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\textsuperscript{[S]}

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ABSTRACT

The present study was planned to investigate the action of pregabalin on voltage-dependent Ca\textsuperscript{2+} channels (VDCCs) and novel targets (fusion pore formed between the secretory vesicle and the plasma membrane, exocytic 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\textsuperscript{2+} current charge density and exocytosis evoked by depolarizing pulses in mouse chromaffin cells. Approximately half of the inhibitory action of pregabalin was rescued by l-isoallevine, showing the involvement of α\textsubscript{2Δ}-dependent and -independent mechanisms. Ca\textsuperscript{2+} channel blockers were used to inhibit Cav1, Cav2.1, and Cav2.2 channels in mouse chromaffin cells, which were unselectively blocked by the drug. Similar values of Ca\textsuperscript{2+} current charge blockade were obtained when pregabalin was tested in human or bovine chromaffin cells, which express very different percentages of VDCC types with respect to mouse chromaffin cells. These results demonstrate that the inhibitory action of pregabalin on VDCCs and exocytosis does not depend on α\textsubscript{1} Ca\textsuperscript{2+} channel subunit types. Carbon fiber amperometric recordings of digitonin-permeabilized cells showed that neither the fusion pore nor the exocytotic machinery were targeted by pregabalin. Mitochondrial Ca\textsuperscript{2+} measurements performed with mitochondrial ratiometric pericam demonstrated that Ca\textsuperscript{2+} uptake or release from mitochondria were not 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

(S)-3-(aminomethyl)-5-methylhexanoic acid (pregabalin; PGB) 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 (VDCCs) after acute application of the drug has been reported previously (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), and 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.

ABBREVIATIONS: PGB, (S)-3-(aminomethyl)-5-methylhexanoic acid (pregabalin); VDCC, voltage-dependent calcium channel; mit-r-pericam, mitochondrial ratiometric pericam; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Nife, nifedipine; ω-Aga IVA, ω-agatoxin IVA; ω-Ctx GVIA, ω-conotoxin GVIA; DRG, dorsal root ganglion.
(Cunningham et al., 2004) and rat neocortical and hippocampal slices (Dooley et al., 2000a), noradrenaline (Dooley et al., 2000b), acetylcholine and serotonin (Dooley et al., 2000a,b; 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 α2δ-1 and α2δ-2 Ca2+ channel subunits (Li et al., 2011). Indeed, α2δ-1 subunits of VDCCs 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). In addition to the action of pregabalin on α2δ subunits to inhibit release (Joshi and Taylor, 2006; Mieheva et al., 2006; Quintero et al., 2011), it has been postulated that an independent mechanism to Ca2+ entry through VDCCs might be involved (Cunningham et al., 2004; McClelland et al., 2004; Mieheva 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 act 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 been 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 (Waldbaur and Patel, 2010a,b; Folbergrová and Kunz, 2012). Several mutations in the electron transport chain associated with epilepsy have been described previously (Shoffner et al., 1990; Tryoen-Toth et al., 2003; Kudin et al., 2009). Given that mitochondria can reach Ca2+ transients of millimolar concentration (Montero et al., 2000), the consequence of those mutations would be the inefficiency to buffer Ca2+ and, therefore, the uncontrolled elevation of cytosolic Ca2+. This increment in the cytosolic Ca2+ 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 VDCCs and also on additional targets such as the fusion pore formed between the plasma membrane and the secretory vesicle, the exocytotic machinery, or the mitochondria. To this purpose, we used chromaffin cells of the adrenal gland, modified ganglionic sympathetic neurons innervated by the splanchic nerve that mainly control the release of adrenaline to the bloodstream, 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 1-Ile, which binds to the α2δ-1 auxiliary subunit of VDCCs. Furthermore, similar percentages of blockade were exerted by pregabalin on the VDCCs of chromaffin cells from murine, human, and bovine species, which express very different percentages of VDCC types. This means that the blockade exerted by pregabalin on VDCCs does not depend on the α1 subunit type of Ca2+ channels, but most probably on their α2δ auxiliary subunits. In addition, no intracellular effects on the fusion pore, the exocytotic machinery, or the mitochondria were observed here, which might explain the selectivity of action of this drug, its reduced adverse effects respect to other antiepileptic drugs, and its safety and tolerability.

