Long-term regulation of voltage-gated Ca^{2+} channels by gabapentin

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Abstract Gabapentin (GBP) is a γ-aminobutyric acid analog effective in the treatment of seizures. A high-affinity interaction between GBP and the $\alpha_2\delta$ subunit of the voltage-gated Ca^{2+} channels has been documented. In this report, we examined the effects of the chronic treatment with GBP on neuronal recombinant P/Q-type Ca^{2+} channels expressed in *Xenopus* oocytes. GBP did not affect significantly the amplitude or the voltage dependence of the currents. Exposure to the drug did, however, slow down the kinetics of inactivation in a dose-dependent fashion. In addition, biochemical analysis showed that the integrity of Ca^{2+} channel complex is not apparently affected by GBP binding, suggesting that chronic treatment with the drug might cause the channel kinetic modification through subtle conformational changes of the protein complex.

Key words: Ca^{2+} channel; Gabapentin; Epilepsy; $Ca_V2.1$ subunit; $\alpha_2\delta$ subunit; ω-Conotoxin

1. Introduction

The influx of Ca^{2+} ions through voltage-gated Ca^{2+} channels contributes to neuronal excitability and is an important feature of epileptogenesis. Ca^{2+} channels contribute significantly to the depolarization shift seen in focal epilepsy and are also implicated in the increase of excitability during the transition to seizures and in the spread of seizure activity [1]. In addition, recent genetic studies have linked Ca^{2+} channel subunits to several animal seizure models and human neurological disorders [2,3]. Ca^{2+} channels are heteromultimers, consisting at least of a main α_1 subunit and auxiliary $\alpha_2\delta$, β and γ subunits. The α_1 subunit is a transmembrane protein that forms the ion-conducting pore and the auxiliary β subunit is an intracellular protein that is thought to play important roles in channel regulation [4]. The auxiliary $\alpha_2\delta$ and γ subunits are transmembrane proteins also involved in the regulation of the basic biophysical properties and surface expression of Ca^{2+} channel [5].

Gabapentin (GBP) is a synthetic drug that has shown anticonvulsant activity in a variety of animal seizure models and is also effective in the treatment of human partial and generalized tonic-clonic seizures [1,6,7]; however, the anticonvulsant mechanism of GBP is not known. Despite its structural relationship to the γ-aminobutyric acid (GABA), GBP does not possess a high affinity for GABA receptors [8], and does not affect the uptake or the degradation of this inhibitory neurotransmitter [6,7]. On the other hand, GBP has been shown to be the substrate for a specific binding site in the brain [8], and is capable of modifying the electrophysiological properties of central neurons in culture after prolonged but not brief exposure at concentrations used in clinical trials [9]. A high-affinity GBP-binding protein purified from brain membranes has been characterized, and identified to be the $\alpha_2\delta$ subunit of the Ca^{2+} channel by N-terminal sequencing [10]. Likewise, transient transfection of the $\alpha_2\delta$ cDNA in COS-7 cells significantly increased GBP binding, consistent with the expression of the $\alpha_2\delta$ protein as measured by Western blotting [10].

In this report we examined the consequences of the chronic treatment with GBP on the plasma membrane Ca^{2+} conductances and on the integrity of the Ca^{2+} channel complex. Our results show an alteration in neuronal P/Q-type Ca^{2+} channel activity after chronic treatment with GBP presumably caused by a direct effect of the drug on the molecular channel configuration.

2. Materials and methods

2.1. Materials and preparation of oocytes

ω-Conotoxin GVIA, ω-Conotoxin MVIIC (Peptides International Inc., Louisville, KY, USA) and GBP [1-(aminomethyl) cyclohexane acetic acid, Neurontin®, Parke-Davis Pharmaceuticals Ltd.] were prepared as stock in distilled water and aliquots were store at −20° C. All other chemicals were of reagent grade. *Xenopus laevis* oocytes were prepared as described previously [14].

