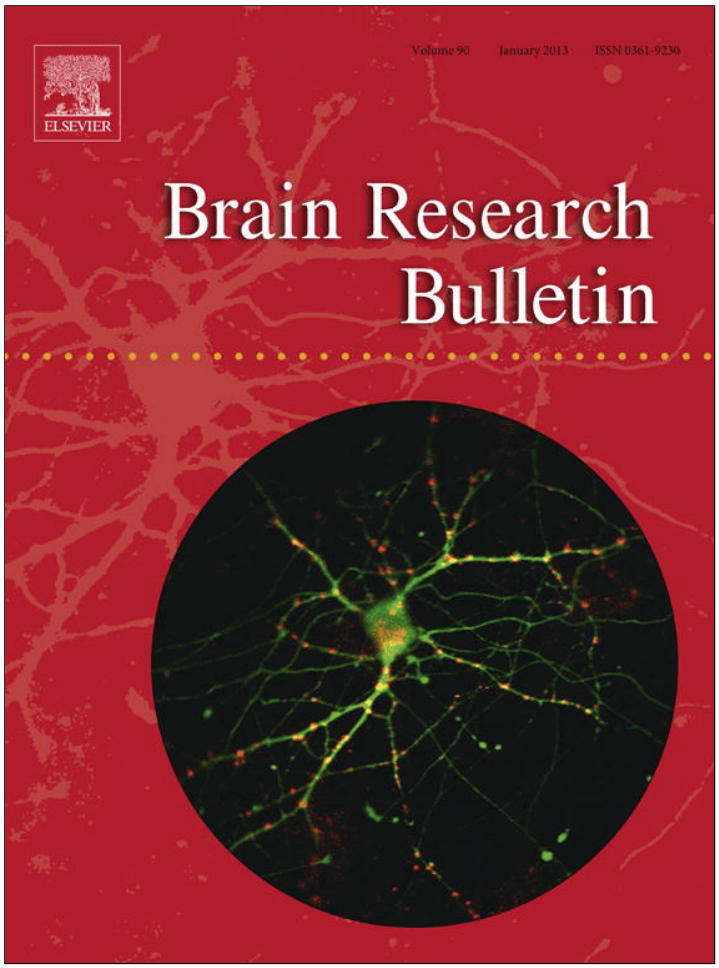

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Research report

Acute effects of pregabalin on the function and cellular distribution of $Ca_V2.1$ in HEK293t cells

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ABSTRACT

We established a cell model to study the acute effects of pregabalin (PGB), a drug widely used in epilepsy and neuropathic pain, on voltage gated $Ca_V2.1$ (P/Q-type) calcium channels function and distribution at the membrane level. HEK293t cells were transfected with plasmids coding for all subunits of the $Ca_V2.1$ channel. We used a $\alpha1$ fused to an eGFP tag to follow its distribution in time and at different experimental conditions.

The expressed channel was functional as shown by the presence of barium-mediated, calcium currents of transfected cells measured by 'whole-cell voltage-clamp' recordings, showing a maximum current peak in the *I–V* curve at +20 mV. The GFP fluorescent signal was confined to the periphery of the cells. Incubation with 500 μM PGB, that binds $\alpha2\delta$ subunits, for 30 min induced changes in localization of the fluorescent subunits as measured by fluorescent time lapse microscopy. These changes correlated with a reversible reduction of barium currents through $Ca_V2.1$ calcium channels under the same conditions. However, no changes in the cellular distribution of the subunits were visualized for cells either expressing another membrane associated protein or after exposure of the $Ca_V2.1$ channels to isoleucine, another $\alpha2\delta$ ligand. Together these results show strong evidence for an acute effect of PGB on $Ca_V2.1$ calcium channels' currents and distribution and suggest that internalization of $Ca_V2.1$ channels might be a mechanism of PGB action.

1. Introduction

Pregabalin (PGB) [S-(+)-3-isobutyl-GABA] has been long used as an anticonvulsant and analgesic drug, though the mechanism of its clinical action is still under investigation. PGB has been described as interacting with the $\alpha2\delta$ auxiliary subunit of voltage-gated calcium ($Ca_V2.1$) channels (Hendrich et al., 2008). $Ca_V2.1$ channels are composed of different subunits: the α_1 principal pore forming unit, and the auxiliary $\alpha_2\delta$, β, and, sometimes, γ auxiliary subunits (Thomas and Smart, 2005). The $\alpha_2\delta$ subunit has been implicated in the trafficking of calcium channels to the plasma membrane (Cantí et al., 2005). The importance of $\alpha_2\delta$ subunits has been further highlighted in recent results by Hoppa et al. The work showed the role of $\alpha_2\delta$ subunits in determining presynaptic calcium channel abundance by affecting a trafficking step of channels from the cell soma to synaptic terminals, thus, allowing synapses to make more efficient use of Ca(2+) entry to drive neurotransmitter release (Hoppa et al., 2012).

Application of PGB has been shown to reduce calcium currents in cultured sensory neurons (Martin et al., 2002; Sutton et al., 2002) and heterologous systems (Hendrich et al., 2008). Our group has recently reported an acute reduction of calcium currents mediated by $Ca_V2.1$ channels in the calyx of Held preparations after 15 min bath application of PGB at 500 μM, the plasma concentration expected as a result of repetitive clinical administrations of PGB (Di Guilmi et al., 2011). However, some controversy arose from other reports failing to reproduce any acute effects of PGB or gabapentin, a related drug, on channel function and electrophysiology in some preparations (Brown and Randall, 2005; Schumacher et al., 1998; Simon Kajaa et al., 2007), and in experiments using recombinant voltage-gated channels (Hendrich et al., 2008).

