Selectivity of action of pregabalin on Ca\textsuperscript{2+} channels but not on fusion pore, exocytotic machinery or mitochondria in chromaffin cells of the adrenal gland

by

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ABBREVIATIONS

PGB: pregabalin; ILE: isoleucine; VDCC: voltage-dependent calcium channels

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ABSTRACT

The present study was planned to investigate the action of pregabalin on voltage-dependent Ca\(^{2+}\) channels (VDCC) and on novel targets (fusion pore formed between the secretory vesicle and the plasma membrane, exocytotic machinery and mitochondria) that would further explain its inhibitory action on neurotransmitter release. Electrophysiological recordings in the perforated-patch configuration of the patch-clamp technique revealed that pregabalin inhibits by 33.4±2.4% and 39±4%, respectively, the Ca\(^{2+}\) current charge density and exocytosis evoked by depolarizing pulses in mouse chromaffin cells. About half of the inhibitory action of pregabalin was rescued by L-isoleucine, showing the involvement of α\(2\)δ-dependent and independent mechanisms. Ca\(^{2+}\) channel blockers were used to inhibit Cav1.2, Cav2.1 and Cav2.2 channels in mouse chromaffin cells, which were unselectively blocked by the drug. Similar values of Ca\(^{2+}\) current charge blockade were obtained when pregabalin was tested in human or bovine chromaffin cells, species that express very different percentage of VDCC types with respect to mouse chromaffin cells. These results demonstrate that the inhibitory action of pregabalin on VDCC and exocytosis does not depend on α1 Ca\(^{2+}\) channel subunit types. Carbon fibre amperometric recordings of digitonin-permeabilized cells showed that neither the fusion pore nor the exocytotic machinery were targeted by pregabalin. Mitochondrial Ca\(^{2+}\) measurements performed with mit-r-Pericam demonstrated that Ca\(^{2+}\) uptake or release from mitochondria were not either affected by the drug. The selectivity of action of pregabalin might explain its safety, good tolerability and reduced adverse effects. In addition, the inhibition of the exocytotic process in chromaffin cells might have relevant clinical consequences.
INTRODUCTION

PBG ((S)-3-(aminomethyl)-5-methylhexanoic acid) is a drug indicated in the treatment of central and peripheral neuropathic pain and generalized anxiety disorder, and in the adjunctive therapy of partial seizures in adults. Its mechanism of action has not been completely clarified. The inhibitory effect of pregabalin on voltage dependent Ca\textsuperscript{2+} channels (VDCC) after acute application of the drug has been reported (Dooley et al., 2002; Fink et al., 2002; McClelland et al., 2004). Pregabalin preferentially blocks Cav2.1 channels in rat neocortical slices (Dooley et al., 2002), human neocortical synaptosomes (Fink et al., 2002) or mice neurons of the calyx of Held (Di Guilmi et al., 2011).

In addition, many studies have documented the effect of pregabalin on neurotransmitter release. Pregabalin inhibits the release of glutamate in rat entorhinal synapses in vitro (Cunningham et al., 2004) and in rat neocortical and hippocampal slices (Dooley et al., 2000a), noradrenaline (Dooley et al., 2000b), acetylcholine and serotonin (Dooley et al., 2000a, 2000b; Brawek et al., 2008) in human and rat neocortical slices, glutamate in rodent neocortical slices (Quintero et al., 2011), GABA in human neocortical synaptosomes (Brawek et al., 2009) and capsaicin-evoked substance P and calcitonin gene-related peptide in rat spinal cord slices (Fehrenbacher et al., 2003). Pregabalin is a potent and selective ligand for \( \alpha_2\delta-1 \) and \( \alpha_2\delta-2 \) \( \text{Ca}^{2+} \) channel subunits (Li et al., 2011). Indeed, \( \alpha_2\delta-1 \) subunits of VDCC have been identified as the molecular target for the analgesic action exerted by pregabalin (Field et al., 2006), as well as for its inhibitory effect on glutamate release in rodent neocortical slices (Quintero et al., 2011). Besides the action of pregabalin on \( \alpha_2\delta \) subunits to inhibit release (Micheva et al., 2006; Joshi et al., 2006; Quintero et al., 2011), it has been postulated that an independent mechanism to \( \text{Ca}^{2+} \) entry through VDCC might be involved (Cunningham et al., 2004; McClelland et al., 2004; Micheva et al., 2006).
In fact, pregabalin uses the system L of amino acid transport across the plasma membrane to access the cytosol (Jezyk et al., 1999; Su et al., 2005), where it might be acting on different targets to generate its neuronal effects. In this sense, pregabalin could reach the axoplasm, act on the exocytotic machinery that controls neurotransmitter release or accumulate into intracellular organelles such as mitochondria. Indeed, antiepileptic drugs such as topiramate have shown to affect the SNARE-associated monoamine exocytotic mechanism (Okada et al., 2005). In addition, there has been increasing evidence supporting the association between mitochondrial oxidative stress and epilepsy (Waldbaum and Patel, 2010a, b; Folbergrová and Kunz, 2011). Several mutations in the electron transport chain associated with epilepsy have been described (Shoffner et al., 1990; TryoenTóth et al., 2003; Kudin et al., 2009). Given that mitochondria can reach Ca\(^{2+}\) transients of millimolar concentration (Montero et al., 2000), the consequence of those mutations would be the inefficiency to buffer Ca\(^{2+}\), and therefore, the uncontrolled elevation of cytosolic Ca\(^{2+}\). This increment in the cytosolic Ca\(^{2+}\) concentration will enhance neurotransmitter release, triggering epileptogenic action potentials at the postsynaptic level.