2.2. Heterologous expression of Ca^{2+} channel subunits

cDNA clones used were as follows: rabbit brain $Ca_V2.1$ ($\alpha_1$2.1; formerly α_{1A}) [11], rat brain $Ca_V\beta_3$ [12], rat brain $Ca_V\alpha_2\delta$-1 [13]. As expression vectors, pcDNA3 (Invitrogen, Carlsbad, CA, USA) was used for $Ca_V\alpha_2\delta$-1, pSP72 (Promega, Madison, WI, USA) for $Ca_V2.1$, and pGEM3 (Promega) for $Ca_V\beta_3$. Linearization of those plasmids, in vitro transcription, cRNA purification, and injection of cRNA were performed as described previously [14]. Injected oocytes were incubated at 18°C for 4 days with or without GBP before electrophysiological recording.

2.3. Electrophysiological recordings and data analyses

Ba^{2+} currents through Ca^{2+} channels were recorded by the two-electrode voltage-clamp (TEV) technique [15] as described previously [14]. Briefly, both voltage and current electrodes were filled with 3 M KCl and had initial tip resistances of 0.5–1.0 MΩ. The recording chamber was filled with recording solution containing 10 $Ba^{2+}(OH)_2$ mM. Test potentials were applied for 500 ms from a holding potential of −90 mV and the results were analyzed by pClamp 6 (Axon Instruments) and SigmaPlot 4.01 (SPSS Inc., Chicago, IL, USA).

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2.4. Partial purification of native Ca^{2+} channels and GBP treatment

Ca^{2+} channel complex was partially purified from rabbit skeletal muscle and brain as described previously [14]. Briefly, from 50 mg of microsomes, the Ca^{2+} channel complexes were extracted from microsomes with solubilization buffer containing 1% digitonin (Biochemica and Synthetica, Switzerland). Out of the solubilized proteins, Ca^{2+} channel complexes were then enriched through wheat germ agglutinin–agarose affinity chromatography (Vector Laboratories, Burlingame, CA, USA). The purified channel complexes were then incubated with or without 1.0 mM GBP at 4°C overnight in a solution containing (in mM) 50 Tris–HCl, pH 7.4, 200 NaCl, a mixture of protease inhibitors, and 0.1% Tween-20 (Fisher Scientific). Similar experiments were also performed with different incubation conditions such as various concentration of GBP, 0.1% digitonin instead of 0.1% Tween-20, and room temperature for 1 h instead of 4°C overnight.

2.5. Antibodies and Western blot analysis

Antibodies, IIID5E1, Guinea Pig 1, Sheep 6, and Guinea Pig 16, specific for the Ca^{2+} channel subunits, $\alpha_1$1.1, α_2, β_{1a}, and γ_1, respectively, have been described previously [16–19]. Western blot analysis was performed as described previously [14].

2.6. Statistical analysis

Each experiment was repeated at least three times using different animals. Data are presented as mean ± S.E.M. and the number of oocytes is indicated in figures and text. Probability (P) of 0.05 or less was considered significant.