We sought to investigate this further in a cell culture system that would allow us to visualize, through a fluorescent *tag*-fused to a subunit of the channel, the acute effects associated to PGB with direct time lapse-microscopy and electrophysiological read outs.

We found that $Ca_V2.1$ channels are affected by the acute application of PGB. Barium current through calcium channels elicited in PGB-treated cells was reduced, and this effect was associated to an increase in internalization of the channel. The mechanism of

Abbreviations: $Ca_V2.1$, $\alpha2\delta$ auxiliary subunit of voltage-gated calcium; PGB, pregabalin; GBP, gabapentin; Ile, isoleucine.

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action described might help understand PGB effects on neuronal excitability and the use of the drug in calcium channel pathologies.

2. Methods

2.1. Cellular and molecular biology

HEK293t cells were cultured as described previously (Thomas, 2005). Cells were transfected with the calcium phosphate method as described in Greenberg protocol using plasmids encoding the different $Ca_V2.1$ calcium channel subunits: flag-eGFP-α_1 (referred to as eGFPα_1, a gift of Erika Piedras-Rentería; Loyola Univ., Chicago, USA), untagged $\alpha_2\delta$-1 and β_3 (a gift from Dr. Jesica Raingo; Lipscomb's lab); and for Venus-P2X_7 plasmid (provided by Fernando Aprile García; Arzt's lab). Cells were used between 3 and 5 days after transfection. Incubation of the cells with pregabalin (Gador S.A., Buenos Aires, Argentina, 0.5 mM, 30 min), isoleucine (Sigma, 1.5 mM, 30 min), and chlorpromazine (Sigma, 100 μM, 30 min), a generous gift from Dr. Fernando Marengo was done in a HEPES/calcium solution (in mM): HEPES, 10; Ca^{2+}, 2; Mg^{2+}, 1; Glucose, 10; Na^{2+} to pH 7.4 with NaOH, at 37 °C.

Cell staining was performed using methanol:acetone (1:1) for 10 min at −20 °C for fixation and permeabilization after the described treatment of the cells cultured on PLL-coated coverslips. Blocking was performed in 1% BSA in PBS (blocking buffer) for 1 h. Coverslips were incubated then with the primary antibody for 1 h in blocking buffer, washed in PBS and incubated with the secondary antibody for 45 min in blocking buffer. After a final wash in PBS, coverslips, were placed onto a slide and covered with mounting medium. The antibodies used were rabbit monoclonal antibody against actin 1:100 (sigma), and a secondary tetramethyl-rhodamine goat anti-rabbit antibody 1:500 (molecular probes).

2.2. Electrophysiology

Whole-cell Ba^{2+} currents were recorded 2–4 days after transfection of cells seeded on PLL coated coverslips, with the whole-cell patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), a Digidata 1200 digitizer (Molecular Devices), and pClamp 9.0 software (Molecular Devices). Patch pipettes were pulled from thin-walled borosilicate glass (GC150F-15; Harvard Apparatus, Kent, UK). Pipettes (~4 MΩ) were filled with a solution containing the following (in mM): 110 mM CsCl, 40 mM Hepes, 10 mM tetraethylammonium (TEA)-Cl, 12 mM Na_2-phosphocreatine, 1 mM EGTA, 2 mM MgATP, 0.5 mM LiGTP, and 1 mM $MgCl_2$. The pH was adjusted to 7.3 with CsOH.

Extracellular solution (ES) contained the following in (mM): 2 $BaCl_2$, 20 tetraethylammonium (TEA) Cl, and 10 HEPES (pH to 7.4 with NaOH 1 M). Before transferring the cells to the ES, the coverslips were washed in a solution like ES except that Ba^{2+} was replaced with 2 Mg^{2+}. $Ca_V2.1$ currents were elicited by either test pulses to +20 mV or 400 ms depolarizing ramps. The current density was calculated according to the capacitance of the cell and expressed in p*A*/p*F*. Percentage of PGB block was expressed as I_{Ba} area change. Whole-cell voltage-clamp recordings were conducted at room temperature; for experiments performed at 4 °C, the ES was kept on ice ensuring it was at 4 °C when reaching the sample via a pump that controlled suction of the solution.

2.3. Imaging and data analysis

Confocal imaging was done using an Olympus FV3000 microscope with a 60× (1.4 NA) oil-immersion, 20× (0.5 NA) water immersion, and 60× (0.9 NA) water immersion objectives. eGFP, and Venus were excited using an Ar/Kr 488 nm laser, and a He/Ne 543 nm laser. Optical sections for stacks were of 2 μm steps for the 20× objective, and 0.5 μm for the 60×. For quantitative experiments, identical laser power and acquisition settings were used. For analysis of subunits internalization, images were imported into ImageJ (NIH); the outline of the cell was manually drawn, and reduced in equal number of pixels using the enlarge function to obtain "total" and "interior" regions of fluorescence, subtraction of which gave a "Peripheral" region, as illustrated in Fig. 1B, where the fluorescence per μm^2 was quantified. For subunits' internalization measurements, about 20 cells were analyzed for each condition. All experiments were preformed three or more times. Normally distributed data were analyzed by Student's *t*-test, ANOVA and Bonferroni post hoc tests using Microcal Origin software.