Materials and Methods

Isolation and Culture of Murine, Human, and Bovine Chromaffin Cells. Mice from 2 to 3 months old were used to obtain the adrenal glands. The mice were housed in the animal facility of the Universidad Autónoma de Madrid (UAM), Madrid Registry number ES-280790000097. The procedure of isolation and culture of cells was performed as reported previously (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 of 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 reported previously (Pérez-Alvarez and Albillos, 2007). Bovine chromaffin cells from the adrenal glands of adult cows were isolated according to the method described previously (Moro et al., 1990).

Electrophysiological Recordings. For the perforated-patch whole-cell recordings, the external solution was 5 mM CaCl2, 100 mM NaCl, 45 mM tetraethylammonium-Cl, 5.5 mM KCl, 1 mM MgCl2, 0.2 mM t-tubocurarine, 0.002 mM tetrodotoxin, 0.0002 mM ampin, 10 mM HEPES, and 10 mM glucose, pH 7.4 adjusted with NaOH. The intracellular solution composition was 145 mM Cs-glutamate, 8 mM NaCl, 1 mM MgCl2, 10 mM HEPES, and 0.5 mM 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 sulfoxide 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 multibarreled glass pipette positioned close to the cell under study, which allowed for the complete exchange of solutions near the cell within 100 ms. The level of the bath fluid was continuously controlled by a home-made fiber optics system coupled to a pump that removed excess fluid.

Electrophysiological measurements were made by using an EPC-10 amplifier and Pulse software (HEKA, Lambrecht/Pfalz, Germany) running on a computer. Pipettes of 2- to 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 (Cm) 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 (Vh) of −80 mV. All toxins used to pharmacologically characterize the Ca2+ channels were purchased from Peptide Institute Inc. (Osaka, Japan), except the dihidropyridine nifedipine (Nif), which was purchased from Sigma-Aldrich. 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 with IGOR Pro software (Wavemetrics, Lake Oswego, OR). The nonspecific background current and Cm, recorded under 200 μM CdCl2 were subtracted off-line from Ca2+ current and Cm traces. Unless otherwise stated, data are given as the mean ± S.E.M. Data were compared by 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 (o.d., 1 mm; i.d., 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 by using an EPC-10 amplifier and Pulse software running on a 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 by using 50 μM adrenaline as standard solution. Only fibers that rendered 200 to 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 of 139 mM K-glutamate, 0.2 mM EGTA, 20 mM PIPES (1,4-piperazinediethanesulfonic acid), 2 mM ATP, and 2 mM MgCl₂, pH 6.5, and the permeabilization solution contained 139 mM K-glutamate, 5 mM EGTA, 20 mM PIPES, 2 mM ATP, 2 mM MgCl₂, 20 μM digitonin, and 10 μM free Ca²⁺, pH 6.5.

Analysis of Amperometric Data. Spike analysis was performed by 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 those references 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 the falling phase were considered as single spikes. Unpaired Student’s t test was used to compare the data.

Mitochondrial Ca²⁺ Measurements. Mitochondrial Ca²⁺ measurements were performed by using mit-r-pericam (Nagai et al., 2001), transduced in chromaffin cells using the pHSVmit-pericam amplicon vector (VAN4), derived from herpes simplex virus type 1. Packaging (Lim et al., 1996) and titering of the amplicon with a titer of 1.02 × 10⁹ infectious vector units × ml⁻¹ was performed as described previously (Chamero et al., 2008). Five microliters of the virus suspension was added to each well containing 600 μl of Dulbecco’s modified Eagle’s medium (DMEM). After gentle shaking, the plate was introduced into the culture chamber for 90 min, and later on 1 ml of DMEM was added to each well. Experiments were performed 12 to 24 h after this procedure.

Results

Pregabalin Inhibits VDCCs and Exocytosis in Mouse Chromaffin Cells. To investigate the possible effect of the acute application of pregabalin on VDCCs and exocytosis in mouse chromaffin cells of the adrenal gland, simultaneous measurements of Ca²⁺ 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 of the VDCCs reported in neurons and Cav1, Cav2.1, Cav2.2, and Cav2.3 channels (Albillos et al., 2000; Aldea et al., 2002), as well as the transcript for α2δ subunits (Garcia-Palomero et al., 2000). We started testing the effect of increasing concentrations of pregabalin, from 3 to 300 μM, on the Ca²⁺ currents elicited by 200-ms depolarizing pulses to the voltage peak current. Pregabalin inhibited VDCCs in a dose-dependent manner (Fig. 1A). Because the plasma membrane concentration reached by a single therapeutic dose of 600 mg of pregabalin is approx-
imately 30 μM (Arroyo et al., 2003; Beydoun et al., 2005) this concentration was chosen for the experiments of this study.