3. Results

3.1. Acute treatment with GBP does not affect Ca^{2+} channel activity

GBP exhibits high-affinity binding activity to the $\alpha_2\delta$ subunit of Ca^{2+} channels; however, the link between GBP binding and channel function remains to be clarified. Though some functional studies have shown a modest inhibitory effect of GBP on neuronal Ca^{2+} currents in mammalian cultured cells, most of previous data are based on native currents where the expression of different isoforms of $\alpha_2\delta$ has produced inconsistent results. We therefore decided to further investigate the actions of GBP on neuronal recombinant Ca^{2+} channels expressed in *Xenopus* oocytes using the TEV technique. We first analyzed the effects of the acute application of GBP on the recombinant P/Q-type currents expressed in oocytes after cRNA injection of Ca^{2+} channel subunits ($\alpha_1$2.1/β_3/$\alpha_2\delta$-1). Fig. 1A shows normalized peak Ba^{2+} current (I_{Ba}) amplitude levels of two oocytes in response to a depolarizing step to 0 mV from a holding potential of −90 mV plotted against time. The effects of GBP were then tested on I_{Ba} elicited by the test potential. As can be seen, GBP at a concentration of 30 μM had no effect on the peak amplitude or in the kinetics of the expressed current (Fig. 1B). Peak current amplitude of the $\alpha_1$2.1/β_3/$\alpha_2\delta$-1 channels was −1952 ± 497 nA (n = 8) and −1908 ± 276 nA (n = 9) with or without GBP treatment, respectively. To ensure that the negative result with GBP was not due to a lack of access of GBP to its binding site, distinct Ca^{2+} channel blockers were added to the recording chamber. Under these circumstances, current amplitude was again unaffected by the N-type Ca^{2+} channel antagonist ω-Ctx GVIA. In contrast, I_{Ba} was significantly reduced by the specific P/Q-type Ca^{2+} channel blocker ω-Ctx MVIIC and by a non-specific blocker ($CdCl_2$) confirming that the recombinant P/Q-type expressed in our system were not sensitive to the acute effects of GBP (Fig. 1A). Lastly, the properties of the current–voltage relation of the I_{Ba} elicited by voltage steps from −40 to +40 mV with a holding potential of −90 mV (not shown) in the presence of GBP were very similar to those in the absence of the drug ($V_{1/2}$ of −12.8 and −13.6 mV, respectively).

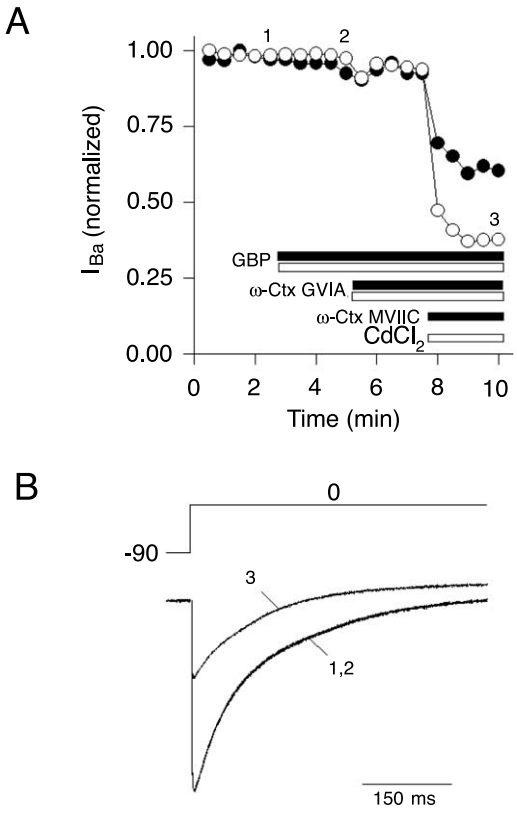

Fig. 1. P/Q-type current in oocytes is insensitive to the acute effects of GBP. A: Representative time courses of block by 200 μM $CdCl_2$ and 1 μM ω-Ctx MVIIC of the P/Q-type ($\alpha_1$2.1/β_3/$\alpha_2\delta$-1) currents expressed in two individual oocytes (open and filled circles). Currents were elicited by stepping from a holding potential of −90 mV to a test potential of 0 mV and normalized to the peak amplitude. Note that in both cases currents were insensitive to the acute application of ω-Ctx GVIA (1 μM) and GBP (30 μM). B: Representative I_{Ba} traces before (1), during application of GBP (2) and after addition of 200 μM of $CdCl_2$ (3) to the external recording solution.

3.2. Chronic effect of GBP on Ca^{2+} channels

Having shown that the acute treatment with GBP does not affect the activity of recombinant Ca^{2+} channels, we next examined the potential effects of the chronic exposure to GBP on the fundamental biophysical properties of channels expressed in oocytes. Treatment with 30 μM GBP for 4 days did not seem to have a major effect on peak current amplitude at all voltages tested (not shown); however, it had a significant impact on the waveform. As shown in Fig. 2, I_{Ba} recorded from oocytes expressing P/Q-type Ca^{2+} channels showed considerable time-dependent inactivation following the peak. To examine a potential effect of GBP treatment on Ca^{2+} channel inactivation, the test pulses to −10 and 0 mV eliciting maximum currents were selected to compare the kinetics of inactivation (Fig. 2A). For every trace, I_{Ba} was normalized to the peak amplitude and the decay was further analyzed by single exponential functions. The results of this analysis showed that in control cells the current during 500 ms pulses decayed with a time constant (τ) of 200 ± 12 and 190 ± 12 ms at −10 and 0 mV, respectively. As shown in