The goal of the present study was to further investigate the effect of pregabalin on VDCC, and also on additional targets such as the fusion pore formed between the plasma membrane and the secretory vesicle, the exocytotic apparatus or the mitochondria. To this purpose, we used chromaffin cells of the adrenal gland, modified postganglionic sympathetic neurons innervated by the splanchnic nerve that mainly control the release of adrenaline to the bloodstream, in order to prepare muscle and cardiovascular systems to a situation of stress. Our study shows that pregabalin unselectively inhibited Cav1, Cav2.1 and Cav2.2 channels. This inhibitory effect was halved by L-isoleucine (ILE), which binds to the \(\alpha\)2\(\delta\)-auxiliary subunit of VDCC. Furthermore, similar percentages of blockade were exerted by pregabalin on the VDCC of chromaffin cells from mice, human and bovine species, which express very different percentages of VDCC types. This means
that the blockade exerted by pregabalin on VDCC is not dependent on the $\alpha_1$ subunit type of Ca$^{2+}$ channels, but most probably on their $\alpha_2\delta$ auxiliary subunits. In addition, no intracellular effects on the fusion pore, the exocytic machinery or the mitochondria were here observed, which might explain the selectivity of action of this drug, its reduced adverse effects respect to other antiepileptic drugs, as well as its safety and tolerability.
MATERIAL AND METHODS

Isolation and culture of mouse, human and bovine chromaffin cells

Mice from 2 to 3 months old were used to obtain the adrenal glands. The procedure of isolation and culture of cells was performed as previously reported (Pérez-Alvarez et al., 2011). The study protocol for the use of human chromaffin cells was approved by the Ethics Committees of the Hospital Ramón y Cajal and Universidad Autónoma de Madrid. Adrenal glands were harvested from two organ donors who had died of cerebral hemorrhage. The method of isolation and culture of the human chromaffin cells was performed as previously reported (Pérez-Alvarez et al., 2007). Bovine chromaffin cells from adrenal glands of adult cows were isolated according to the method reported by Moro and coworkers (1990).

Electrophysiological recordings

For the perforated-patch whole-cell recordings, the external solution was (in mM): 5 CaCl₂, 100 NaCl, 45 TEACl, 5.5 KCl, 1 MgCl₂, 0.2 d-tubocurarine, 0.002 TTX, 0.0002 apamin, 10 HEPES and 10 glucose (pH 7.4 adjusted with NaOH). The intracellular solution composition was (in mM): 145 Cs-glutamate, 8 NaCl, 1 MgCl₂, 10 HEPES and 0.5 amphotericin B (Sigma-Aldrich, Madrid, Spain) and the pH was adjusted to 7.2 with CsOH. An amphotericin B stock solution was prepared every day at a concentration of 50 mg/mL in dimethyl sulphoxide (DMSO) and kept protected from light. The final concentration of amphotericin B was prepared by ultrasonicating 10 µL of stock amphotericin B in 1 mL of Cs-glutamate internal solution in the dark. Pipettes were tip-dipped in amphotericin-free solution for several seconds and back-filled with freshly mixed intracellular amphotericin solution.

The perfusion system for drug application consisted of a multi-barrelled glass pipette positioned close to the cell under study, which allowed the complete exchange of solutions near the cell within
100 ms. The level of the bath fluid was continuously controlled by a home-made fibre optics system coupled to a pump that removed excess fluid.

Electrophysiological measurements were made using an EPC-10 amplifier and PULSE software (HEKA Elektronik, Lambrecht, Germany) running on a PC computer. Pipettes of 2-3 MΩ resistance were pulled from borosilicate glass capillary tubes, partially coated with wax and fire polished. Only recordings in which the leak current and access resistance were lower than 20 pA and 20 MΩ, respectively, were accepted. Cell membrane capacitance \( (C_m) \) changes as an index of exocytosis were estimated by the Lindau-Neher technique implemented in the “Sine+DC” feature of the “PULSE” lock-in software. A 1 kHz, 70 mV peak-to-peak amplitude sinewave was applied at a holding potential \( (V_h) \) of -80 mV.

All toxins used to pharmacologically characterize the Ca\(^{2+}\) channels were purchased from Peptide Institute Inc., Osaka, Japan, except the dihidropyridine nifedipine which was purchased from Sigma. Pregabalin was always perfused for at least 15 min in every experiment.

Experiments were performed at room temperature (22-24°C). Analysis of data was conducted using IGOR Pro software (Wavemetrics, Lake Oswego, Oregon). The nonspecific background current and \( C_m \) recorded under 200 μM CdCl\(_2\) were subtracted off-line from Ca\(^{2+}\) current and \( C_m \) traces. Unless otherwise stated, data are given as the mean±S.E.M. Data were compared using paired or unpaired Student’s t-test.

**Amperometric recordings**

Carbon fiber electrodes were prepared by cannulating a 10 μm-diameter carbon fiber in polyethylene tubing (diameter: outer, 1 mm; inner, 0.5 mm). The carbon fiber tip was glued into a
glass capillary for mounting on a patch-clamp headstage, and back filled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. Amperometric currents were recorded using an EPC-10 amplifier and PULSE software running on a PC computer. The sampling rate was 14.5 kHz. Samples were digitally filtered at 2 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments using 50 µM adrenaline as standard solution. Only fibers that rendered 200-300 pA of current increment after 50 µM adrenaline pulse were used for the experiments. The tip of the fiber was recut for each experiment and calibrated again.

The bath solution was composed by (in mM): 139 K-glutamate, 0.2 EGTA, 20 PIPES, 2 ATP and 2 MgCl₂ (pH 6.5) and the permeabilitation solution contained (in mM): 139 K-glutamate, 5 EGTA, 20 PIPES, 2 ATP, 2 MgCl₂, 20 µM digitonin and 10 µM of free Ca²⁺ (pH 6.5).

Analysis of amperometric data

Spike analysis was performed using IGOR Pro software and macros that allow the analysis of single events and the rejection of overlapping spikes (Segura et al. 2000; Mosharov and Sulzer, 2005). The macros from Borges’s and Sulzer’s groups were used to analyze the amperometric spikes and “foot” of the spikes, respectively. A threshold of 4.5 times the first derivative of the noise standard deviation was calculated to clearly detect amperometric events. Then, among the events whose first derivative was above this threshold, only those showing one peak and one rising and falling phase, were considered as single spikes. To minimize variability among cells, the overall mean of average spike values recorded in several single cells was used. Unpaired Student’s t-test was used to compare our data.
Mitochondrial Ca\(^{2+}\) measurements

Mitochondrial Ca\(^{2+}\) measurements were performed using mit-r-pericam (Nagai et al., 2001), transduced in chromaffin cells using the pHSVmit-pericam amplicon vector (VAN4), derived from the Herpes Simplex Virus type 1 (HSV-1). Packaging (Lim et al., 1996) and titering of the amplicon with a titer of 1.02 \times 10^7 infectious vector units (ivu) \times ml^1 was performed as previously described (Chamero et al., 2008). 5 \mu l of the virus suspension was added to each well containing 600 \mu l of DMEM. After gentle shaking, the plate was introduced into the culture chamber for 90 min, and later on 1 ml DMEM was added to each well. Experiments were performed 12-24 hrs after this procedure.

