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Parabens inhibit hNa$_V$ 1.2 channels

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ABSTRACT

Propylparaben, a commonly used antimicrobial preservative, has been reported as an anticonvulsant agent targeting neuronal Na^+ channels (Na$_V$). However, the specific features of the Na$_V$ channel inhibition by this agent have so far not been extensively studied. Moreover, it is still unclear if it shares this pharmacological activity with other parabens. Here, we fully characterized the mechanism of action of the inhibitory effect that propylparaben and benzylparaben induce on human Na$_V$ 1.2 channel isoform (hNa$_V$1.2). We established a first approach to know the parabens structural determinants for this channel inhibition. The parabens effects on hNa$_V$1.2 channel mediated currents were recorded using the patch-clamp whole-cell configuration on hNa$_V$1.2 stably transfected HEK293 cells. Propylparaben induced a typical state-dependent inhibition on hNa$_V$1.2 channel carried current, characterized by a left-shift in the steady-state inactivation curve, a prolongation in the time needed for recovery from fast inactivation and a frequency-dependent blocking behavior. The state-dependent inhibition is increased for butylparaben and benzylparaben and diminished for methylparaben, ethylparaben and p-hydroxybenzoic acid (the major metabolite of parabens hydrolysis). Particularly, butylparaben and benzylparaben shift the steady-state inactivation curve 2- and 3-times more than propylparaben, respectively. Parabens are blockers of hNa$_V$1.2 channels, sharing the mechanism of action of most of sodium channel blocking antiseizure drugs. The potency of this inhibition increases with the size of the lipophilic alcoholic residue of the ester group. These results provide a basis for rational drug design directed to generate new potential anticonvulsant agents.

1. Introduction

Epilepsy is a chronic neurological disorder that affects more than 50 million people worldwide. It is characterized by different levels of neuronal hyperexcitability producing recurrent seizures. Despite the wide diversity of available antiepileptic drugs (AEDs), there is still a high percentage (close to 30%) of therapeutic failure, caused by the lack of efficacy of available drugs to control seizures; furthermore, the occurrence of side effects frequently leads to treatment suspension [1,2]. This situation raises the need to find new compounds capable of controlling epilepsy, with improved efficacy and tolerability.

The pharmacological treatment of epilepsy involves, for most AEDs, the prevention of seizure onset and propagation by inhibition of neuronal hyperexcitability. An established anticonvulsant mechanism involves the inhibition of voltage-operated Na^+ channels (Na$_V$) expressed in the central nervous system (particularly the isoforms Na$_V$ 1.1, Na$_V$ 1.2, Na$_V$ 1.3, and Na$_V$ 1.6) which have a key role in the initiation and propagation of neural action potential discharge [3–5] Particularly, AEDs such as phenytoin, carbamazepine, lamotrigine, oxcarbazepine, eslicarbazepine, zonisamide, and lacosamide exert their pharmacological effect by a state-dependent inhibition of neuronal Na$_V$ channels due to their higher affinity for the open and inactivated channel states [6]. Moreover, interaction with these drugs stabilizes the channel in the inactivated state, making difficult their transition to the closed resting state and consequently reducing the action potential discharge and neuronal excitability [7].

Parabens are a homologous series of esters of p-hydroxybenzoic acid (including methyl, ethyl, propyl, butyl, and benzyl paraben, among others), used singly or in combination as antimicrobial preservatives in foods, drugs, and cosmetics for over 50 years. They are safe to use (i.e. relatively non-irritating, non-sensitizing, and low- toxic) and there have been several safety assessments on these substances by a diversity of

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agencies, including FAO/WHO, FDA and FEMA [8–10].

In a previous work, Talevi and col. (2007) reported that propylparaben (PPB) and methylparaben (MPB) elicited anticonvulsant effects in the Maximal Electroshock Seizure (MES) test in mice [11]. Later, it was shown that PPB blocks the Na_Vcurrents in rat brain slices and prevents the 4AP-induced epileptogenic discharges of hippocampus CA1 pyramidal neurons [12]. Besides, PPB also showed anticonvulsant effects against pilocarpine-induced seizures and reduced the neuronal damage and the excitability produced by status epilepticus and spontaneous recurrent seizures in rats [13].