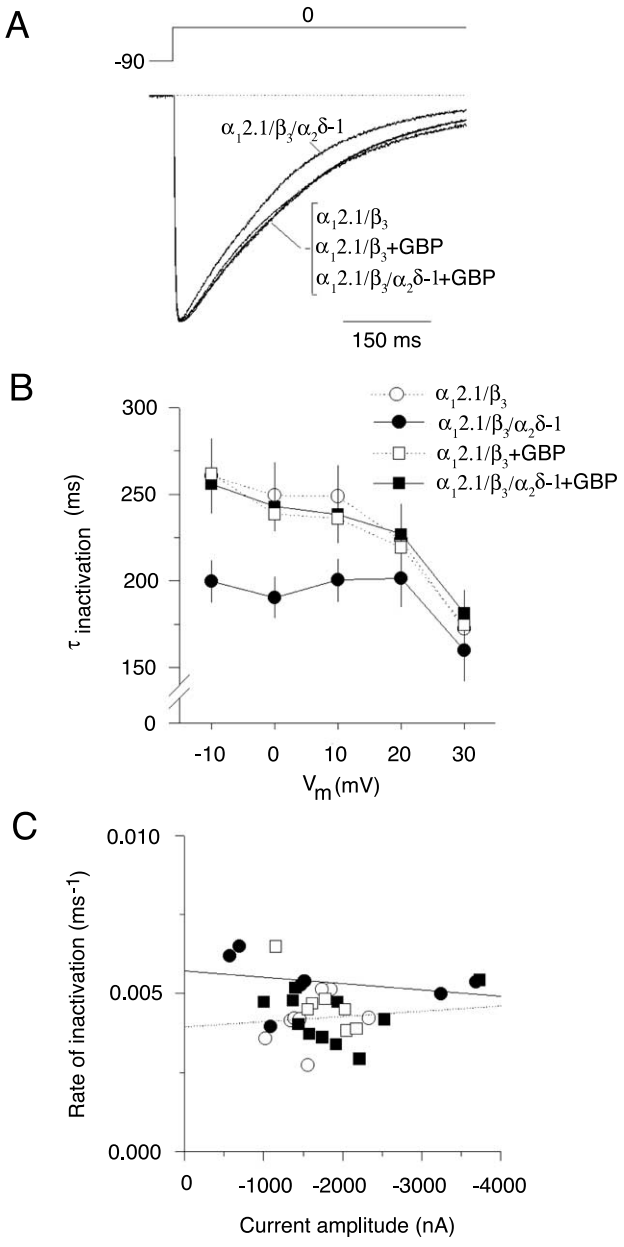

Fig. 2. Evidence for regulation of P/Q-type Ca^{2+} channels current in oocytes after chronic treatment with GBP. A: Representative traces of I_{Ba} through recombinant P/Q-type channels recorded in the presence and absence of GBP at 0 mV and scaled to the same amplitude. B: A single exponential was used to fit I_{Ba} decay to generate the relationship between the time constant, τ, of inactivation and the test potential. The curve was obtained by applying 500 ms voltage pulses in the range of from −10 to +30 mV. C: Comparison of the rates of inactivation ($1/\tau$) plotted with respect to peak current at 0 mV in oocytes expressing different Ca_V subunit composition: ○ $\alpha_1 2.1/\beta_3$; ● $\alpha_1 2.1/\beta_3/\alpha_2\delta$; □ $\alpha_1 2.1/\beta_3$+GBP; ■ $\alpha_1 2.1/\beta_3/\alpha_2\delta$+GBP.