3. Results

3.1. HEK293t cells can express functional fluorescently tagged $Ca_V2.1$ channels

Acute effects of PGB on $Ca_V2.1$ calcium channels were examined in HEK293t transfected cells. For this purpose, we used plasmids encoding the different subunits (α_1, $\alpha_2\delta$, and β_3) of the channel with a fluorescent *tag* (eGFP) present at the α_1 subunit (eGFPα1). When expressed in HEK293t cells, calcium channels showed a peripheral distribution at the plasma membrane (Fig. 1A, left). Fluorescence was not present in the nucleus (Fig. 1A, middle) as opposed to the homogenous distribution and fluorescence at the nucleus observed for just eGFP-expressing cells (Fig. 1A, right). Fluorescence of eGFPα1 + $\alpha_2\delta$ + β_3-transfected HEK293t cells was predominant at the periphery of the cells as expected for a membrane protein (Fig. 1B).

The expressed calcium channels were functional as demonstrated by the observed barium currents (Fig. 1C; using depolarizing square voltage pulses in eGFPα1-positive HEK293t cells under 'whole-cell voltage-clamp' configuration) and in agreement with current peaks reported for this model in the literature (Aromolaran et al., 2007; Eroglu et al., 2009; Hendrich et al., 2008). No currents were detected neither in eGFP positive cells (Fig. 1C), nor in eGFPα1-positive cells after cadmium incubation (cadmium 200 μM; $n = 3$, not shown).

3.2. Acute effect of PGB on eGFPα1 + $\alpha_2\delta$ + β_3-transfected HEK293t cells channels distribution and currents

We used the described system to test the acute effect of PGB on $Ca_V2.1$ calcium channels' cellular distribution and currents using a 500 μM concentration previously described to acutely reduce barium currents through $Ca_V2.1$ channels in brainstem slices (Di Guilmi et al., 2011).

PGB changed the distribution of membrane-expressed calcium channels, increasing the fluorescence signal at the cellular cytoplasm and decreasing the membrane signal at the periphery of the cell (Fig. 2A) by about 30% (Fig. 2B). Fluorescence could be visualized to the interior of the cell (Fig. 2C). PGB was tested under whole-cell patch-clamp configuration, and showed a blocking effect of barium currents through $Ca_V2.1$ calcium channels. PGB blocking effect on I_{Ba} was independent of the voltage protocol used, showing similar percentage of blocking effect when voltage ramps (Fig. 2D), or multiple depolarizing pulses (*I–V* curves) were used in HEK293t cells. We found that the HEK293t $Ca_V2.1$ eGFP-barium currents through $Ca_V2.1$ calcium channels currents area values were reduced by a 54.1 ± 7.8% ($n = 6$ cells) in the presence of 500 μM PGB (Fig. 2E). This effect was reversible, recovering the control current up to 93 ± 18% after 20 min of external solution perfusion (Fig. 2E, lower panel, washout). Furthermore, an example of fluorescence recovery of cells incubated with PGB after a washing step can be visualized in Fig. 2F. In conclusion, the acute effect of PGB on the currents supports the effects of internalization visualized by fluorescence microscopy.

3.3. Specificity of PGB action

We next set out to test the specificity of the PGB–$\alpha_2\delta$-$Ca_V2.1$ interaction. In a new set of experiments, we tested the effect of the interaction of Isoleucine (Ile), another $\alpha_2\delta$ ligand of equal affinity for the same binding site but lacking any effect on the analyzed calcium currents (Cunningham et al., 2004; Micheva et al., 2006). Isoleucine had no effect on calcium channels 'cellular distribution (Fig. 3A), thus, suggesting a specific effect of PGB. In addition, we tested the specificity of PGB-calcium channels interactions on HEK293t cells transfected with a membrane-expressed receptor, the P2X_7 purinoceptor. A lack of effect on the distribution of the fluorescent-channels was observed (Fig. 3B), and confirmed the specificity of the PGB–$Ca_V2.1$ interaction.

3.4. Acute mechanism of action of PGB

To determine the mechanism of channel internalization, we checked whether the application of PGB at 4 °C affected the channels' distribution, and thus determined the energy dependency