When 30 μM 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 to 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. Figure 1 shows the time course of Ca\(^{2+}\) charge blockade and exocytosis induced by pregabalin (B) and the original recordings of Ca\(^{2+}\) currents (C) and exocytosis (D) under control conditions, pregabalin, and CdCl\(_2\) perfusion.

The Inhibition of Pregabalin on VDCCs Is Mediated by α2δ Auxiliary Subunits in Mouse Chromaffin Cells. Next, experiments were conducted to investigate whether the observed inhibitory effect of pregabalin on VDCCs in mouse chromaffin cells was exerted through its interaction with α2δ subunits, as reported previously in other cell systems (Thurrow et al., 1993; Cunningham et al., 2004; Joshi and Taylor, 2006; Micheva et al., 2006; Quintero et al., 2011). To achieve that purpose, L-Ile, which binds to the α2δ subunit of VDCCs (Brown et al., 1998; Dooley et al., 2007; Brawek et al., 2009), was tested. The time course of Ca\(^{2+}\) charge blockade and the original recordings of the Ca\(^{2+}\) currents after perfusion of L-Ile in the presence of pregabalin are shown in Fig. 2, A and B, respectively. L-Ile diminished the Ca\(^{2+}\) charge density by 30 ± 4.8% (n = 11). The subsequent addition of pregabalin in the presence of L-Ile exerted still another additional blockade of 15.3 ± 1.1% (n = 8), which might be caused by an α2δ-independent inhibitory mechanism. However, the effect of pregabalin on VDCCs after L-Ile treatment was significantly diminished with respect to its action in the absence of L-Ile (33.4 ± 2.4%; data from Fig. 1A), showing that pregabalin inhibits VDCCs 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 Ca\(^{2+}\) channel type and to analyze the interaction of pregabalin with α2δ subunits, different selective Ca\(^{2+}\) channel blockers were perfused before pregabalin. Nife (3 μM), 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, because SNX-482, the selective Ca\(^{2+}\) channel blocker available to inhibit Cav2.3 channels, also inhibits Cav2.1 channels in chromaffin cells (Arroyo et al., 2003).

After Ca\(^{2+}\) currents inhibited by the blockers reached the steady state, pregabalin was added to the perfusion solution to evaluate whether the inhibitory effect of the drug on Ca\(^{2+}\) currents and exocytosis had been modified by the corresponding blocker. The effect of pregabalin after the perfusion of the Ca\(^{2+}\) channel blocker was compared with its effect when it was first perfused in a different set of experiments (data from Fig. 1). Figure 3 shows the original traces of Ca\(^{2+}\) currents (A–C) and \(C_m\) (D–F) obtained under each condition. After perfusion of Nife, ω-Ctx GVIA, and ω-Aga IVA, the additional inhibition exerted by pregabalin on Ca\(^{2+}\) currents and exocytosis was 10 ± 1% (n = 9) and 12.4 ± 2% (n = 3), 10 ± 2% (n = 8) and 8.6 ± 4% (n = 4), and 20.5 ± 4% (n = 9) and 28.6 ± 4.6% (n = 7), respectively, which notably differ from the inhibition achieved by pregabalin when it was first applied (33.4 ± 2.4% and 39 ± 4% for Ca\(^{2+}\) currents and exocytosis, respectively).

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. Martin et al. (2002) reported that the sensitivity of gabapentin, an structural and functional analog of pregabalin, to Ca\(^{2+}\) subunit of VDCCs, was used to investigate whether the action of pregabalin is mediated through α2δ auxiliary subunits. Time course of Ca\(^{2+}\) charge blockade yielded by 30 μM L-Ile and by L-Ile in the presence of 30 μM pregabalin in mouse chromaffin cells. B, original recordings of Ca\(^{2+}\) current traces recorded under control conditions or after perfusion of L-Ile, L-Ile coperfused with pregabalin, or 200 μM CdCl\(_2\). C, bar graph of the Ca\(^{2+}\) charge density blockade obtained after perfusion with L-Ile (filled bars) and after coperfusion of L-Ile and pregabalin (striped bars). 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 < 0.001. a, control; b, L-Ile; c, L-Ile + PGB; d, CdCl\(_2\)
(Pérez-Alvarez et al., 2011), human chromaffin cells, which could be sorted into two groups of similar size according to the 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+ channels, in cells with predominance of Cav2.1 and Cav2.2, respectively (Pérez-Alvarez et al., 2008). Bovine chromaffin cells show percentages of Ca2+ channel types that approach more like the human species, accounting for approximately 15, 30, 40, and 15% for Cav1, Cav2.1, Cav2.2, and Cav2.3 channels, respectively (Albillos et al., 1993, 1996; García-Palomero et al., 2000).