Fig. 2B, current inactivation in GBP treated cells was significantly slower at all voltages tested (e.g. $\tau = 256 \pm 13$ and 249 ± 14 ms at −10 and 0 mV). Notably, the time course of the currents recorded in cells expressing recombinant channels lacking $\alpha_2\delta$ was not affected by GBP treatment, suggesting that $\alpha_2\delta$ is essential for the effect of the drug on channel inactivation (Fig. 2A,B). In addition, one of the concerns when analyzing macroscopic currents in the oocyte system is that the density of the expressed channels may alter the kinetics of the currents. Fig. 2C summarizes the results obtained in a number of control and GBP treated oocytes from different batches, and expressing a different Ca^{2+} channel subunit composition. At 0 mV, I_{Ba} amplitudes had a six-fold range (−571 to −3730 nA); however, these cells with large differences in channel expression had similar rates of inactivation ($1/\tau$), suggesting that at least over this range of current, the rate of inactivation is independent of the channel density.

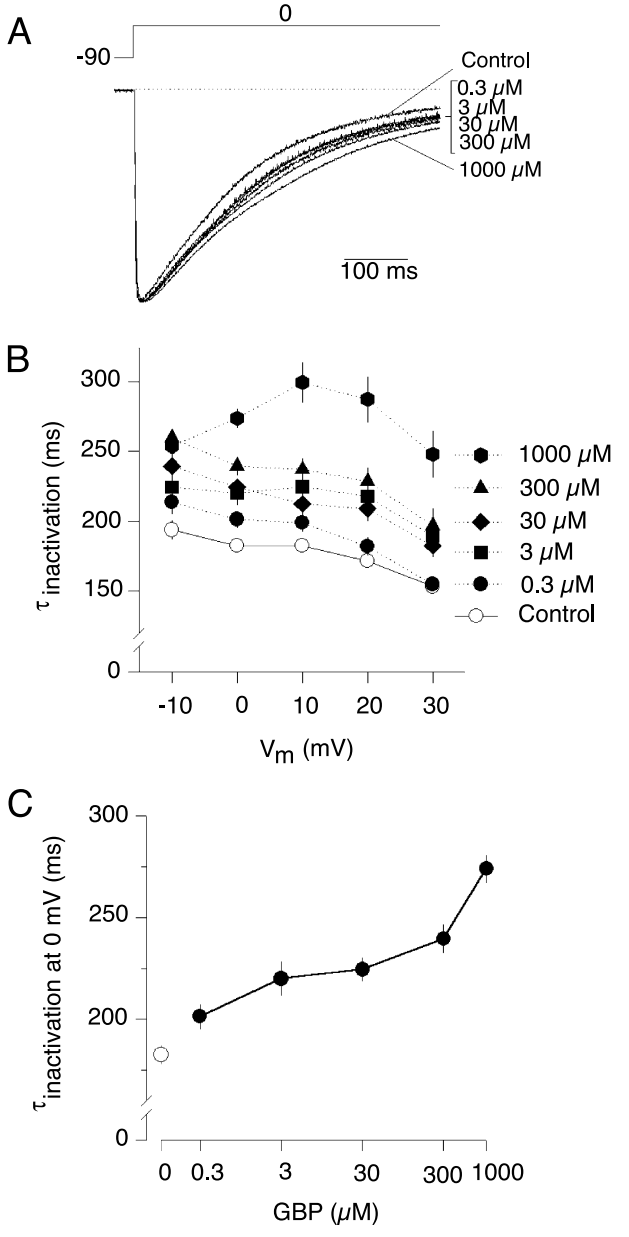

Fig. 3. The decrease in current inactivation by GBP was dose dependent. A: Representative traces of I_{Ba} through recombinant P/Q-type Ca^{2+} channels ($\alpha_1 2.1/\beta_3/\alpha_2\delta$-1) recorded at 0 mV and scaled to the same amplitude. Oocytes were treated for 4 days with GBP as listed. B: Averaged voltage dependence of I_{Ba} inactivation kinetics in cells chronically treated with different concentrations of GBP. Time constants were determined by fitting I_{Ba} traces sampled in the range of −10 to +30 mV and then plotted as function of test potentials. C: Concentration/response curve for the slowing of I_{Ba} inactivation by GBP (mean ± S.E.M., $n = 6$–9). The current was evoked by a voltage pulse from the holding potential of −90 to 0 mV.