Cells expressing mit-r-pericam were placed in the perfusion chamber in standard medium containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM glucose, and 10 mM HEPES, pH 7.4. Standard medium containing 0.5 mM EGTA instead of CaCl\(_2\) was then perfused for 1 min, followed by a 1-min perfusion of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM MgCl\(_2\), 1 mM K\(_3\)PO\(_4\), 0.2 mM EGTA, 1 mM ATP, 20 \mu M ADP, 2 mM succinate, 20 mM HEPES, pH 7) containing 20 \mu M digitonin. Intracellular medium without digitonin but with 30 \mu M of free Ca\(^{2+}\) was perfused 5 min. In the experiments where the effect of pregabalin was evaluated, all the solutions contained 30 \mu M of this drug.

To measure mitochondrial Ca\(^{2+}\) transients, the Metafluor software (Molecular Devices, CA, USA), a Nikon eclipse TE2000-S microscope with a Nikon S-Fluor objective (40X, NA 1.30), coupled to a monochromator (Cairn Research, Kent, UK) and a cooled Orca ER digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) were used. A 506 dicroic mirror along with a 536/40 emission filter were also employed. After appropriate regions of interest were selected, changes in mitochondrial Ca\(^{2+}\) signal were monitored taking alternative epifluorescence images at 415 and 485
nm excitation wavelengths, and calculating the 485/415 ratio. The rate of image acquisition was 2 Hz.
RESULTS

Pregabalin inhibits VDCC and exocytosis in mouse chromaffin cells

To investigate the possible effect of the acute application of pregabalin on VDCC and exocytosis in mouse chromaffin cells of the adrenal gland, simultaneous measurements of Ca\(^{2+}\) currents and C\(_m\) were performed in the “perforated-patch” configuration of the “patch-clamp” technique. Mouse chromaffin cells have been shown to possess all the VDCC reported in neurons, Cav1, Cav2.1, Cav2.2 and Cav2.3 channels (Albillos et al., 2000; Aldea et al., 2002), as well as the transcript for \(\alpha_2\delta\) subunits (García-Palomero et al., 2000). We started testing the effect of increasing concentrations of pregabalin, from 3 to 300 \(\mu\)M, on the Ca\(^{2+}\) currents elicited by 200 ms depolarizing pulses to the voltage peak current. Pregabalin inhibited VDCC in a dose-dependent manner (Fig. 1A). Since the plasma membrane concentration reached by a single therapeutic dose of 600 mg pregabalin is about 30 \(\mu\)M (Arroyo et al., 2003, Beydoun et al., 2005), this concentration was chosen to perform the experiments of this study.

When 30 \(\mu\)M pregabalin was perfused on top of voltage-clamped mouse chromaffin cells, the inhibition of Ca\(^{2+}\) currents and exocytosis developed gradually, reaching a stable value after 10-15 min. The percentage of inhibition exerted by pregabalin on the Ca\(^{2+}\) charge density and C\(_m\) was 33.4±2.4\% (n=38) and 39±4\% (n=36), respectively, in mouse chromaffin cells. Fig. 1 shows the time course of Ca\(^{2+}\) charge blockade and exocytosis induced by pregabalin (panel B), and the original recordings of Ca\(^{2+}\) currents (panel C) and exocytosis (panel D) under control conditions, pregabalin and CdCl\(_2\) perfusion.
The inhibition of pregabalin on VDCC is mediated by α2δ auxiliary subunits in mouse chromaffin cells

Next experiments were conducted to investigate whether the observed inhibitory effect of pregabalin on VDCC in mouse chromaffin cells was exerted through its interaction with α2δ subunits, as reported in other cell systems (Thurlow et al., 1993; Cunningham et al., 2004; Micheva et al., 2006; Joshi et al., 2006; Quintero et al., 2011). To achieve that purpose, L-isoleucine (ILE), which binds to the α2δ subunit of VDCC (Brown et al., 1998; Dooley et al. 2007; Brawek et al., 2009), was tested. The time course of Ca2+ charge blockade and the original recordings of the Ca2+ currents after perfusion of ILE or ILE in the presence of pregabalin are shown in Fig. 2A and B, respectively. ILE diminished the Ca2+ charge density by 30±4.8% (n=11). The subsequent addition of pregabalin in the presence of ILE exerted still an additional blockade of 15.3±1.1% (n=8), which might be due to an α2δ-independent inhibitory mechanism. However, the effect of pregabalin on VDCC after ILE treatment was significantly diminished with respect to its action in the absence of ILE (33.4±2.4%, data from Fig. 1A), showing that pregabalin inhibits VDCC in chromaffin cells through α2δ-dependent and independent mechanisms.