The anticonvulsant effect of PPB in rodents, together with the valuable information about its safety and pharmacokinetics and their versatile chemistry, makes the parabens interesting candidates to investigate possible therapeutic applications as well as their mechanism of action. Here, using the patch-clamp technique, we fully characterized the mechanism of action of PPB on human Na_V 1.2 channels (hNa_V 1.2) expressed in HEK293 cells. Besides, we tested the effect of the other p-hydroxybenzoic acid esters of the series: MPB, ethylparaben (EtPB), butylparaben (BuPB) and benzylparaben (BePB), for a preliminary insight on a structure-activity relationship for this class of compounds. Finally, p-hydroxybenzoic acid, the major metabolite of parabens, was also investigated.

2. Materials and methods

2.1. Cell culture

All experiments were performed in HEK293 cells stably expressing $hNa_V1.2$ α-subunits (a kind gift of GlaxoSmithKline, Stevenage, UK) that have previously been described [14–16] and used in our lab [17,18]. HEK293 cells were cultured in minimum essential medium (DMEM HIGH), containing 10% fetal bovine serum, and 0.5% geneticin G418 sulfate, in a 5% CO_2 atmosphere at 37 °C.

2.2. Whole-cell voltage-clamp recordings

The cells were detached from the culture flask and settled in a glass-bottom experimental chamber with 3 mL extracellular solution containing (in mM): NaCl 50, N-methyl-D-glucamine 90, $CaCl_2$ 2, MgCl2 1, HEPES 10 and glucose 11; pH was adjusted to 7.4 with HCl. The patch electrodes were filled with pipette solution containing (in mM): CsF 100,CsCl 40, EGTA 10, HEPES 10, NaCl 5, $MgCl_2$ 2; Na_2ATP 4; the pH was adjusted to 7.3 with CsOH. Cells were observed with a mechanically stabilized inverted microscope (Telaval 3, Carl Zeiss, Jena, Germany) equipped with a 40X objective lens. The test solutions were applied through a multi-barreled pipette, by gravity and in continuous flux, positioned close to the target cell. After each experiment on a single cell, the experimental chamber was replaced by another one containing a new sample of cells. All experiments were performed at room temperature (~ 22 °C). The standard tight-seal whole-cell configuration of the patch-clamp technique was used to record macroscopic currents [19]. Glass pipettes were drawn from GB150T-10 glass on a microprocessor-controlled vertical pipette puller (PUL-100, World Precision Instrument, Sarasota, FL. USA) and pipette resistance ranged from 2 to 3 MΩ. Voltage-clamp protocols were applied by the computer-controlled Axopatch 200A amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA). Currents were filtered with a 4-pole lowpass Bessel filter at 2 kHz and digitized (Digidata 1440, Molecular devices) at a sampling frequency of 200 kHz. Compensation circuitry was used to reduce the series resistance error by at least 80%. The currents were corrected offline for linear nonspecific leak and residual capacitive current. The experimental recordings were stored on a computer hard disk for later analysis.

Na_V current amplitude and series resistance stability were evaluated with a 15 ms-voltage-clamp step from a holding potential of − 80 mV to a test potential of − 20 mV repeated each 10 s. The time needed for the current stabilization was variable. Once the stability was achieved, the same voltage protocols (see below) were applied in the control conditions (vehicle) and in the presence of parabens, dissolved in 0.1% dimethylsulfoxide. Each protocol was performed at least twice in each extracellular solution to check stability.