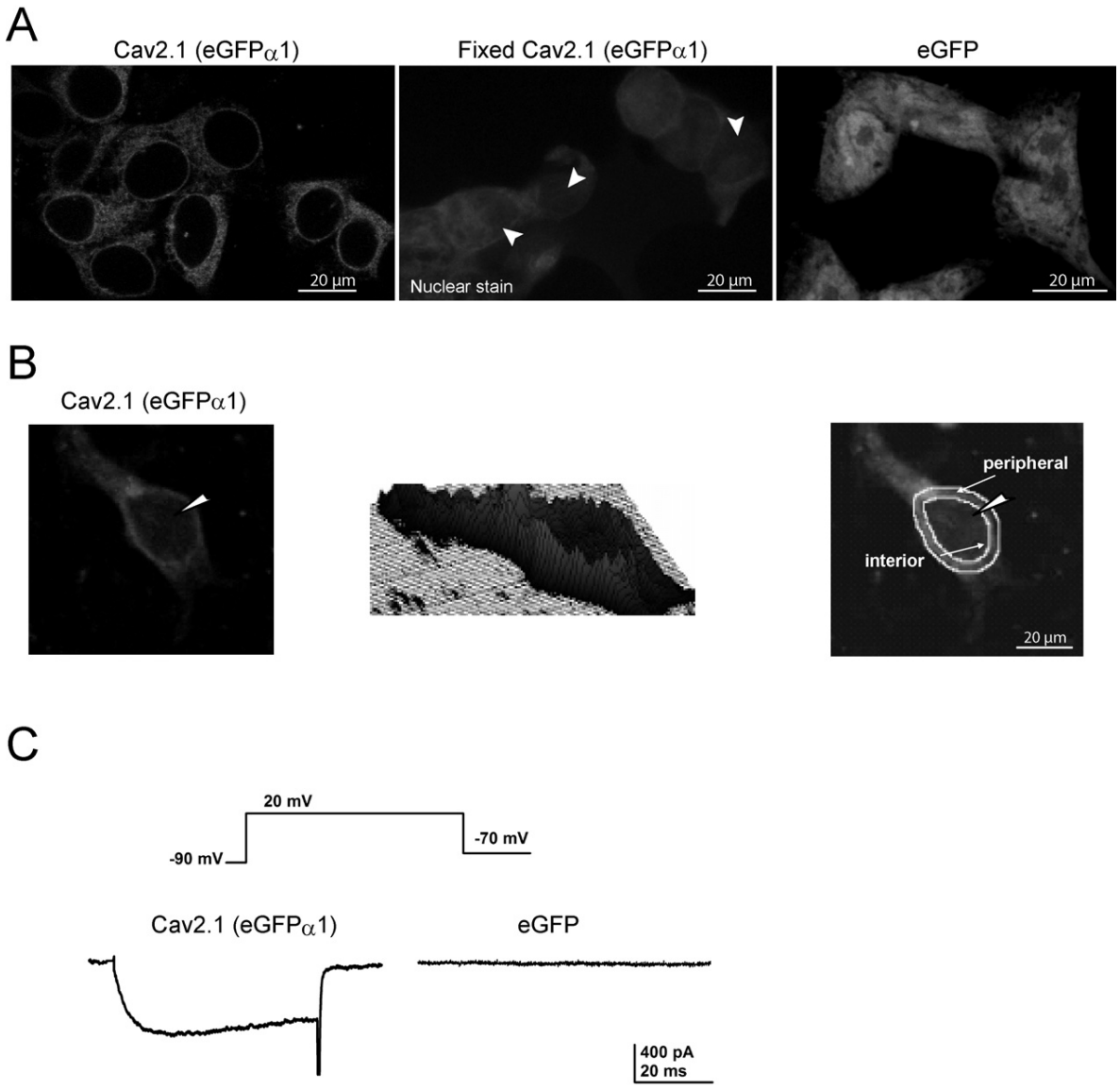

Fig. 1. HEK293t cells can express functional fluorescently tagged $Ca_V2.1$ channels. (A) Peripheral, membrane distribution and functionality of $Ca_V2.1$ channels expressed in HEK293t cells. Representative confocal micrographs of HEK293t cells expressing eGFPα1 + untagged $\alpha_2\delta + \beta_3$ subunits (left and middle) or eGFP alone (right). An average of 3 middle sections from a stack of images is shown. Confocal micrographs of fixed eGFPα1 + untagged $\alpha_2\delta + \beta_3$ subunits transfected HEK293t cells stained with Hoechst dye to show the cell Nuclei localization (middle). Notice the difference of fluorescence distribution for eGFP-expressing cells (high nuclear signal) vs. fluorescent-$Ca_V2.1$-expressing cells (mostly peripheral signal). (B) A Micrograph of a HEK293t cell transfected with fluorescent $Ca_V2.1$ channels is shown on the left and processed with the *Surface plot* function in ImageJ, and the resulting plot with regions of increased fluorescent intensity is displayed (B, middle). Arrowhead points at the interior region of the cell as opposed to the high fluorescence signal clearly detected at the peripheral region of the cell delineated with white lines (Fig. 1C, right) as explained in Section 2.3. Scale bar, 20 μm; micrographs are an average of 3 middle sections of images obtained with a confocal microscope with a 20× objective. (C) 'Whole-cell voltage-clamp' recordings of barium current through $Ca_V2.1$calcium channels for the examples in (A). 'whole-cell voltage-clamp' barium current through $Ca_V2.1$calcium channels elicited using 400 ms depolarizing square pulses in eGFPα1 + untagged $\alpha_2\delta + \beta_3$ subunits (left) or eGFP (right, $n = 3$) cells (for details see Section 2.2).