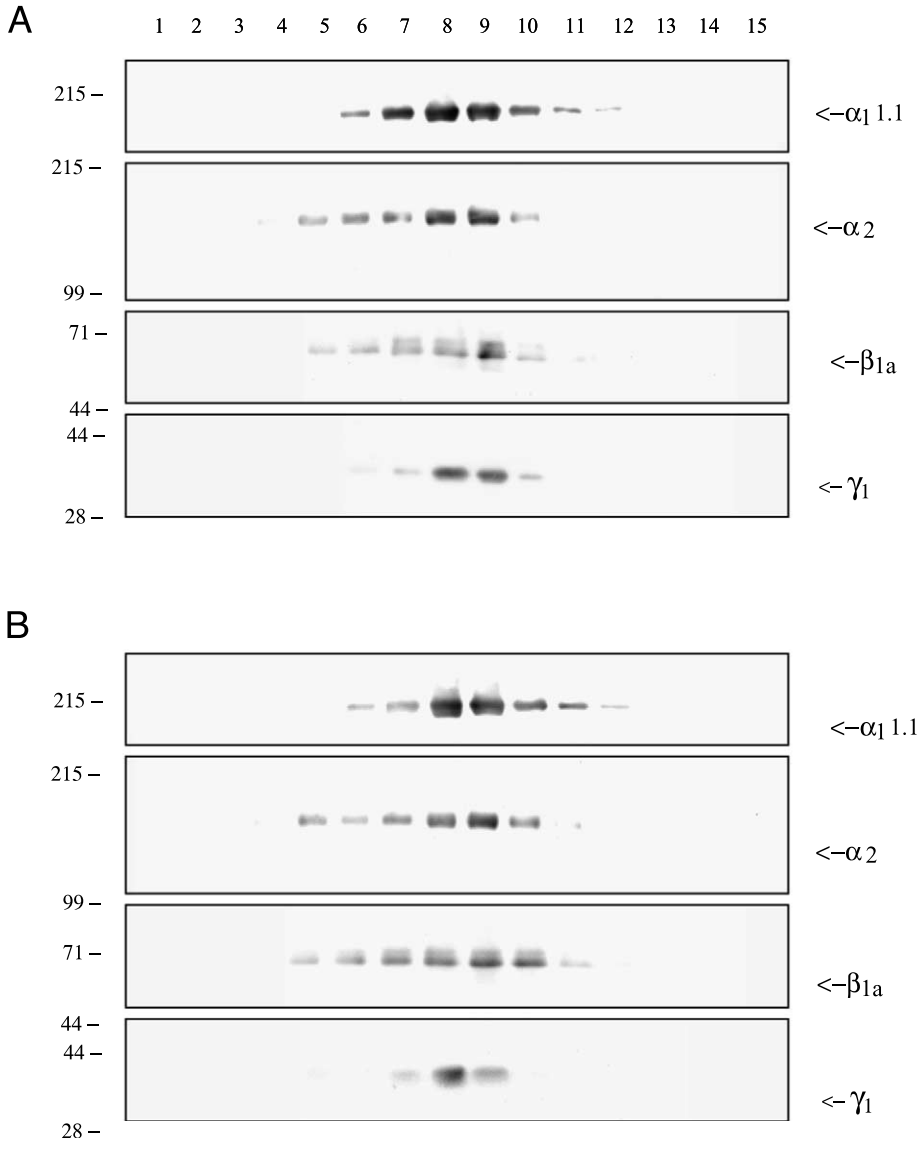

Fig. 4. GBP treatment does not alter the integrity of the Ca^{2+} channel complex. A,B: Sucrose gradient fractionation of partially purified skeletal Ca^{2+} channels with (B) or without (A) 1 mM GBP treatment. Equal volume of proteins (100 μl) were loaded in each lane. After transfer of gels into membranes, the blots were cut and probed separately with affinity-purified antibodies against the subunits of the Ca^{2+} channel. The numbers at the top indicate the fraction of the sucrose gradient from top to bottom. The identity of each band is indicated at the right side of panels. Molecular mass standards ($\times 10^{-3}$) are indicated on the left.