Pregabalin blocks Cav1, Cav2.1 and Cav2.2 channels in mouse chromaffin cells

To assess whether pregabalin exhibits any specificity to inhibit a certain Ca2+ channel type, and to analyze the interaction of pregabalin with α2δ subunits, different selective Ca2+ channel blockers were perfused before pregabalin. 3 µM nifedipine (Nife), 200 nM ω-agatoxin IVA (ω-Aga IVA) and 1 µM ω-conotoxin GVIA (ω-Ctx GVIA) were used to block Cav1, Cav2.1 and Cav2.2 channels, respectively. The action of pregabalin on Cav2.3 channels could not be investigated, since SNX-482, the selective Ca2+ channel blocker available to inhibit Cav2.3 channels, also inhibit Cav2.1 channels in chromaffin cells (Arroyo et al., 2003).
After Ca\textsuperscript{2+} currents inhibited by the blockers reached the steady state, pregabalin was added to the perfusion solution in order to evaluate whether the inhibitory effect of the drug on Ca\textsuperscript{2+} currents and exocytosis had been modified by the corresponding blocker. The effect of pregabalin after the perfusion of the Ca\textsuperscript{2+} channel blocker was compared with its effect when it was first perfused in a different set of experiments (data from Fig. 1). Fig. 3 shows the original traces of Ca\textsuperscript{2+} currents (panels A-C) and C\textsubscript{m} (D-F) obtained under each condition. After perfusion of Nife, \(\omega\)-Ctx GVIA and \(\omega\)-Aga IVA, the additional inhibition exerted by pregabalin on Ca\textsuperscript{2+} currents and exocytosis was 10\(\pm\)1\% (n=9) and 12.4\(\pm\)2\% (n=3), 10\(\pm\)2\% (n=8) and 8.6\(\pm\)4\% (n=4), and 20.5\(\pm\)4\% (n=9) and 28.6\(\pm\)4.6\% (n=7), respectively, which notably differ from the inhibition achieved by pregabalin when it was first applied (33.4\(\pm\)2.4\% and 39\(\pm\)4\% for Ca\textsuperscript{2+} currents and exocytosis, respectively).

**Identical percentages of Ca\textsuperscript{2+} charge density and exocytosis blockade are achieved by pregabalin in human and bovine chromaffin cells with respect to mouse chromaffin cells**

Martin and coworkers (2002) reported that the sensitivity of gabapentin, an structural and functional analog of pregabalin, to Ca\textsuperscript{2+} currents depends on the relative abundance of accessory Ca\textsuperscript{2+} channel subunits, in particular \(\beta\)2 and \(\alpha\)2\δ\-2 subunits, expressed in dorsal root ganglion neurons (DRG). \(\alpha\)1 Ca\textsuperscript{2+} channel subunits did not seem to influence the inhibition of Ca\textsuperscript{2+} currents by gabapentin in these cells. Gabapentin exerted an unselective inhibition on VDCC types in DRG (Sutton et al., 2002), similarly as it was obtained with pregabalin in the present study. Thus, we found interesting to investigate whether also pregabalin inhibited Ca\textsuperscript{2+} channels in chromaffin cells independently of the \(\alpha\)1 Ca\textsuperscript{2+} channel type. Therefore, we analized the inhibitory effect of pregabalin on chromaffin cells that express different percentages of Ca\textsuperscript{2+} channel types to those of the murine species, such as human or bovine species. While mouse chromaffin cells express 45\%, 14\%, 25\% and 16\% for Cav1, Cav2.1, Cav2.2 and Cav2.3 channels (Pérez-Alvarez et al., 2011), human chromaffin cells, which could be sorted into two groups of similar size according to their predominance of either
Cav2.1 or Cav2.2 channels, express 14.5% and 17.7% for Cav1 and Cav2.3 channels, respectively, and 46% and 20%, or 18% and 51% for Ca2.1 and Cav2.2 channels, in cells with predominance of Cav2.1 and Cav2.2, respectively (Pérez-Alvarez et al., 2008). Bovine chromaffin cells show percentages of Ca\textsuperscript{2+} channel types that approach more the human species, accounting for about 15%, 30%, 40% and 15% for Cav1, Cav2.1, Cav2.2 and Cav2.3 channels, respectively (Albillos et al., 1993; Albillos et al., 1996; García-Palomero et al., 2000).

The time course of Ca\textsuperscript{2+} charge blockade exerted by 30 \(\mu\)M pregabalin and the original traces of the Ca\textsuperscript{2+} currents under control and pregabalin conditions in human and bovine chromaffin cells are shown Fig. 4A and B, respectively. The blockades of the Ca\textsuperscript{2+} charge density obtained were similar to those achieved in mouse, amounting to 33.6±7% and 32.6±4%, respectively, in human (n=7) and bovine (n=9) chromaffin cells. Also, the exocytotic process was blocked at a similar extent in human (36.7±12.5%, n=7) and bovine (40±7%, n=6) chromaffin cells. These data suggest that the inhibitory action of pregabalin on VDCC does not either depend on \(\alpha_1\) Ca\textsuperscript{2+} channel types, as previously reported for gabapentin in DRG neurons.

**Pregabalin does not act on the fusion pore or the exocytotic machinery in mouse chromaffin cells**

Carbon fibre amperometric recordings were performed to assess the action of pregabalin on the fusion pore or the intracellular exocytotic machinery. Chromaffin cells possess all the same elements that build the secretory apparatus in neurons (Neher 1998) and the released catecholamines can be detected at the single level using the carbon fibre amperometric technique (Wightman et al., 1991). Each vesicle that exhibits exocytosis generates a spike with kinetic properties that can be determined. The release of neurotransmitter molecules through the narrow fusion pore formed after the fusion of a chromaffin vesicle with the plasma membrane appears as a
“foot” signal that precedes the main body of the amperometric spikes (Chow et al., 1992; Albillos et al., 1997). If pregabalin interacts with some element of the exocytotic apparatus, this would be reflected in the number or in the kinetic parameters of the spikes or “foot” signals.

To avoid any effect derived from blockade of pregabalin on VDCC, cells were permeabilized with digitonin in a 10 µM free Ca\(^{2+}\) solution to allow Ca\(^{2+}\) entry into the cytosol. Original recordings of the spikes obtained under control conditions, and after incubation with 30 µM pregabalin for 1 h are shown in Fig. 5A and B, respectively. Typical spikes recorded under both conditions (marked with an asterisk) are displayed at the right of the panels.

The average number of “foot” signals obtained in control and pregabalin-treated cells was similar (Table 1). The following parameters were determined for each “foot” signal: \(I_{\text{max}}\) (maximal amplitude), \(t\) (duration), \(Q\) (charge, expressed as pC or as number of molecules) and \(n_{\text{foot}}\) (number of “foot” signals). The parameters of the amperometric “foot” signals of the spikes obtained in the digitonin-treated cells were unchanged in the presence of pregabalin, as shown in Table 1. This means that pregabalin does not act on the fusion pore formed between the secretory vesicle and the plasma membrane to release the neurotransmitter content stored at the chromaffin vesicle.