of this process (Schachter et al., 2008). Our results showed that PGB-mediated calcium channels' internalization is a temperature-dependent process, being absent at 4 °C (Fig. 4A). Indeed, the fluorescent signal at the cell periphery was higher than at control conditions in which constitutive endocytosis processes occur (Fig. 4D). Patch-clamp experiments with PGB (500 μM; 15–20 min incubation) at 4 °C also interfered with the effect of PGB on the currents measured ($n = 3$; Fig. 4A, bottom). Additionally, barium-mediated calcium currents presented activation kinetics which were significantly faster at room temperature compared to the 4 °C condition. Fitting a single exponential to the time course of I_{Ba} activation in response to +25 mV square pulses yielded mean tau values (in ms) of 4.8 ± 3.9 ms at room temperature ($n = 8$) vs. 16.6 ± 4.2 ms at 4 °C ($n = 7$; Tukey's test, $q = 2.9$; $p < 0.05$). To analyze the endocytosis mechanism further, we tested whether actin filaments were involved in the internalization process. We found that actin filaments, involved in clathrin mediated endocytosis (Yarar et al., 2005), showed a different distribution after PGB application. A greater actin-fluorescent signal was detected, within fixed cells after PGB treatment in comparison to a control; accompanying the channel's accumulation within cells (Fig. 4B). To analyze the participation of clathrin in the endocytic mechanism activated by PGB, we pre-incubated transfected cells with chlorpromazine, an inhibitor of clathrin-dependant endocytosis at conditions previously used in HEK293t cells, that showed no major effects in cells (Tulapurkar et al., 2005) as visualized in Fig. 4C ("control 2")