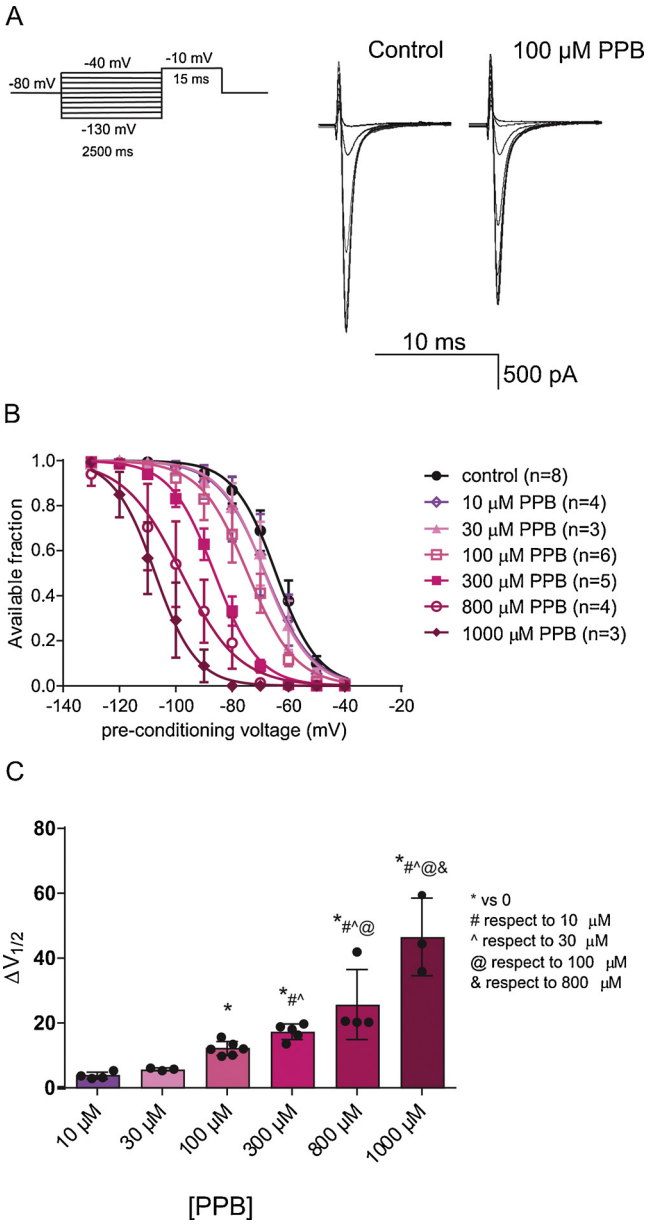

Fig. 1. PPB induces a left-shift in steady-state inactivation curves of $hNa_V1.2$ channels in a concentration-dependent manner. A) Superimposed typical traces of the $hNa_V1.2$ channel mediated currents obtained in HEK293 cells at the test pulse of the steady-state inactivation curves (protocol given as inset), before and after 100 µM PPB perfusion. B) Mean steady-state inactivation curves obtained from recordings at different PPB concentrations. The available fraction was calculated as the peak current observed on each test pulse normalized by the one corresponding to the −130 mV pre-conditioning pulse (I/Imax). Solid lines represent the fit of the points of each condition with a Boltzmann equation. C) Mean $V_{1/2}$ displacement values at different PPB concentrations. The comparison with the control was made using one-sample *t*-test against a hypothetical value of zero. To compare different concentrations of PPB one-way ANOVA was used followed by a post hoc Tukey´s multiple comparisons test. (N = 3-6).