The time course of Ca2+ charge blockade exerted by 30 μM pregabalin and the original traces of the Ca2+ currents under control and pregabalin conditions in human and bovine chromaffin cells are shown in Fig. 4, A and B, respectively. The blockades of the Ca2+ 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. In addition, 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 VDCCs does not depend on α1 Ca2+ channel types, as reported previously for gabapentin in DRG neurons (Martin et al., 2002).

**Pregabalin Does Not Act on the Fusion Pore or the Exocytotic Machinery in Mouse Chromaffin Cells.** Carbon fiber amperometric recordings were performed to assess the action of pregabalin on the fusion pore or intracellular exocytotic machinery. Chromaffin cells possess all of the same elements that build the secretory apparatus in neurons (Neher, 1998), and the released catecholamines can be detected at the single level by using the carbon fiber 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 (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 (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 (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 (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 (Wightman et al., 1991). Each vesicle that exhibits exocytosis generates a spike with kinetic properties that can be determined.
To avoid any effect derived from the blockade of pregabalin on VDCCs, cells were permeabilized with digitonin in a $10^{-5}/H_9262M$ free $Ca^{2+}/H_11001$ solution to allow $Ca^{2+}/H_11001$ entry into the cytosol. Original recordings of the spikes obtained under control conditions and after incubation with $30^-5/H_9262M$ pregabalin for $1\ h$ are shown in Fig. 5, A and B, respectively. Typical spikes recorded under both conditions (marked by *) are displayed at the right of the figure.

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 number of molecules), and $n$ 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 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 by using the anti-SNARE tetanus toxin. This toxin has been shown to inhibit exocytosis in chromaffin cells (Penner et al., 1986; Bittner and Holz, 1988; 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 Fig. 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 (Jezzyk 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 by 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: \(I_{\text{max}}\) (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), \(n\) (number of amperometric spikes), and \(n\) cells (number of cells). Recordings were obtained in digitonin-permeabilized chromaffin cells under control conditions and after PGB treatment for 1 h.

### Table 2

<table>
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<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>4.9 ± 0.3</td>
<td>20.4 ± 2.0</td>
<td>0.1 ± 0.0</td>
<td>2.8 ± 7 ± 2.9e-8</td>
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<tr>
<td>PGB</td>
<td>4.5 ± 0.3</td>
<td>20.6 ± 3.5</td>
<td>0.1 ± 0.0</td>
<td>3.2 ± 7 ± 6.0e-8</td>
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In the present study, high-resolution techniques were used to investigate possible functional interactions of pregabalin with VDCCs, fusion pore, exocytotic machinery, and mitochondria. The main findings in the present study are: 1) pregabalin inhibited VDCCs and exocytosis in murine, human, and bovine chromaffin cells of the adrenal gland, thus limiting the release of catecholamines to the bloodstream; 2) the inhibition of pregabalin on VDCCs is partially mediated by α2δ auxiliary subunits of Ca\(^{2+}\) channels; 3) pregabalin inhibition of VDCCs does not depend on α1 Ca\(^{2+}\) channel types; and 4) 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 VDCCs, we first investigated whether it was mediated through α2δ auxiliary subunits. Transcripts for α2δ auxiliary subunits have been reported in bovine chromaffin cells (Garcia-Palomero et al., 2000). The partial retrieval of blockade achieved with L-Ile in mouse chromaffin cells, also reported in other cell systems (McClelland et al., 2004; Di Guilmì et al., 2011), shows that pregabalin acts through α2δ-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 Guilmì et al., 2011). In the present study we found that pregabalin inhibits Cav1, Cav2.1, and Cav2.2 channel types. However, Martin et al. (2002) reported that the inhibition of gabapentin in DRG neurons depended on the expression of β2 and α2δ-2 subunits, but not α1 sub-