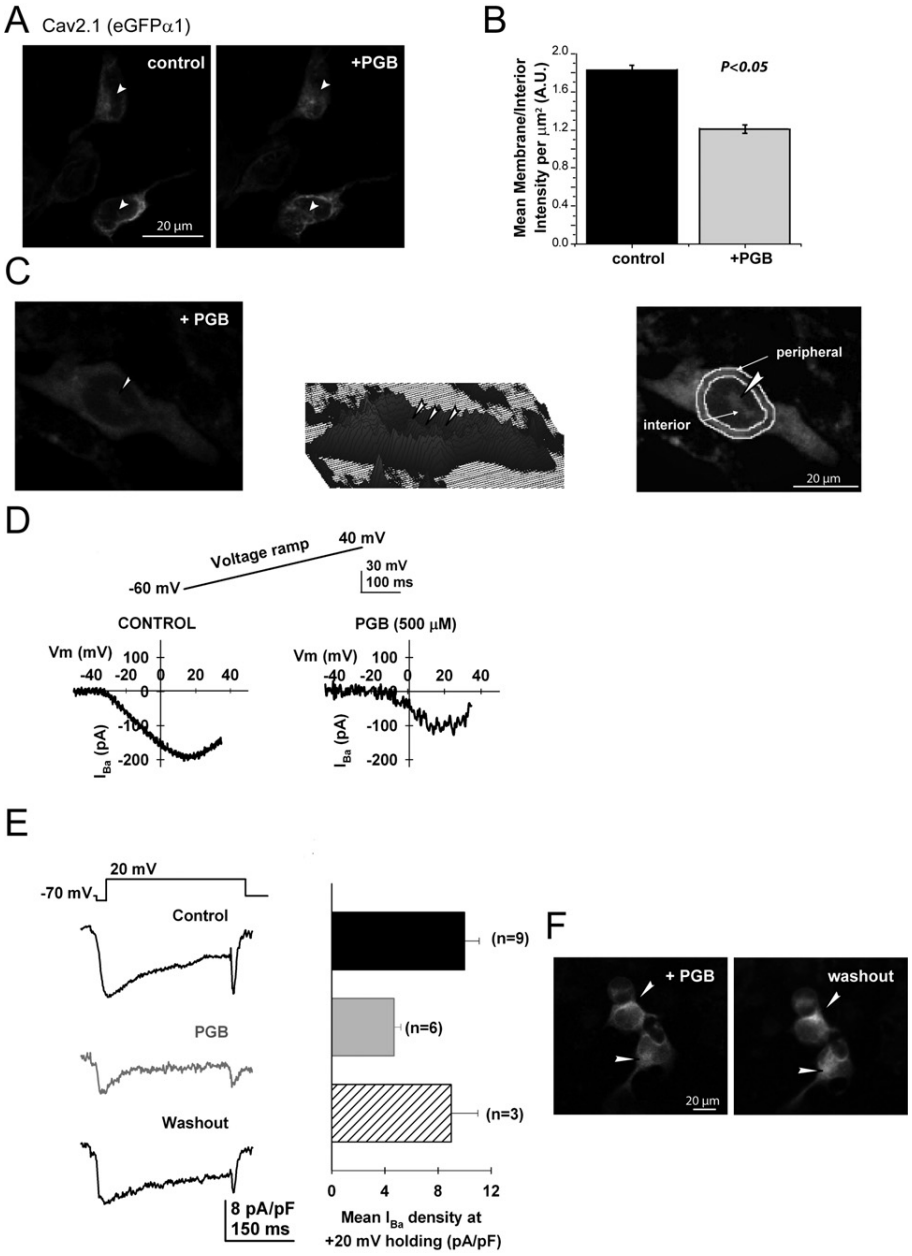

Fig. 2. Acute effect of PGB on eGFPα1 + α2δ + β3-transfected HEK293t cells. (A) Representative confocal micrographs of the same eGFPα1 + α2δ + β3-transfected HEK293t cells before (left) and re-localized after (right) a 30 min-incubation period with PGB at 500 μM. An average of 3 middle sections from a stack of images is shown. Notice the presence (PGB treated) or absence (control) of intracellular accumulations pointed by arrowheads. Scale bar, 20 μm. (B) Mean fluorescence intensity per μm^2 of peripheral to interior regions (see Section 2.3 for details) for $n = 19$ cells before and after 500 μM PGB treatment, $p < 0.05$ using Student's t-test. (C) Micrograph of a HEK293t cell transfected with fluorescent $Ca_V2.1$ channels after PGB treatment (500 μm for 30 min) is shown (left panel). The same cells depicted on the left panel was processed with the *Surface plot* function in ImageJ, and the resulting plot with regions of increased fluorescent intensity is shown (middle panel). Arrowheads points at the interior region of the cell where an increase of fluorescence is clearly detected. The peripheral and interior region of the cells is shown on the right panel delineated with white lines (Section 2.3) and the arrowhead points at accumulations of fluorescence in the interior. Scale bar, 20 μm; micrographs are an average of 3 middle sections of images obtained with a confocal microscope with a 20× objective. (D) 'Whole-cell voltage-clamp' barium current through $Ca_V2.1$ calcium channels (I_{Ba}) elicited using a 400 ms depolarizing ramp before (left panel), and after (right panel) bath application of 500 μM PGB showing that the effects were not affected by membrane potential. Similar effects were observed in other two HEK293t cells. (E) 'Whole-cell voltage-clamp' barium current through $Ca_V2.1$ calcium channels elicited using 400 ms depolarizing square pulses (on the left) before (upper panel), and after (middle panel) bath application of 500 μM PGB, and 20 min after washout (lower panel). On the right panel, a bar graph of the mean barium current density before (black bar, $n = 9$), after PGB (500 μM, gray bar $n = 6$), and after washout (dashed bar; $n = 3$). Area values of I_{Ba} currents were significantly reduced by a 54.1 ± 7.8% ($P < 0.05$). (F) Micrograph of the same HEK293t cells transfected with fluorescent $Ca_V2.1$ channels and treated with PGB (500 μM) (left) and 30 min after washing with normal solution ("washout", right). Arrowheads point to regions in which fluorescence recovery after the washing step can be easily distinguished.

and Supplementary Fig. 2. In addition, perfusion of cells with CPZ (100 μM) in patch-clamp experiments showed that CPZ was able to totally block I_{Ba} after 5 min perfusion (Suplementary Fig. 2A) as previously reported (Ogata and Yoshii, 1990). Cells treated with CPZ followed by PGB treatment resulted in the lack of $Ca_V2.1$eGFP internalization (Fig. 4C, right). Quantification of the fluorescent signal at the cell periphery showed that the signal at the periphery increased for cells treated with CPZ and then with PGB, to similar levels as was the case for cells incubated at 4 °C, which inhibits all forms of *endocytosis* (Fig. 4D).