The graphs on Fig. 3 show the effect of different concentrations of GBP (300 nM to 1 mM) on the time constant of current inactivation. In Fig. 3A, representative normalized current traces to determine the time course of inactivation are compared at the potential of 0 mV, and the values obtained for the inactivation time constants in different cells are plotted versus membrane voltage in Fig. 3B. The τ for I_{Ba} in control cells was in the range of ~160–200 ms and showed no apparent voltage dependence in the −10–30 mV range. Notably, a decrease of the decay kinetics of the current seemed to be an important effect of the drug treatment, as reflected in the concentration/response curve in Fig. 3C, which shows how the time constant for the inactivating component of I_{Ba} at 0 mV increased as a function of increasing GBP concentrations from a value of 201 ± 6 ms in the cells treated with 300 nM to 274 ± 7 ms in the cells exposed to 1 mM of the drug (Fig. 3C).

3.3. GBP seems not to affect the integrity of the Ca^{2+} channel complex

Since the inactivation kinetics of GBP treated cells closely resembled that of cells expressing channels lacking $\alpha_2\delta$, we hypothesized that GBP binding could cause an alteration in the integrity of the Ca^{2+} channel complex. To test this hypothesis, we investigated the potential dissociation of subunits including $\alpha_2\delta$ from the channel complex through partial purification, GBP treatment (1.0 mM), and sucrose gradient fractionation. Western blot analysis of sucrose gradient fractions with anti-$\alpha_1$1.1, α_2, β_{1a}, and γ_1 antibodies showed that the co-sedimentation of all these subunits in fractions 7–10 without GBP treatment (Fig. 4A). Interestingly, as shown in Fig. 4B, the co-sedimentation of Ca^{2+} channel subunits was not significantly affected by GBP treatment. None of the Ca^{2+} channel subunits including $\alpha_2\delta$ was apparently dissociated from the complex by the treatment, suggesting that GBP

binding does not disrupt the channel complex. We also tried different incubation conditions of GBP treatment such as various concentrations of the drug, incubation time, and temperature. In none of these experimental conditions, we could observe any appreciable change in the Ca^{2+} channel complex integrity by the drug treatment (data not shown). Lastly, similar experiments using partially purified Ca^{2+} channel complexes from rabbit brain showed that the treatment with GBP has no effect also on the integrity of native neuronal Ca^{2+} channels (data not shown).

4. Discussion

In spite of the demonstrated direct interaction between GBP and the Ca^{2+} channel $\alpha_2\delta$ subunit [10], controversial results regarding the functional effects of the drug on Ca^{2+} channels have been reported. No appreciable effects of the acute treatment with GBP on native currents recorded from human hippocampal granulose cells [20] and PC12 cells [21] have been found. On the other hand, acute application of GBP has been shown to inhibit L-type Ca^{2+} currents in isolated rat neurons [22], mouse skeletal myotubes [23], and neuronal N-type currents in cultured rat dorsal root ganglion neurons [24].

Most of the previous functional assays studying the effects of GBP on Ca^{2+} channel activity have been performed after acute exposure to the drug. Nevertheless, previous animal investigations have suggested that GBP should be transported across the plasma membrane of the neurons to exert its effect, and interestingly, the antiepileptic effect of GBP peaked at a time when the brain interstitial level of the drug, as well as its plasma concentration, had declined significantly [25]. Furthermore, previous studies have shown that GBP is actively absorbed by the cells via the system L-amino acid transporter [26]. In this context, the present study focused on testing the potential chronic effects of GBP on neuronal Ca^{2+} channel activity.

