom of each bar; $^*P<0.05$, compared to the corresponding controls without gabapentin.

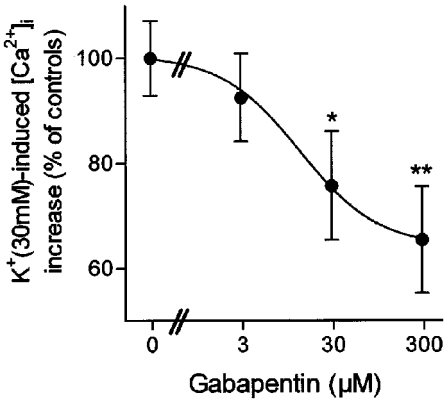

Figure 1 Inhibition by gabapentin of the K^+-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2-loaded rat neocortical synaptosomes. For depolarization K^+ concentration in the PSS was elevated by 30 mM. The effects on the K^+-induced $[Ca^{2+}]_i$ increase, determined 10 s after exposure to the K^+ stimulus, are presented as percentage of control. Means±s.e.mean of 4–14 experiments in duplicate; $^*P<0.05$ or $^{**}P<0.01$ compared to corresponding controls without gabapentin.

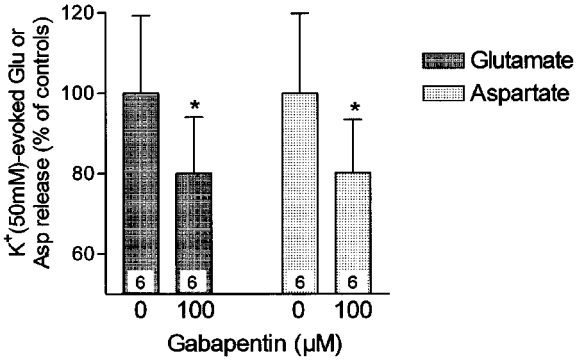

Figure 3 Effect of GBP on K^+ (50 mM)-evoked endogenous aspartate and glutamate release from rat neocortical slices. Release of either amino acid varied substantially between the animals but the inhibition by GBP was consistently found in tissue preparations from each individual animal. Means±s.e.mean of n experiments in quadruplicate; n is indicated at the bottom of each bar; $^*P<0.05$ (paired t-test), compared to the corresponding controls without GBP. Adequate pairing was tested by calculating the Pearson correlation coefficient r; r=0.9775, $P<0.0004$ for glutamate release and r=0.9736, $P<0.0005$ for aspartate release.

minus basal release) and, for aspartate these values were 8 ± 1 and 41 ± 8, respectively.

Effect of GBP on K^+-evoked [^{3}H]-noradrenaline release from neocortical slices and synaptomes

GBP produced a concentration-dependent inhibition of K^+ (25 mM)-evoked [^{3}H]-noradrenaline release from neocortical slices ($IC_{50} = 48$ μM, $CI_{95} = 20 - 114$ μM; maximal calculated inhibition 46%), but was inactive on K^+ (15 mM)-evoked release of [^{3}H]-noradrenaline from neocortical synaptosomes (Figure 4).

Effect of GBP in combination with glutamate receptor antagonists on K^+-evoked [^{3}H]-noradrenaline release from neocortical slices

The AMPA receptor antagonist NBQX produced a concentration-dependent inhibition of K^+ (25 mM)-evoked [^{3}H]-noradrenaline release from neocortical slices ($IC_{50} = 5$ μM, $CI_{95} = 3.9 - 11$ μM; Figure 5B), whereas the competitive NMDA receptor antagonist CGP37849 did not affect this release (Figure 6A). In the presence of NBQX (30 μM), which by itself caused an inhibition by 25%, GBP did not cause further significant decrease (Figure 5C). Similarly, in the presence of the less selective AMPA receptor antagonist CNQX (10 μM) which by itself inhibited release by 21% GBP did not inhibit [^{3}H]-noradrenaline release (Figure 5A). GBP inhibited [^{3}H]-noradrenaline release in the presence of 100 μM CGP37849 ($IC_{50} = 53$ μM, $CI_{95} = 20 - 163$ μM; Figure 6B) and 300 μM CGP37849 ($IC_{50} = 59$ μM, $CI_{95} = 17 - 270$ μM; Figure 6B). GBP did not inhibit [^{3}H]-noradrenaline release in the presence of CNQX (30 μM) plus CGP37849 (300 μM) (Figure 6C), which by themselves produced an inhibition by 25%, presumably due to the effect of this CNQX concentration.