The average number of spikes obtained in control or pregabalin-treated cells was identical. The kinetic parameters of the individual amperometric spikes were not either modified after pregabalin treatment (Table 2). The following parameters were determined: \(I_{\text{max}}\) (peak amplitude), \(Q\) (charge), \(m\) (ascending slope, calculated from the linear portion of the trace between 25% and 75% of the \(I_{\text{max}}\)), \(t_{1/2}\) (half-width or duration of the amperometric signal at 50% of its peak amplitude) and \(t_p\) (time-to-peak, time from the start of the spike until the peak in seconds) (Fig. 6A). In addition, the frequency histograms of the different parameters under both conditions did not vary after the
treatment with the drug, showing that pregabalin did not interfere with any component of the exocytotic machinery to modulate neurotransmitter release (Fig. 6B).

Positive control experiments of the negative effect of pregabalin on the exocytotic apparatus were performed using the anti-SNARE tetanus toxin. This toxin has been shown to inhibit exocytosis in chromaffin cells (Penner et al., 1986; Bittner and Holz, 1998; Xu et al., 1998). Digitonin-permeabilized cells were incubated for 3 min with 300 nM tetanus toxin, resulting in a marked reduction of the number of amperometric spikes (227 spikes in control versus 96 spikes in tetanus toxin-treated cells, n= 6 cells, p=0.002) (Supplemental Figure 1).

**Pregabalin does not interfere with the mitochondrial Ca\(^{2+}\) fluxes in mouse chromaffin cells**

Pregabalin uses the system L of amino acid transport across the plasma membrane to generate its neuronal effects (Jezyk et al., 1999; Su et al., 2005). Thus, pregabalin might enter the cell and interact with mitochondria, an organelle implied in epilepsy. Mitochondrial Ca\(^{2+}\) transients were monitored using mit-r-Pericam. Digitonin-permeabilized cells were perfused with a solution containing 30 \(\mu\)M Ca\(^{2+}\), to analyze how mitochondria uptakes and releases Ca\(^{2+}\) under control conditions (n=14 cells) and after 20 min pretreatment with pregabalin (n=15 cells) (Fig. 7).

The following kinetic parameters of the mitochondrial Ca\(^{2+}\) signal were determined: \(t_{\text{peak}}\) (time-to-peak, time from the start of the rise until the maximal value of the fluorescence ratio in seconds), *increase* (increment of the fluorescence ratio from the basal line to the maximal value), *decrease at 150 s* (decrement of the fluorescence ratio from the maximal value until 150 seconds later) and *tau* (time constant) (Fig. 7A).
The release of Ca$^{2+}$ from mitochondria could be well fitted by a double exponential. None of the kinetic parameters of the mitochondrial Ca$^{2+}$ signal were modified after pregabalin treatment (Table 3), reflecting that pregabalin does not affect either mitochondrial uptake nor release. Thus, mitochondria is not affected by pregabalin to inhibit neurotransmitter release.

Positive control experiments of the negative effect of pregabalin on the mitochondrial Ca$^{2+}$ fluxes were performed using Ru-360, a selective inhibitor of the mitochondrial Ca$^{2+}$ uniporter (Matlib et al., 1998; Kirichok et al., 2004; Santo-Domingo and Demaurex, 2010). Digitonin-permeabilized cells that expressed mit-r-Pericam were perfused for 15 min with 1 μM Ru-360 (n=7), which completely abolished the mitochondrial Ca$^{2+}$ uptake observed in control cells (n=4) (Supplemental Figure 2).
DISCUSSION

The mechanism of action of pregabalin to inhibit Ca\(^{2+}\) channels and consequently neurotransmitter release has been reported to be mediated by the \(\alpha 2\delta\)-1 subunit of VDCC (Field et al., 2006; Quintero et al., 2011). Other targets different to these auxiliary subunits have been posed (Cunnigham et al., 2004; McClelland et al., 2004; Micheva et al., 2006). Indeed, some intracellular additional mechanism might explain its inhibitory action on neurotransmitter release since pregabalin uses the system L of amino acid transport across the plasma membrane to enter into the cytosol (Su et al., 2005). Therefore, it is plausible that pregabalin interacts with cellular structures such as the fusion pore, the exocytotic machinery or intracellular organelles such as the mitochondria, involved in the epileptogenic processes, to reduce neurotransmitter release.

In the present study, high-resolution techniques were used to investigate possible functional interactions of pregabalin with VDCC, fusion pore, exocytotic machinery and mitochondria. The main findings achieved in the present study are: i) pregabalin inhibited VDCC and exocytosis in mouse, human and bovine chromaffin cells of the adrenal gland, thus limiting the release of catecholamines to the bloodstream; ii) the inhibition of pregabalin on VDCC is partially mediated by \(\alpha 2\delta\) auxiliary subunits of Ca\(^{2+}\) channels; iii) pregabalin inhibition of VDCC does not depend on \(\alpha 1\) Ca\(^{2+}\) channel types; iv) pregabalin does not interfere with the fusion pore, the exocytotic machinery or the handling of Ca\(^{2+}\) by mitochondria.

In relation to the inhibitory effect of pregabalin on VDCC, we first investigated whether it was mediated through \(\alpha 2\delta\) auxiliary subunits. Transcripts for \(\alpha 2\delta\) auxiliary subunits have been reported in bovine chromaffin cells (García-Palomero et al., 2000). The partial retrieval of blockade achieved with ILE in mouse chromaffin cells, also reported in other cells systems (McClelland et
al., 2004; Di Guilmi et al., 2011), shows that pregabalin acts through $\alpha_2\delta$-dependent and independent mechanisms to regulate Ca$^{2+}$ channels and neurotransmitter release.