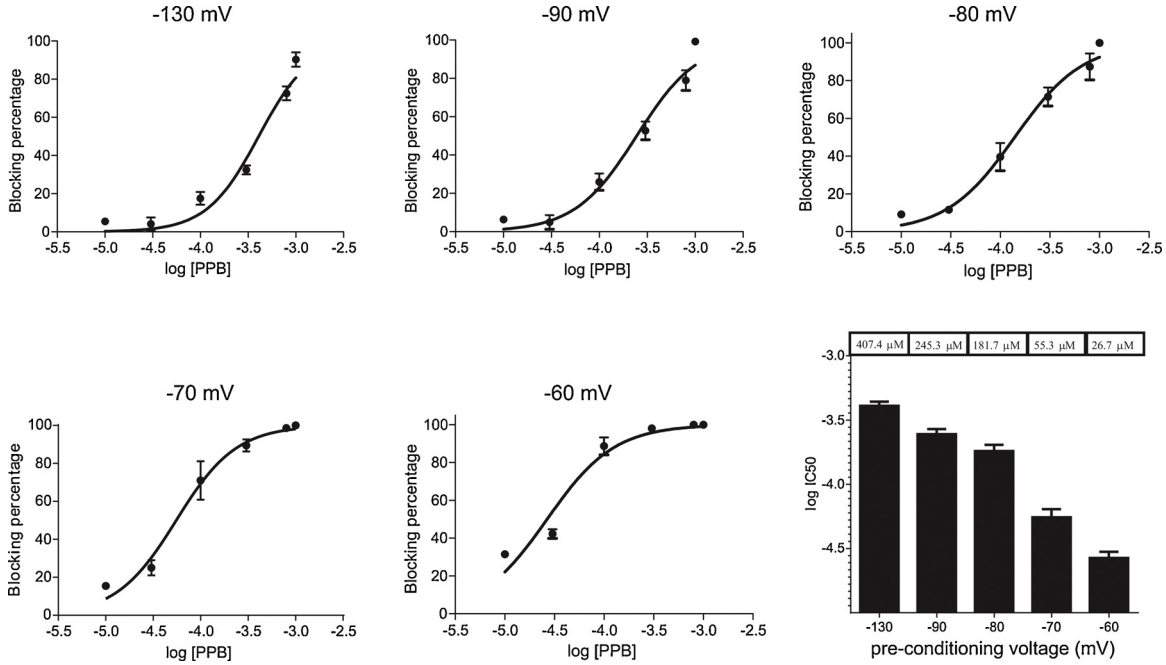

Fig. 2. Voltage-dependence of PPB-induced hNa$_V$1.2 channel inhibition. PPB concentration-response curves of hNa$_V$1.2 channel mediated current inhibition to pre-conditioning pulses of −130 mV, −90 mV, −80 mV, −70 mV and −60 mV. (N = 3-6). Solid lines represent the fit of the points of each condition with a Hill equation. Bar graph represents the IC_{50} values obtained from curves adjustments corresponding to mentioned above pre-conditioning potentials.

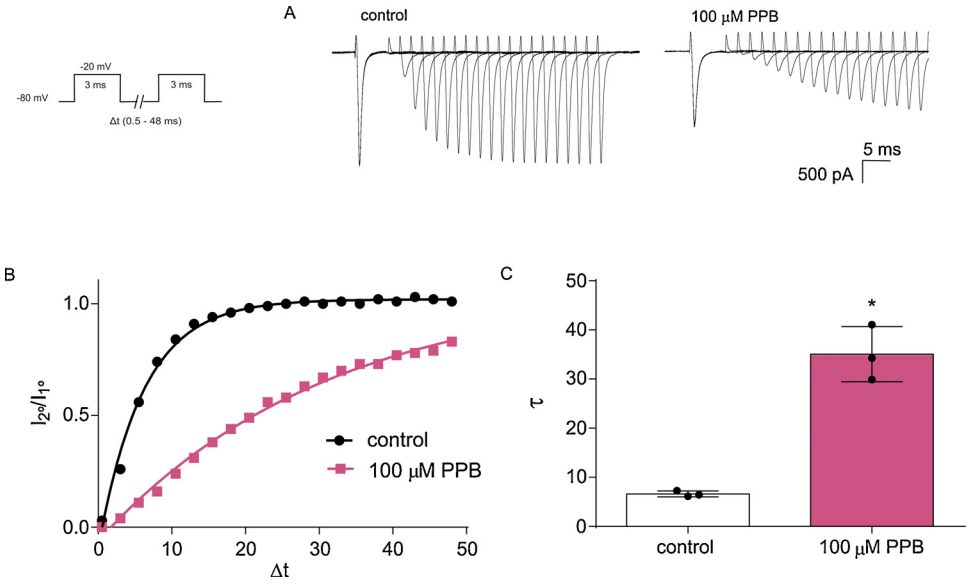

Fig. 3. PPB increases the hNa$_V$1.2 channel time for recovery from inactivation. A) Superimposed typical traces of the hNa$_V$1.2 channel mediated currents obtained from the double-pulse protocol before and after 100 µM PPB perfusion. B) Current ratio between the second and the first pulse as a function of the time elapsed between them for one of the typical cells. Solid lines represent the fit of the points of each condition with an exponential relationship. C) Mean time constant values (τ) obtained for control and 100 µM PPB group. Paired t-test (N = 3).

2.3. Voltage-clamp protocols

The effect of parabens on the inactivated state of hNaV1.2 channels was evaluated by using a typical double voltage step protocol. From a resting potential of -80 mV, we applied a series of pre-conditioning steps during 2500 ms (from -130 to −40 mV), each followed by a test pulse of 25 ms to −10 mV. The magnitude of peak current evoked by the test pulse to −10 mV depends on the available channels to be opened after each pre-conditioning pulse. The available fraction at each potential was calculated as the relationship between the peak current measured in the test pulse preceded by each pre-conditioning step (I) and the one evoked after a pulse of −130 mV where in our recordings the current was the maximal (Imax). Then, plotting the available fraction (I/Imax) against the voltage of the pre-conditioning voltage step, allows obtaining the h curve which was fitted with the Boltzmann equation to obtain the $V_{1/2}$ (the potential of half-maximal inactivation) and the slope k parameter (Eq. 1).