Our results show that GBP deeply affected the kinetics of channel inactivation after chronic exposure to the drug. Notably, several studies have shown that alteration in the inactivation properties of Ca^{2+} channels is an important mechanism for $\alpha_2\delta$ regulation of ionic current [27,28]. In addition, conversely to what happens with the acute GBP treatment where no alterations in the activity of the channels was observed, the data of the chronic treatment may be better explained by an intracellular interaction between $\alpha_2\delta$ and GBP during the process of channel assembly, possibly modifying the association of the auxiliary subunit with the channel complex. Therefore, by using partially purified Ca^{2+} channels, we tested whether GBP could alter the integrity of the native Ca^{2+} channel complex. However, as shown in Fig. 4, none of the Ca^{2+} channel subunits including $\alpha_2\delta$ was dissociated from the complex already formed by the overnight treatment with GBP. Together, the electrophysiological and biochemical analyses in this study suggest that the chronic effects of GBP on the inactivation kinetics of Ca^{2+} channel could be explained by an alteration of the $\alpha_2\delta$ actions on the properties of the Ca^{2+} channels possibly by the induction of a conformational change in the channel, although this alteration might not be large enough to cause the dissociation of $\alpha_2\delta$ from the channel complex.

Our results, however, do not rule out the presence of acute effects of GBP on neuronal Ca^{2+} channels. Acute GBP treatment might introduce subtle alterations on the properties of the channels that are not apparent under some experimental conditions or are not easily detected in large currents such as those expressed in the oocytes system. Therefore, a combination of intracellular and extracellular interaction (after chronic and acute treatment, respectively) between $\alpha_2\delta$ and GBP could be seen as complementary mechanisms of channel regulation. In addition, we could speculate that the change of inactivation kinetics by chronic treatment with GBP could not be observed in cultured mammalian cells [21] due to the fact that they are generally keep in L-glutamine-containing culture media. The concentration of L-glutamine could be high enough to saturate the amino acid transporter system in the cells and GBP may not be efficiently translocated to the interior of the cells. Actually, it was recently reported that GBP could compete with some amino acids for the transportation by the L-type amino acid transporter 1 [29] that has been shown to be a pathway for concentrating GBP intracellularly [26]. In contrast, oocytes are kept in culture in an amino acid-free medium. In this case, the availability of the transporter would be high and the translocation of the drug to the cytoplasm would occur normally. Furthermore, the discrepancy between the data obtained in mammalian cells and oocytes might be also explained by the fact that only $\alpha_2\delta$-1 and $\alpha_2\delta$-2 isoforms (but not $\alpha_2\delta$-3) bind to GBP [30,31] but all three isoforms are expressed differentially in the brain [32]. This may indicate the presence of an exquisite sensitivity to GBP that is dependent on neuronal type and/or localization as well as in Ca^{2+} channel $\alpha_2\delta$ isoform expression.

Lastly, being GBP the first ligand that interacts with the $\alpha_2\delta$ subunit, demonstration of a direct regulation of neuronal Ca^{2+} channels by the drug may be important to the understanding of its antiepileptic properties. Experimental evidence indicates that the epileptic discharges, generated when a physiological tonic mode of neuronal firing shifts to a burst-firing mode [33], are produced by aberrant thalamocortical oscillations [34]. P/Q-type Ca^{2+} channels are present and play a role in neurotransmitter release in thalamic neurons [35] providing a potential mechanistic link between Ca^{2+} currents and thalamocortical circuits. Interestingly, a reduction of excitatory synaptic transmission in the thalamus of epileptic mice has been documented [36] as a result of reduced Ca^{2+} influx, and it was proposed that a net enhanced GABAergic input in thalamocortical neurons may synchronize them into a burst-firing mode. In addition, several studies have revealed that some paroxysmal disorders including epilepsy are linked to Ca^{2+} channel mutations leading to reduced Ca^{2+} currents in cerebellar neurons of mice and humans [2,3]. Therefore, it is tempting to speculate that one of the consequences of the chronic treatment with GBP may be to revert this increasing Ca^{2+} influx by slowing down neuronal Ca^{2+} channel inactivation, though further studies will be needed to unveil the precise molecular mechanisms of GBP antiepileptic action.

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