Extensive research has been performed on the VDCC type targeted by gabapentin. This drug was found to preferentially inhibit Cav1 (Stefani et al., 2001), Cav2.1 (Bayer et al., 2004) and Cav2.2 (Sutton et al., 2002) channels. Pregabalin has been reported to inhibit Cav2.1 channels (Dooley et al., 2002; Fink et al., 2002; Di Guilmi et al., 2011). In the present study we found that pregabalin inhibits Cav1, Cav2.1 and Cav2.2 channel types. However, Martin and coworkers (2001) reported that the inhibition of gabapentin in DRG neurons depended on the expression of $\beta_2$ and $\alpha_2\delta$-2 subunits, but not on $\alpha_1$ subunits. The unselective action of pregabalin in chromaffin cells prompted us to investigate whether, indeed, the effect of this drug did not depend on the $\alpha_1$ types of Ca$^{2+}$ channels. Therefore, we evaluated the effect of pregabalin on species of chromaffin cells that express very different percentages of Ca$^{2+}$ channel types. Mouse, human and bovine chromaffin cells, where Cav1, Cav2.1 or Cav2.2, and Cav2.2 channels, respectively, predominate, were challenged with 30 $\mu$M pregabalin, exhibiting identical amounts of Ca$^{2+}$ charge density blockade. Therefore, the data obtained in the present study further support the idea that pregabalin action mostly depends on the amount and type of expressed $\alpha_2\delta$, but not $\alpha_1$ subunits.

The experiments designed to investigate the Ca$^{2+}$ channel type targeted by pregabalin, perfusing the Ca$^{2+}$ channel blocker first, reflect that once the $\alpha_1$ subunit is targeted by the Ca$^{2+}$ antagonist, the action of the $\alpha_2\delta$ subunit ligand is mostly prevented. In the case of Cav1.2 channels, this idea is supported by previous data showing that $\alpha_2\delta$ subunits bind to the binding site for dihydropyridines in the loop S5-S6 of the $\alpha_1$Cav1.2 channel (Gurnett et al., 1997). Thus, the blockade of the channel by Nife would further prevent the regulatory action of an $\alpha_2\delta$ subunit already bound to pregabalin.
On the other hand, the possibility that pregabalin may be acting on different targets to Ca\(^{2+}\) channels have been previously proposed (Cunningham et al., 2004; McClelland et al., 2004; Micheva et al., 2006). In the present study, the action of pregabalin on the fusion pore formed between the plasma membrane and the secretory vesicle, the exocytotic apparatus or the mitochondria were also evaluated. Indeed, the anticonvulsivant topiramate has been shown to affect the SNARE-associated monoamine exocytotic mechanism (Okada et al., 2005). To investigate the action of pregabalin on the fusion pore and the exocytotic machinery, cells were treated with digitonin to avoid the effect of pregabalin on VDCC, so that Ca\(^{2+}\) would access the cytosol through pores formed by the detergent in the plasma membrane. If the fusion pore or any protein of the exocytotic machinery would be affected by the drug, a significant change in the number or the kinetic parameters of “foot” signals or spikes, recorded with the carbon fibre amperometric technique in single cells would be detected. However, this was not the case, reflecting that pregabalin does not act on these cellular structures.

It has been reported that mitochondria is largely involved in epilepsy (Folbergrová and Kunz, 2011). This organelle can transiently store high Ca\(^{2+}\) concentrations (Montero et al., 2000), and therefore, its dysfunction would trigger an increase of cytosolic Ca\(^{2+}\) and the enhancement of neurotransmitter release. This idea prompted us to investigate the possible functional interaction between pregabalin and the mitochondria, which might be affecting the mitochondrial handling of Ca\(^{2+}\). However, the Ca\(^{2+}\) uptake or release by mitochondria, measured with mit-r-Pericam in permeabilized cells challenged with 30 \(\mu\)M free Ca\(^{2+}\) was identical, showing that pregabalin does not act on the mitochondrial Ca\(^{2+}\) uniporter or the Na\(^+\)/Ca\(^{2+}\) exchanger.

Our study reports the effect of pregabalin on VDCC of chromaffin cells of the adrenal gland, the main source of adrenaline released to the bloodstream, an effect that might have clinical consequences. Indeed, it has been reported that pregabalin treatment improved heart rate variability.
(HRV) in patients with painful diabetic neuropathy (Jiang et al., 2011). An increased resting heart rate is frequently observed in diabetic patients, most likely due to vagal cardiac neuropathy that results in increased cardiac sympathetic activity. The tachycardia may be followed by a decrease in heart rate and, ultimately, by a fixed heart rate due to progressive dysfunction of the cardiac sympathetic nervous system. Therefore, pregabalin, by decreasing the exocytotic process, and consequently the adrenaline release, would initially diminish reflex tachycardia, thus stabilizing heart rate.

On the other hand, a decompensation of chronic heart failure associated with pregabalin in patients with neuropathic pain has been observed, probably due not only to the effect of pregabalin on VDCC of myopathic ventricles (Murphy et al., 2007), but also, as shown in the present study, to the decrease of the exocytotic process, and consequently of adrenaline release, yielded by the drug in human chromaffin cells.

In conclusion, our data show that pregabalin inhibits exocytosis by blocking Cav1, Cav2.1 and Cav2.2 channels through α2δ-dependent and independent pathways. These mechanisms lead to the inhibition of Ca\(^{2+}\) channels to a certain extent, independently of the amount and type of α1 Ca\(^{2+}\) channel types. The inhibition of Ca\(^{2+}\) channels by pregabalin provokes the inhibition of the exocytotic process, which might possess clinical relevance. Finally, pregabalin does not act on other targets related with exocytosis or Ca\(^{2+}\) homeostasia such as the fusion pore, the exocytotic machinery and the mitochondria. This selective mechanism of action of the drug may contribute to its safety, good tolerability and lack of adverse effects.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Albillos

Conducted experiments: Hernández-Vivanco, Pérez-Alvarez, Caba-González, Moreno-Ortega, Cano-Abad, Ruiz-Nuño, Carmona-Hidalgo

Contributed new reagents or analytic tools: Alonso

Performed data analysis: Hernández-Vivanco

Wrote or contributed to the writing of the manuscript: Albillos
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FOOTNOTES