### Discussion

The mechanism of action of pregabalin to inhibit Ca\(^{2+}\) channels and consequently neurotransmitter release has been reported to be mediated by the α2δ-1 subunit of VDCCs (Field et al., 2006; Quintero et al., 2011). Other targets different from these auxiliary subunits have been posed (Cunningham et al., 2004; McClelland et al., 2004; Micheva et al., 2006). Indeed, some intracellular additional mechanism might explain pregabalin’s inhibitory action on neurotransmitter release, because 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 following paragraphs, we describe our findings and discuss their potential implications for the mechanisms of action of pregabalin and related drugs.
units. 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. Murine, 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 and exhibited identical amounts of Ca$^{2+}$ charge density blockade. Therefore, the data obtained in the present study further support the idea that pregabalin action depends primarily 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 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 has been proposed previously (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 anticonvulsant topiramate has been shown to affect the SNARE-associated

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**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 ($Q$; considered 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 and C, frequency histograms of the kinetic parameters of individual amperometric spikes obtained in cells under control conditions (B) or after treatment with 30 $\mu$M pregabalin (C). Data are pooled from 1256 (control) and 1113 (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 mit-r-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 s later) and $\tau$ (time constant). B and C, original recordings of the mitochondrial Ca$^{2+}$ signal recorded under control conditions (B) or in the presence of 30 $\mu$M pregabalin (after 20-min pretreatment) (C).
monooamine exocytic 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 VDCCs, so that Ca<sup>2+</sup> would access the cytosol through pores formed by the detergent in the plasma membrane. If the fusion pore or any protein of the exocytic 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 fiber 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, 2012). This organelle can transiently store high Ca<sup>2+</sup> concentrations (Montero et al., 2000), and therefore, its dysfunction would trigger an increase of cytosolic Ca<sup>2+</sup> 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<sup>2+</sup>. However, the Ca<sup>2+</sup> uptake or release by mitochondria, measured with mit-r-Pericam in permeabilized cells challenged with 30 M free Ca<sup>2+</sup>, was identical, showing that pregabalin does not act on the mitochondrial Ca<sup>2+</sup> uniporter or the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger.

Our study reports the effect of pregabalin on VDCCs 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 in patients with painful diabetic neuropathy (Jiang et al., 2011). An increased resting heart rate is frequently observed in diabetic patients, most likely because of 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 caused by progressive dysfunction of the cardiac sympathetic nervous system. Therefore, pregabalin, by decreasing the exocytic process, and consequently the adrenaline release, would initially diminish reflex tachycardia, thus stabilizing heart rate.

On the other hand, a decomposition of chronic heart failure associated with pregabalin in patients with neuropathic pain has been observed, probably caused not only by the effect of pregabalin on VDCC of myopathic ventricles (Murphy et al., 2007), but also, as shown in the present study, by 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<sup>2+</sup> channels to a certain extent, independently of the amount and type of α1 Ca<sup>2+</sup> channel types. The inhibition of Ca<sup>2+</sup> 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<sup>2+</sup> homeostasis 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.


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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**TABLE 3**

Kinetic parameters of the mitochondrial Ca<sup>2+</sup> signal

The following kinetic parameters of the mitochondrial Ca<sup>2+</sup> 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 (decrease of the fluorescence ratio from the maximal value until 150 s later), and $r$ (time constant), where $r$ 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 PGB treatment for 20 min.

<table>
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<th>$t_{\text{peak}}$ (s)</th>
<th>Increase</th>
<th>Decrease at 150 s</th>
<th>$r$ 1 (s)</th>
<th>$r$ 2 (s)</th>
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<td>Control PGB Control PGB Control PGB Control PGB Control PGB</td>
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<td>3 ± 0.6 (n = 14) 2.9 ± 0.4 (n = 15)</td>
<td>1.1 ± 0.1 (n = 14) 1.3 ± 0.2 (n = 15)</td>
<td>0.6 ± 0.1 (n = 14) 0.8 ± 0.1 (n = 15)</td>
<td>118.4 ± 16.4 (n = 14) 101.3 ± 25 (n = 15)</td>
<td>22.8 ± 4.2 (n = 14) 17.9 ± 2.6 (n = 15)</td>
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Selectivity of action of pregabalin on Ca\(^{2+}\) channels but not on fusion pore, exocytotic machinery or mitochondria in chromaffin cells of the adrenal gland

by

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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\(^{2+}\) uptake in mouse chromaffin cells

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