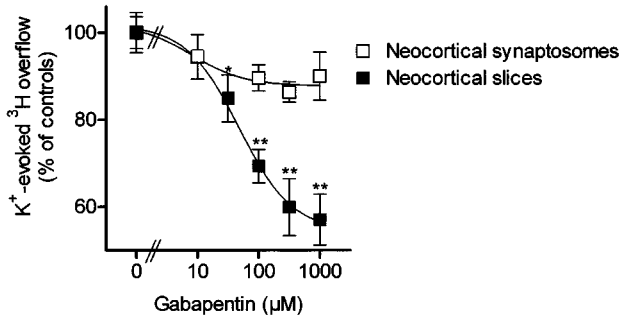

Figure 4 Effect of GBP on K^+-evoked [^{3}H]-noradrenaline release from rat neocortical slices (25 mM K^+) and synaptosomes (15 mM K^+). The effects on tritium overflow are presented as percentage of control without gabapentin. In controls the K^+-evoked [^{3}H]-noradrenaline release in slices amounted to 11.23 ± 0.52% of tissue tritium, corresponding to 3.80 ± 0.25 nCi and from synaptosomes to 6.38 ± 0.23% of synaptosomal tritium, corresponding to 1.29 ± 0.12 nCi. Means $\pm$ s.e.mean of 8–11 experiments (slices) and 9–11 experiments in quadruplicate (synaptosomes); $^*P < 0.05$, $^{**}P < 0.01$, compared to the corresponding controls without gabapentin.

Discussion

The antiepileptic drug GBP has been reported to reduce stimulation-evoked catecholamine release in the rat neocortex (Schlicker *et al*., 1985; Helmer & Dooley, 1998; Pugsley *et al*., 1998). Although GBP has structural similarities to the neurotransmitter γ-aminobutyric acid, the effects are apparently not directly mediated by GABA receptors (Reimann, 1983; Schlicker *et al*., 1985). There is also uncertainty as to whether there is any relationship between modulation of catecholamine release by the drug and its antiepileptic efficacy. The binding of [^{3}H]-GBP to rat brain membranes *in vitro* has been suggested to reflect binding to the VGCC $\alpha_2\delta$ subunit (Gee *et al*., 1996); this binding occurs at a relatively high affinity ($K_D = 38$ nM; Suman-Chauhan *et al*., 1993). The present study was intended to provide functional evidence for GBP effects on VGCC and signal-transmission processes upstream of catecholamine release.

We have shown here that GBP, effecting a concentration-dependent inhibition of K^+-induced $[Ca^{2+}]_i$ increase, was ineffective to inhibit this $[Ca^{2+}]_i$ increase in the presence of ω-agatoxin IVA. ω-Agatoxin IVA (0.2 μM) given alone caused an inhibition of 45% in rat neocortical synaptosomes, a finding consistent with previous reports (Hillyard *et al*., 1992; Tareilus *et al*., 1992; Luebke *et al*., 1993; Meder *et al*., 1997). In the presence of ω-conotoxin GVIA (0.1 μM; Meder *et al*., 1997),