$$\frac{I}{Imax} = \frac{1}{1 + e^{\frac{V_{1/2} - V}{k}}} \quad (1)$$

The ability of parabens to modify the time required for channel recovery from the inactivated state, was tested by using a double-pulse protocol where two depolarizing voltage steps from −80 mV to −20 mV were applied separated by a variable time interval at −80 mV (from 0.5–48 ms), during which the current was allowed to recover from the inactivation induced by the first voltage step. Then, the ratio between the peak current values of the second pulse and the one corresponding to the first pulse was plotted against the time between the two pulses. The points were fitted with an exponential function (Eq. 2)

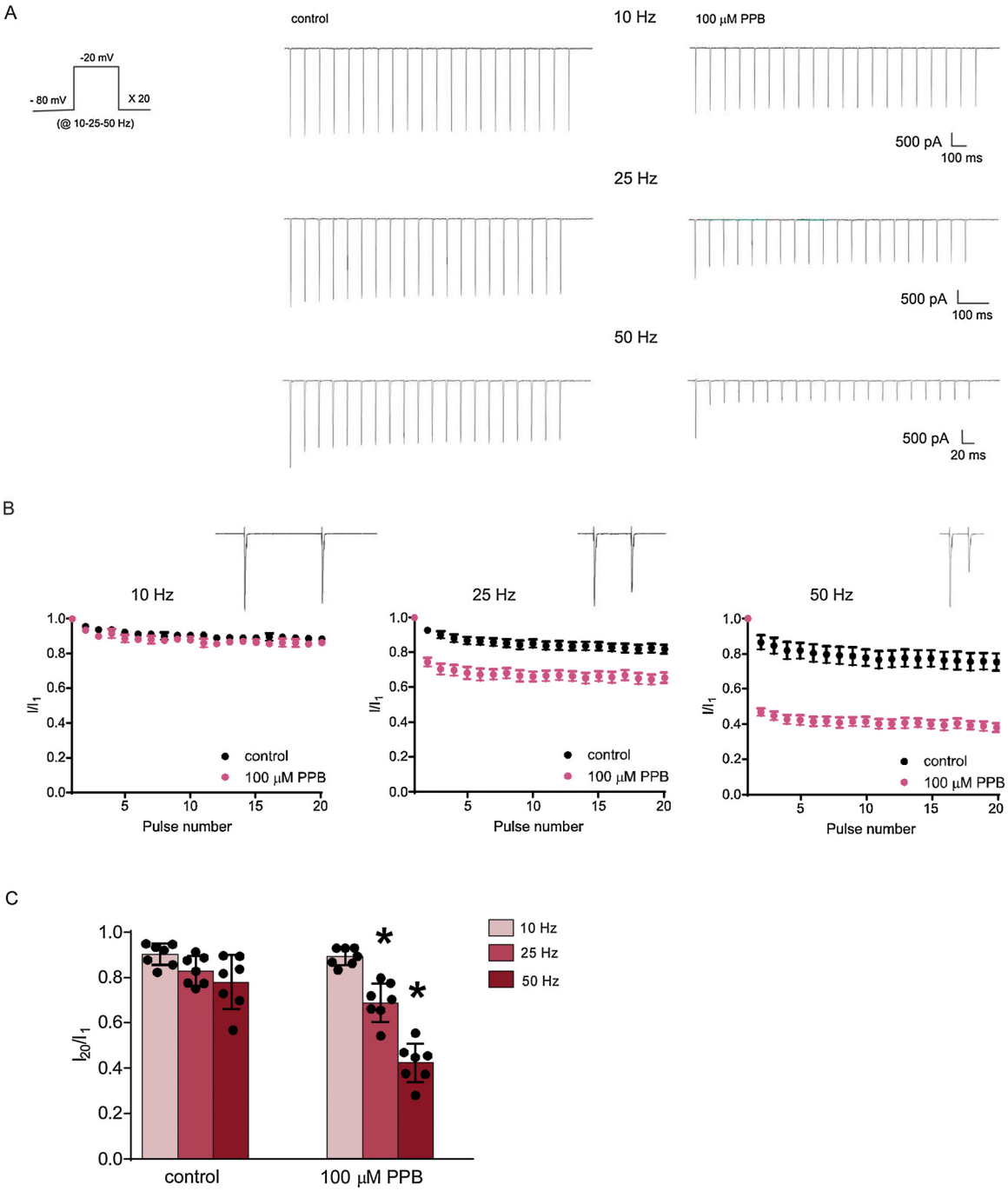

Fig. 4. PPB promoted a frequency-dependent inhibition on hNa$_V$1.2 channels. A) Typical traces of the hNa$_V$1.2 channel mediated currents evoked by a train voltage pulses before and after 100 µM PPB perfusion. B) Mean current ratio between each pulse and the first pulse vs. pulse number (N = 7). The inset graphs represent the recordings of the first and the last pulses obtained in the presence of 100 µM PPB. C) Mean current ratio between the last and the first pulse obtained at frequency of 10 Hz, 25 Hz and 50 Hz. The asterisk means that currents are significantly affected stimulatory frequency and PPB (two-way ANOVA, N = 7, Bonferroni post-test).

to obtain the temporal parameter of the Na$_V$ channels recovery from inactivation under control and treated conditions.

$$I_{2^\circ}/I_{1^\circ} = 1 - e^{-\Delta t/\tau} \quad (2)$$

where I2°/I1° represents the available fraction of recovered channels and τ is the time constant of this process.

The frequency-dependent block of parabenswas studied applying 20 identical voltage pulses of 3 ms at −10 mV, from a holding potential of -80 mV, at a constant frequency (10 Hz, 25 Hz and 50 Hz). The ratio between the peak current values (I_x) evoked by each pulse with the one corresponding to the first pulse (I_{1°) was plotted against the number of pulse. The frequency dependence was measured as a reduction in the ratio of current values at the 20th pulse when the tested frequency increases.

Furthermore, the data obtained from h curves were also useful to analyze if parabens were able to bind to the closed channel state. When the pre-conditioning applied potential was −130 mV the closed channel state fraction was maximal in all conditions evaluated, so, we measured the magnitude of Na$_V$ peak current occurring at the corresponding test pulse (I control) in control condition, and after incubation