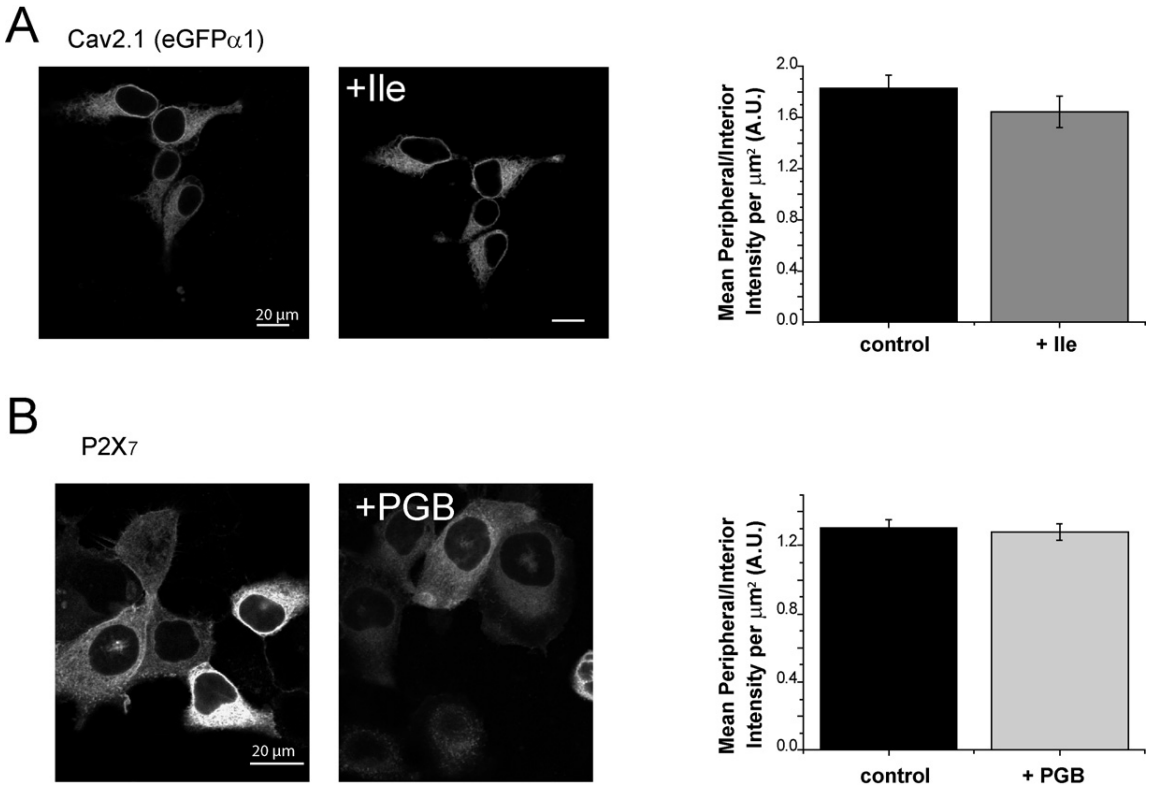

Fig. 3. Specificity of PGB action. (A) Representative confocal micrographs of the same HEK293t cells transfected with eGFPα1 + $\alpha_2\delta$ + β_3 before (left) and after (right) 30 min incubation with the $\alpha_2\delta$ natural ligand isoleucine (Ile, 1.5 mM). On the right panel, the mean fluorescence intensity per μm^2 of Peripheral to interior regions is quantified; n = 16–19 cells per example (see Section 2.3 for details). (B) Representative confocal micrographs of HEK293t cells transfected with the purinergic $P2X_7$ channel before (left) and after (right) PGB treatment. On the right panel, the mean fluorescence intensity per μm^2 of Periphery to intracellular regions (right) is given. Images acquired with a 60× water immersion objective, n = 14 for each condition. An average of 3 middle sections from a stack of images is shown.

4. Discussion

In this study we showed that acute PGB application in a heterologous expression system mediated the internalization of $Ca_V2.1$ channels in a clathrin dependent manner, concomitantly with a reduction of barium currents through $Ca_V2.1$ calcium channels that was observed to be independent of the holding potential, in HEK293t cells as well as in calyx of Held terminals (Supplementary Fig. 1). Thus, at a real synapse as well as in a heterologous system, PGB applied acutely determined the reduction of $Ca_V2.1$ mediated barium currents. The difference in magnitude of the effect might well be explained by the nature of the preparations (slices versus cell lines) where the channels interact with a different environment (Di Guilmi et al., 2011). Also, accessibility to PGB differs in either preparation, with and expected stronger effect in individual HEK293t cells compared to slices. Recently, Hendrich et al. (2008) tested the effect of gabapentin (GBP), a drug that interacts with the $\alpha_2\delta$ subunit, structurally related to PGB on HEK293t cells (Taylor et al., 2007). Their model failed to reproduce any acute effects on the $Ca_V2.1$ exogenously expressed channel, and only "chronic" (after 40 h incubation) inhibitory effects on calcium currents were evidenced. It is worth noting that Martin et al. (2002) showed that some preparations do not respond to GBP as well as to other gabapentoids (Martin et al., 2002). In addition, they showed that sensitivity to GBP was associated to a relatively lower $\alpha_2\delta$-2 channel subunit expression which they proposed could explain why some studies show sensitivity of channels to GBP and some do not. Therefore changes in the relative proportion of type 1and type 2 $\alpha_2\delta$ subunits could account for a change in sensitivity of preparations to the drug. Additionally, studies by Davies et al. (2007) documented the difference of affinity in GBP binding to the different subunit isoforms, showing a higher (2.5-fold) affinity for $\alpha_2\delta$ type-1 than for type 2. This is of great importance since the type of $\alpha_2\delta$ used by Hendrich et al. (2008) and Tran-Van-Minh and Dolphin (2010) is type 2, while we used the type 1 isoform in our study. The expression, and over-expression of the $\alpha_2\delta$-1 subunit is of great relevance since its up-regulation is physiologically found in neuropathic pain (Davies et al., 2007), a clinical condition for which gabapentoids are used. Additionally, it should be considered that the solution used by Hendrich et al. for GBP incubation is culture media in which other aminoacids are present, such as Ile which competes for the $\alpha_2\delta$ binding site. In our hands, PGB effects were evidenced after 30 min, both through electrophysiological recordings and changes in fluorescent distribution throughout the cells. The use of proteins fused to fluorescent *tags* allowed us to follow $Ca_V2.1$ channel trafficking in time before and after PGB application, without the need of fixation methods, giving us the possibility to analyze changes at the very same cells (Figs. 2A and 4A and C). This methods, as well as biochemical assays that use HA-tags have been used extensively in the literature to measure internalization (Altier et al., 2006; Hendrich et al., 2008; Tulapurkar et al., 2005).

Acute effects of drugs on receptors has been documented previously: Tulapurkar et al. (2005) were able to show endocytosis of the metabotropic P2Y2 receptor after a 30-min incubation with the UTP agonist, mediated by clathrin and the actin cytoskeleton (Tulapurkar et al., 2005). Moreover, this type of internalization mechanism has also been documented for $Ca_V2.2$ (N-type) calcium channels after opioid receptor agonist application concomitantly with a decrease in N-type channel-mediated calcium entry, suggested by the authors as a key mechanism for long term down