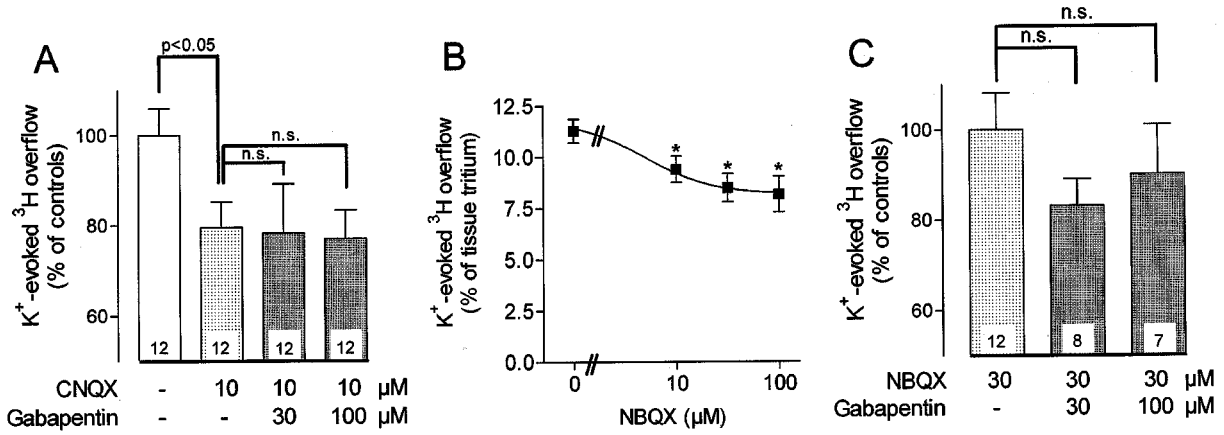

Figure 5 Effect of GBP in combination with CNQX (A) or NBQX (C), or effect of NBQX alone (B) on K^+ (25 mM)-evoked [^{3}H]-noradrenaline release from rat neocortical slices. The effects on tritium overflow are presented as percentage of control in the absence of CNQX and gabapentin (A), or absence of NBQX (B), or absence or gabapentin (C). In controls the K^+-evoked [^{3}H]-noradrenaline release in slices amounted to 10.5 ± 0.88% of tissue tritium, corresponding to 4.28 ± 0.44 nCi. Means $\pm$ s.e.mean of n experiments; n is indicated at the bottom of each bar (A, C), $n = 4 - 12$ (B); $^*P < 0.01$ compared to corresponding controls in the absence of NBQX.