Table 1
Effect of 100 μM parabens on $Na_V1.2$ peak current evoked at -20 mV from a holding potential of -80 mV. The inhibitory effect was statistically significant for all tested parabens (paired *t*-test). Additionally, one way ANOVA (on Tukey's) was used to compare the level of inhibition between the different parabens: * represents differences respect to MPB, while $^{+}$ respect to EtPB, $^{@}$ respect to PPB and $^{\#}$ respect to BuPB.

Paraben (100 μM)	blocking percentage	SEM	N
MPB	11.4	1.0	4
EtPB	15.0	2.9	3
PPB	39.7*	6.2	6
BuPB	$72.2^{*+@}$	6.3	6
BePB	$93.8^{*+@\#}$	3.4	5

of 1 − 5 min with the drug. The ratio of these magnitudes allow us to quantify the inhibitory effect of parabens on closed or resting channel state (Eq.3)

$$closedchannelinhibition = I_{max}^{paraben} \Big/ I_{max}^{control} \quad (3)$$

Also, using the data from *h* curves, we constructed the concentration-response curves of the inhibitory effect of PPB and BePB on $hNa_V1.2$ currents elicited at −10 mV, after the pre-conditioning pulses of −130 mV, −90, −80, −70 or −60 mV. Then, the relationships were fitted with a Hill relationship (Eq. 4) to calculate the IC50.

$$blocking\ percentage = \frac{100}{1 + 10^{[(\log IC_{50} - X) \times Hill\ Slope]}} \quad (4)$$

where X is the concentration if the tested paraben. As we defined above, the curve corresponding to the pre-conditioning pulse of −130 mV represents the concentration dependence of the closed state block, while the remaining curves show addition of the voltage-dependent and -independent inhibition.

2.4. Data and statistical analysis

Statistical analyses were carried out using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Data are given as the mean ± SEM. Multiple groups were compared using a one-way or two-way ANOVA followed by a *post hoc*Tukey´s and Bonferroni tests, respectively for multiple comparisons test. Paired Student's *t*-test was used for the direct comparison of two conditions. Statistical significance of the change in the $V_{1/2}$ parameter induced by parabens was tested by one-sample *t*-test against a hypothetical value of zero.Statistical significance of percentage of closed channel blockage was tested by one-sample *t*-test against a hypothetical value of 1. $P < 0.05$ was considered to indicate a significant difference.