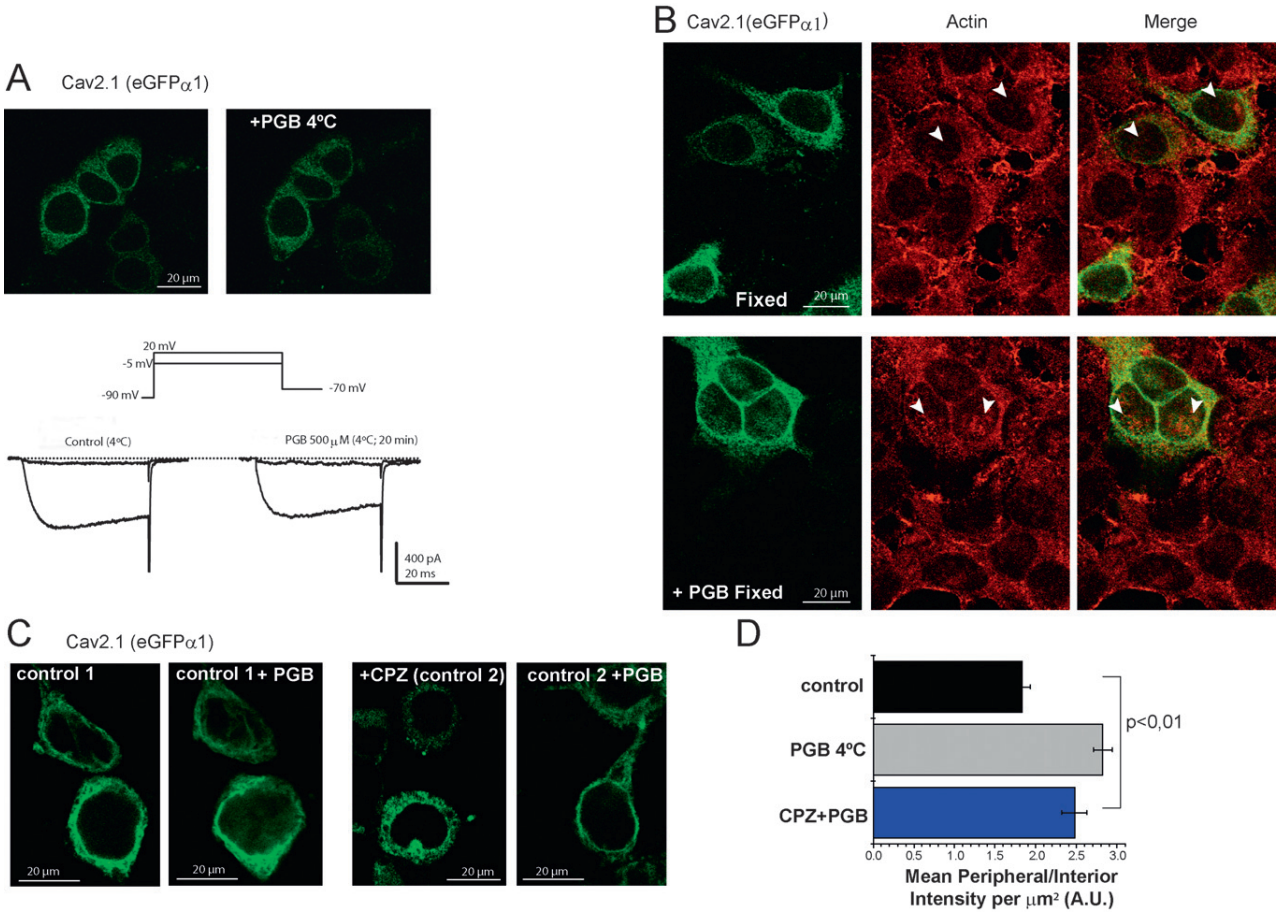

Fig. 4. Mechanism of internalization of $Ca_V2.1$ channels in HEK293t transfected cells. Micrographs of the same HEK293t cells transfected with eGFP$\alpha1 + \alpha_2\delta + \beta_3$ before (left) and after (right) incubation with PGB 500 µM at 4 °C; 60× water immersion objective (top) and 'whole-cell voltage-clamp' barium current measured at 4 °C before (bottom, left) and after (bottom, right) incubation of transfected cells with PGB. (B) Micrographs of single sections from a stack of images of fixed $Ca_V2.1$-expressing cells stained against actin filaments before (upper panels) and after (lower panel) PGB treatment to show eGFP fluorescence (in green) indicative of the channel presence, or actin (rabbit anti-actin antibody followed by goat tetramethyl-rhodamine anti-rabbit antibody; in red) and both channels (merge, in yellow); 60× oil immersion objective. (C) Micrograpsh of cells before and after PGB treatment (left); and treated with the endocytosis inhibitor chlorpromazine alone, or treated with CPZ for 30 min before PGB treatment (right). (D) Quantification of mean fluorescence intensity per μm^2 of Peripheral to intracellular regions (see Section 2.3 for details) for the different conditions used in (A), (C) and controls, $n = 15–22$ cells per condition. Scale bar 20 µm. Each micrograph is an average of middle sections of a stack of confocal images. Data analyzed using ANOVA and Bonferroni post hoc tests.

regulation of N-type channel activity in the pain pathway (Altier et al., 2006). We were able to show that the mechanism of PGB is associated to an endocytic process as demonstrated through patch-clamp as well as microscopy experiments performed at 4 °C (Fig. 4A).

Our work, combining the use of electrophysiology and fluorescent microscopy of fluorescently-tagged channels, favor also this type of mechanism for channels also relevant for a pain condition (Li et al., 2006), and give further support to an acute mechanism of action of PGB. The present work will help understand the pharmacological action of the drug to be used in different pathologies involving calcium channels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.brainresbull.2012.10.001.

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