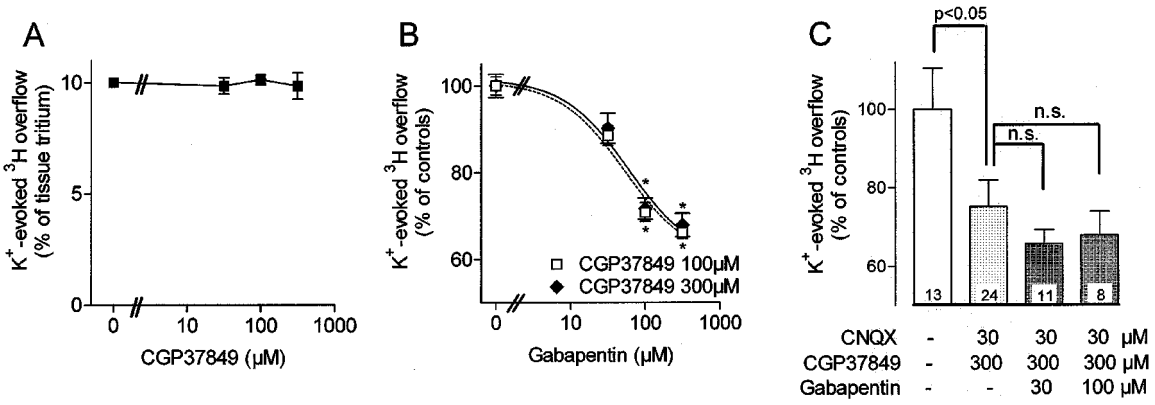

Figure 6 Effect of CGP37849 (A), of GBP in the presence of CGP37849 (B), and of GBP in the presence of CNQX (30 μM) plus CGP37849 (300 μM) (C) on K^+(25 mM)-evoked [^{3}H]-noradrenaline release from rat neocortical slices. The effects on tritium overflow are presented as percentage of control in the absence of drugs. In controls the K^+-evoked [^{3}H]-noradrenaline release in slices amounted to $7.49 \pm 0.78\%$ of tissue tritium, corresponding to 2.14 ± 0.24 nCi. Means $\pm$ s.e.mean of n experiments; $n = 5-32$ and 5–22 (A and B, respectively); n is indicated at the bottom of each bar (C); $^*P < 0.05$ compared to corresponding controls in the absence of drugs.

which also produced an inhibitory effect if applied alone, GBP still inhibited the K^+-induced $[Ca^{2+}]_i$ increase. This result suggests that the inhibition effects of GBP on Ca^{2+} influx are mainly mediated by P/Q-type rather than N-type VGCC. If binding of GBP to the $\alpha_2\delta$ subunit of VGCC (Gee *et al*., 1996) alters channel function, it should have similar effects across VGCC subtypes because the $\alpha_2\delta$ subunit appears to be expressed and assembled in all VGCC (Hofmann *et al*., 1994; Isom *et al*., 1994). Since this was not the case, other factors appear to be involved such as different splice variants of the $\alpha_2\delta$ subunit (Klugbauer *et al*., 1999) which could lead to different binding affinities for, and functional responses to, GBP at different VGCC (Gee *et al*., 1996; Wang *et al*., 1999). Alternatively, binding of GBP to the $\alpha_2\delta$ subunit may not have any functional consequences. Finally, GBP has minimal if any effect on Ca^{2+} release from intracellular Ca^{2+} stores in synaptosomes since presynaptic Ca^{2+} signals are mainly generated by Ca^{2+} influx from the extracellular medium (Okada *et al*., 1989; Mulkey & Zucker, 1991).

Electrophysiological reports on the effects of GBP appear to be contradictory. GBP did not affect high-voltage activated (HVA) Ca^{2+} currents recorded from cultured rodent neurons (Rock *et al*., 1993), but inhibited Ca^{2+} currents in pyramidal neocortical cells dissociated from adult rat brain (Stefani *et al*., 1998); this latter finding is in agreement with our data. In another study, GBP did not alter HVA Ca^{2+} currents recorded from acutely dissociated hippocampal granule cells from patients undergoing selective amygdalahippocampectomy for otherwise intractable epilepsy (Schumacher *et al*., 1998). Although all these studies used different experimental paradigms, HVA Ca^{2+} currents from patch-clamped neurons represent Ca^{2+} flux through somadendritically located L- and N-type VGCC, whereas we have studied presynaptic N- and P/Q-type VGCC. Hippocampal granule cells from epileptic patients (Schumacher *et al*., 1998) may have been altered by seizures. These different findings are compatible with each other if P/Q-type VGCC are also involved in Ca^{2+} currents recorded from pyramidal neocortical cells as used by Stefani *et al*. (1998).

Since neocortical synaptosomal preparations, as used for the cytosolic Ca^{2+} measurements, contain mostly glutamatergic (in addition to GABAergic) terminals (Nieuwenhuys, 1994), we determined if GBP would also inhibit K^+-evoked EAA release as a functional correlate of the decreased Ca^{2+} influx. In fact, the release of both glutamate and aspartate, another EAA, was inhibited 20% by GBP (100 μM). This suggests that GBP inhibited Ca^{2+} influx into glutamatergic terminals by acting on P/Q-type VGCC which are predominant on these terminals (Figure 7; Luebke *et al*., 1993; Burke *et al*., 1993). The inhibition of EAA release conceivably leads to reduced postsynaptic excitability, providing one reasonable explanation for the antiepileptic efficacy of the drug.