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FIGURE LEGENDS

Fig.1. *Pregabalin inhibits VDCC and exocytosis in mouse chromaffin cells*

The effect of pregabalin on VDCC was investigated in the perforated-patch configuration of the patch-clamp technique, applying 200 ms depolarizing pulses to the peak current voltage to mouse chromaffin cells. A) Bar graph of the $Ca^{2+}$ charge density blockade yielded by increasing concentrations of pregabalin (from 3 to 300 $\mu$M). Numbers of cells are shown in parentheses. Bars represent average values ± SEM values. B) Time course of the blockade exerted by 30 $\mu$M pregabalin on the $Ca^{2+}$ current charge (black circles) and exocytosis (white circles) elicited by 200 ms depolarizing pulses to the peak current voltage. C and D) Original recordings of the $Ca^{2+}$ current traces (C) and exocytosis (D) under control conditions, after perfusion of pregabalin or CdCl$_2$ corresponding to the cell of panel B.

Fig.2. *The inhibition of pregabalin on VDCC is mediated by $\alpha 2\delta$ auxiliary subunits in mouse chromaffin cells*

ILE, which binds to the $\alpha 2\delta$ subunit of VDCC, was used to investigate whether the action of pregabalin is mediated through $\alpha 2\delta$ auxiliary subunits. A) Time course of $Ca^{2+}$ charge blockade yielded by 30 $\mu$M ILE and by ILE in the presence of 30 $\mu$M pregabalin in mouse chromaffin cells. B) Original recordings of $Ca^{2+}$ current traces recorded under control conditions, or after perfusion of ILE, ILE coperfused with pregabalin, or 200 $\mu$M CdCl$_2$. C) Bar graph of the $Ca^{2+}$ charge density blockade obtained after perfusion with ILE (black bar) and after coperfusion of ILE and pregabalin (white bar). The blockade exerted by pregabalin alone (data obtained from Fig. 1A) was also shown for comparison. Bars represent average values and asterisks show statistical significance (* P<0.05; ** P<0.01, *** P<0.001).
Fig. 3. *Pregabalin blocks Cav1, Cav2.1 and Cav2.2 channels*

The effect of pregabalin on VDCC types was investigated perfusing selective Ca$^{2+}$ channels blockers of Cav1, Cav2.1 and Cav2.2 before application of pregabalin. The Ca$^{2+}$ channel blockers used were 3 µM nifedipine (Nife), 200 nM ω-agatoxin IVA (ω-Aga IVA) and 1 µM ω-conotoxin GVIA (ω-Ctx GVIA) to block Cav1, Cav2.1 and Cav2.2 channels, respectively. Original Ca$^{2+}$ current (panels A-C) and exocytosis (D-F) traces recorded under control conditions, after application of the different Ca$^{2+}$ channel blockers and after coperfusion of the blocker with 30 µM pregabalin. Cm traces were simultaneously recorded to Ca$^{2+}$ current traces obtained in the corresponding cell of the left panels.

Fig. 4. *Identical percentages of Ca$^{2+}$ charge density and exocytosis blockade are achieved by pregabalin in human and bovine chromaffin cells with respect to mouse chromaffin cells*

The effect of pregabalin on VDCC was investigated in the perforated-patch configuration of the patch-clamp technique, applying 200 ms depolarizing pulses to the peak current voltage to voltage-clamped human or bovine chromaffin cells. A and B) Time course of the Ca$^{2+}$ current charge (top) and the original current and capacitance traces (bottom) obtained under control conditions and after perfusion of 30 µM pregabalin or CdCl$_2$ in human (A) and bovine (B) chromaffin cells.

Fig. 5. *Pregabalin does not act on the fusion pore or the exocytotic machinery in mouse chromaffin cells*

Amperometric spikes elicited by a solution containing 10 µM free Ca$^{2+}$ in digitonin-permeabilized mouse adrenal chromaffin cells. Original recordings obtained under control conditions (A) and after incubation with 30 µM pregabalin for 1 h (B). Typical spikes recorded under both conditions are displayed at the right of both panels.
**Fig. 6. Pregabalin does not act on the fusion pore or the exocytotic machinery in mouse chromaffin cells**

A) Scheme of the analyzed kinetic parameters of the amperometric spikes. These parameters include the peak amplitude ($I_{\text{max}}$), charge ($Q$; considering as the integral of individual amperometric spikes), ascending slope ($m$; calculated from the linear part of the trace located between 25 and 75% of the $I_{\text{max}}$), half-width ($t_{1/2}$; duration of the amperometric signal at 50% of its peak amplitude) and time-to-peak ($t_p$; determined as the time from the start of the spike until the peak in seconds). B-C) Frequency histograms of the kinetic parameters of individual amperometric spikes obtained in cells under control conditions (B) or after treatment with 30 µM pregabalin (C). Data are pooled from 1,256 (control) and 1,113 (pregabalin) individual secretory spikes obtained from the experiments shown in Fig. 5.

**Fig. 7. Pregabalin does not interfere with mitochondrial Ca$^{2+}$ fluxes in mouse chromaffin cells**

A) Scheme of the analyzed kinetic parameters of the mitochondrial Ca$^{2+}$ signal measured with mitr-Pericam. The following kinetic parameters were determined: $t_{\text{peak}}$ (time-to-peak, time from the start of the rise until the maximal value of the fluorescence ratio in seconds), increase (increment of the fluorescence ratio from the basal line to the maximal value), decrease at 150 s (decrement of the fluorescence ratio from the maximal value until 150 seconds later) and $\tau$ (time constant). B-C) Original recordings of the mitochondrial Ca$^{2+}$ signal recorded under control conditions (B) or in the presence of 30 µM pregabalin (after 20 min pretreatment) (C).
Table 1. Kinetic parameters of the “foot” signals recorded from the individual amperometric spikes. The following parameters were determined for each “foot” signal: $I_{\text{max}}$ (maximal amplitude), $t$ (duration), $Q$ (charge, expressed as pC or as number of molecules) and $n_{\text{foot}}$ (number of “foot” signals). Recordings were obtained in digitonin-permeabilized chromaffin cells under control conditions or after pregabalin (PGB) treatment for 1 h.