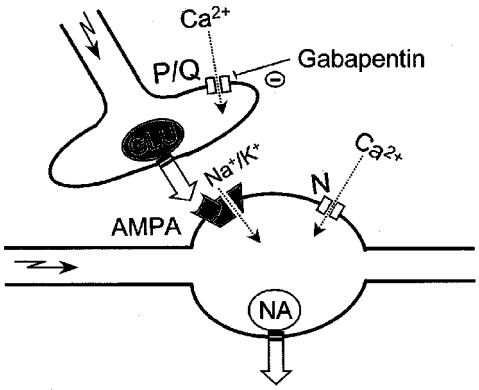

Figure 7 Proposed mechanism of action of GBP. GBP reduces Ca^{2+} influx into glutamatergic terminals, which is mainly mediated by P/Q-type voltage-gated Ca^{2+} channels, thus inhibiting glutamate (GLU) release. Indirectly it reduces noradrenaline (NA) release stimulated by AMPA receptors on noradrenergic varicosity chains.

Glutamate-evoked noradrenaline release is well established in both rat and human neocortical slices and synaptosomes (Fink *et al*., 1989; 1992; Pittaluga & Raiteri, 1992); in the presence of Mg^{2+}, this effect is mediated mainly by AMPA/kainate receptors. We report here that GBP inhibits noradrenaline release in slice preparations but not from synaptosomes, i.e. isolated terminals which are predominantly endowed with N- rather than P/Q- or L-type VGCCs (Figure 7; Feuerstein *et al*., 1990; Gaur *et al*., 1994; Sabria *et al*., 1995). The discrepancy between the GBP effect in slices and synaptosomes is compatible with the hypothesis that in slices glutamate (released in response to K^+ depolarization) stimulates noradrenaline release, an effect which is reduced by GBP due to its inhibitory effect on glutamate release; in superfused synaptosomes such a mechanism cannot be involved because any endogenous neurotransmitter released

(in this case glutamate) is flushed away so rapidly by the superfusion stream that its concentration in the biophase of a receptor on a neighbouring synaptosome is not sufficient to induce a stimulation (Starke *et al*., 1989). If this hypothesis is true, blockade of the glutamate receptors involved in slices should interrupt the signal cascade necessary for unmasking an inhibitory effect of GBP, thus mimicking the lack of effect in synaptosomes. In fact, this hypothesized signal transmission cascade in which stimulation of AMPA receptors on noradrenergic axon terminals by endogenous glutamate is a crucial component, was confirmed by the use of NMDA and/or AMPA receptor antagonists in slice experiments. CGP 37849, a competitive NMDA receptor antagonist, even at concentrations as high as 300 μM (K_i = 220 nM to inhibit L-[^{3}H]-glutamate binding to rat brain membranes; Fagg *et al*., 1990) neither altered K^+-evoked noradrenaline release nor the inhibition of this release by GBP. In contrast, the AMPA receptor antagonists CNQX and NBQX inhibited K^+-evoked noradrenaline release at concentrations consistent with other reports (Jin, 1997), and abolished the inhibitory effect of GBP.

Taken together, it may be concluded that in intact tissue the following chain of events provides an explanation for the inhibitory effects of GBP on the various parameters (Figure 7): GBP inhibits Ca^{2+} influx *via* P/Q-type VGCC which decreases EAA release with subsequent reduction of noradrenaline release, due to attenuated activation of AMPA receptors. The effective concentrations (e.g., IC_{50} values) of GBP which inhibited K^+-induced $[Ca^{2+}]_i$ increase, K^+-evoked EAA release, and K^+-evoked catecholamine release are in the range of the therapeutically relevant plasma concentrations of 10–100 μM (Vollmer *et al*., 1986; Goa & Sorkin, 1993; D. Welty, personal communication). The reduced cytosolic Ca^{2+} concentrations and decreased EAA release may be assumed to attenuate neuronal excitability, and thereby contribute to or underlie the anticonvulsant activity and other therapeutic effects of GBP.

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