<table>
<thead>
<tr>
<th></th>
<th>$I_{\text{max}}$ (pA)</th>
<th>$t$ (ms)</th>
<th>$Q$ (pC)</th>
<th>$Q$ (molec)</th>
<th>$n_{\text{foot}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.9±0.3</td>
<td>20.4±2.0</td>
<td>0.1±0.0</td>
<td>2.8e-7±2.9e-8</td>
<td>133</td>
</tr>
<tr>
<td>PGB</td>
<td>4.5±0.3</td>
<td>20.6±3.5</td>
<td>0.1±0.0</td>
<td>3.2e-7±6.0e-8</td>
<td>110</td>
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</table>
Table 2. Kinetic parameters of the individual amperometric spikes. The following parameters were determined: $I_{\text{max}}$ (peak amplitude), $Q$ (charge), $m$ (ascending slope: calculated from the linear portion of the trace between 25% and 75% of the $I_{\text{max}}$), $t_{1/2}$ (half-width or duration of the amperometric signal at 50% of its peak amplitude), $t_p$ (time-to-peak: time from the start of the spike until the peak in seconds), $n$ spikes (number of amperometric spikes) and $n$ cells (number of cells). Recordings were obtained in digitonin-permeabilized chromaffin cells under control conditions and after pregabalin (PGB) treatment for 1 h.

<table>
<thead>
<tr>
<th></th>
<th>$I_{\text{max}}$ (pA)</th>
<th>$Q$ (pC)</th>
<th>$m$ (nA/s)</th>
<th>$t_{1/2}$ (ms)</th>
<th>$t_p$ (ms)</th>
<th>$n$ spikes</th>
<th>$n$ cells</th>
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<td>Control</td>
<td>41.3±2.8</td>
<td>0.7±0.1</td>
<td>23.3±2.3</td>
<td>10.9±1.5</td>
<td>8.6±1.4</td>
<td>1256</td>
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<tr>
<td>PGB</td>
<td>39.4±2.6</td>
<td>0.6±0.1</td>
<td>25.7±3.4</td>
<td>10.2±0.9</td>
<td>8.3±1.2</td>
<td>1113</td>
<td>37</td>
</tr>
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</table>
Table 3. Kinetic parameters of the mitochondrial Ca$^{2+}$ signal. The following kinetic parameters of the mitochondrial Ca$^{2+}$ signal were determined: $t_{peak}$ (time-to-peak, time from the start of the rise until the maximal value of the fluorescence ratio in seconds), $increase$ (increment of the fluorescence ratio from the basal line to the maximal value), $decrease$ at 150 s (decrement of the fluorescence ratio from the maximal value until 150 seconds later) and $tau$ (time constant), where $tau$ 1 and 2 are the slow and fast time constant values, respectively, obtained from the double exponential fit. Recordings were obtained under control conditions and after pregabalin (PGB) treatment for 20 min.

<table>
<thead>
<tr>
<th>t peak (s)</th>
<th>Increase</th>
<th>Decrease at 150 s</th>
<th>Tau 1 (s)</th>
<th>Tau 2 (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>PGB</td>
<td>Control</td>
<td>PGB</td>
<td>Control</td>
</tr>
<tr>
<td>3±0.6</td>
<td>2.9±0.4</td>
<td>1.1±0.1</td>
<td>0.6±0.1</td>
<td>118.4±16.4</td>
</tr>
<tr>
<td>(n=14)</td>
<td>(n=15)</td>
<td>(n=14)</td>
<td>(n=14)</td>
<td>(n=14)</td>
</tr>
<tr>
<td>1.3±0.2</td>
<td>0.8±0.1</td>
<td>101.3±25</td>
<td>22.8±4.2</td>
<td>17.9±2.6</td>
</tr>
<tr>
<td>(n=14)</td>
<td>(n=15)</td>
<td>(n=14)</td>
<td>(n=14)</td>
<td>(n=15)</td>
</tr>
</tbody>
</table>
Figure 2

A

![Graph showing Ca\(^{2+}\) current over time.](image)

- Control
- ILE 30 μM
- ILE 30 μM + PGB 30 μM
- Cd

B

![Graph showing current traces.](image)

- Control (a)
- ILE (b)
- ILE + PGB (c)
- Cd (d)

200 pA

50 ms

C

![Bar graph showing charge density blockade percentages.](image)

- PGB
- ILE + PGB

***
Figure 4

A. HUMAN

- Control
- PGB 30 μM
- Cd

Calcium charge (pC)

B. BOVINE

- Control
- PGB 30 μM
- Cd

Calcium charge (pC)
Figure 6

A

![Graph showing current (pA) over time (s). Parameters include t_{1/2}, t_p, Q, and Imax.]

B

**Control**

- Imax
- Q
- m
- t_{1/2}
- t_p

C

**PGB 30 µM**

- Imax
- Q
- m
- nA/s
- ms
- ms

Histograms showing the distribution of various parameters before and after PGB treatment.
Selectivity of action of pregabalin on $\text{Ca}^{2+}$ channels but not on fusion pore, exocytotic machinery or mitochondria in chromaffin cells of the adrenal gland

by

Alicia Hernández-Vivanco, Alberto Pérez-Alvarez, José Carlos Caba-González, María Teresa Alonso, Ana José Moreno-Ortega, María Cano-Abad, Ana Ruiz-Nuño, Beatriz Carmona-Hidalgo and Almudena Albillos
Supplemental Figure 1. Tetanus toxin inhibits exocytosis in single mouse chromaffin cells

Amperometric recordings were obtained in digitonin-permeabilized cells under control conditions (A) or after 3 min preincubation with 300 nM tetanus toxin (B).
Supplemental Figure 2. Ru-360 inhibits mitochondrial Ca\textsuperscript{2+} uptake in mouse chromaffin cells

Mitochondrial Ca\textsuperscript{2+} signal was recorded in digitonin-permeabilized cells expressing mitr-Pericam under control conditions (A) or after 15 min treatment with 1 µM Ru-360 (B).