2.5. Materials

PPB (Parafarm, Bs As, Argentina) and BePB (Merck, Darmstast, Germany) were dissolved in DMSO (Merck, Darmstast, Germany) to make stock solutions of 3, 10, 30, 60, 100, 300 and 1000 mM. EtPB (Merck, Darmstast, Germany), MPB (Parafarm, Bs As, Argentina), BuPB (Merck, Darmstast, Germany),) and p-hydroxybenzoic acid (Fluka) were dissolved in DMSO to make a stock solution of 100 mM. An appropriate amount of DMSO was added to all control solutions.

3. Results

3.1. Inhibitory effect of PPB on hNaV1.2 channels

We first evaluated the ability of PPB to block $hNa_V1.2$ channels in a state-dependent manner by applying the double voltage step protocol described in the Materials and Methods section. In Fig. 1 (B and C) are depicted the data showing that PBB induces a significant left-shift of the *h* curve, quantified by the change in the $V_{1/2}$ measured for each cell, before and after a stable effect of PPB at different concentration ($\Delta V_{1/2}$). This effect suggests that PBB in a concentration-dependent way can bind to the inactivated state of hNav1.2 channels and stabilize this conformation. Moreover, is possible to observe in Fig. 1B that, after the pre-conditioning potential of −130 mV the available fraction of $hNa_V1.2$ channels (at the closed state) was maximal at all tested PPB concentrations. However, the current amplitude in the test pulse is visibly reduced when compared with the magnitude of the control current evoked after the same pre-conditioning potential (Fig. 1A). This fact can be explained by considering that PBB, not only blocks the inactivated state of the channel, like the left shift of *h* curve demonstrates, but also it is able to block the closed state of the hNa1.2v channel decreasing the number of channels available to open during the test potential.

After this analysis, we can observe that PBB is able to inhibit both, the closed and the inactivated state of Nav1.2 channels. Thus, we perform additional analysis to evaluate the weight of the two components in the global inhibitory effect, which is presented in Fig. 2, where the percentage of PBB blocking effect on Na + current magnitude (evoked by the test pulse after different preconditioning potentials) was plotted versus PBB concentration. Then, the relationships were fitted with Eq.4. Particularly, the IC_{50} obtained at the preconditioning pulse of −130 mV exclusively depends on the binding of PPB to the Nav1.2 channels closed state. However, we observed an increase in drug affinity (a reduction in the obtained IC_{50}s) when the pre-conditioning potential was more depolarized and the proportion of channels at the inactivated state increases. This result suggests that PPB affinity for the inactivated state is higher respect for the closed state, which is clearly evident at lower concentrations (e.g.: 10 and 30 μM) where the inhibitory effect at the closed state (−130 mV of preconditioning pulse) is null, but there is a clearly blocking at preconditioning potentials where the channels can adopt the inactivated states.

3.2. hNa_V 1.2 channel recovery from fast inactivation is delayed by PPB

To evaluate the effect of PPB on hNa_V 1.2 channel recovery from fast inactivation, we performed a standard protocol where two depolarizing steps (to -20 mV) separated by a variable recovery time at -80 mV was applied before and after 100 μM PPB perfusion (Fig. 3). The temporal course of the recovery from inactivation state is shown in Fig. 3B for both conditions, where it is possible to observe that PPB significantly increases the rate constant of the process (τ: 35.1 ± 3.2 ms for PPB vs 6.6 ± 0.3 ms for the control group). This result indicates that $hNa_V1.2$ channels in the presence of PPB need more time to recover from the inactivated state, which is consistent with the stabilizing effect induced by PPB and evidenced before (Fig. 1).

3.3. Frequency-dependent blockage induced by PPB

Most of the AEDs that bind to the inactivated state of the Na_V channels and prolong the time of recovery from fast inactivation are able to promote a frequency-dependent blocking effect. We evaluated if PPB shares this property by measuring the $hNa_V1.2$ current activated by a train of 3 ms depolarizing steps, at either 10, 25, and 50 Hz, in control conditions and the presence of 100 μM PPB. As shown in a typical $hNa_V1.2$ current recording performed at different frequencies of pulse stimulation (Fig. 4A), already in the control condition, is possible to observe that the magnitude of peak currents shows a decay during pulsing, and this is increased in the presence of 100 μM PPB (Fig. 4 B). The effect was frequency-dependent since the same number of test pulses induced a significant higher block when applied at frequencies of 25 and 50 Hz (Fig. 4C). These results reinforce the idea that PPB, likely by inducing stabilization of $hNa_V1.2$ channel inactivated state, can reduce the resting state available fraction of channels that could be activated by a second depolarizing stimulus when cells are exposed at a

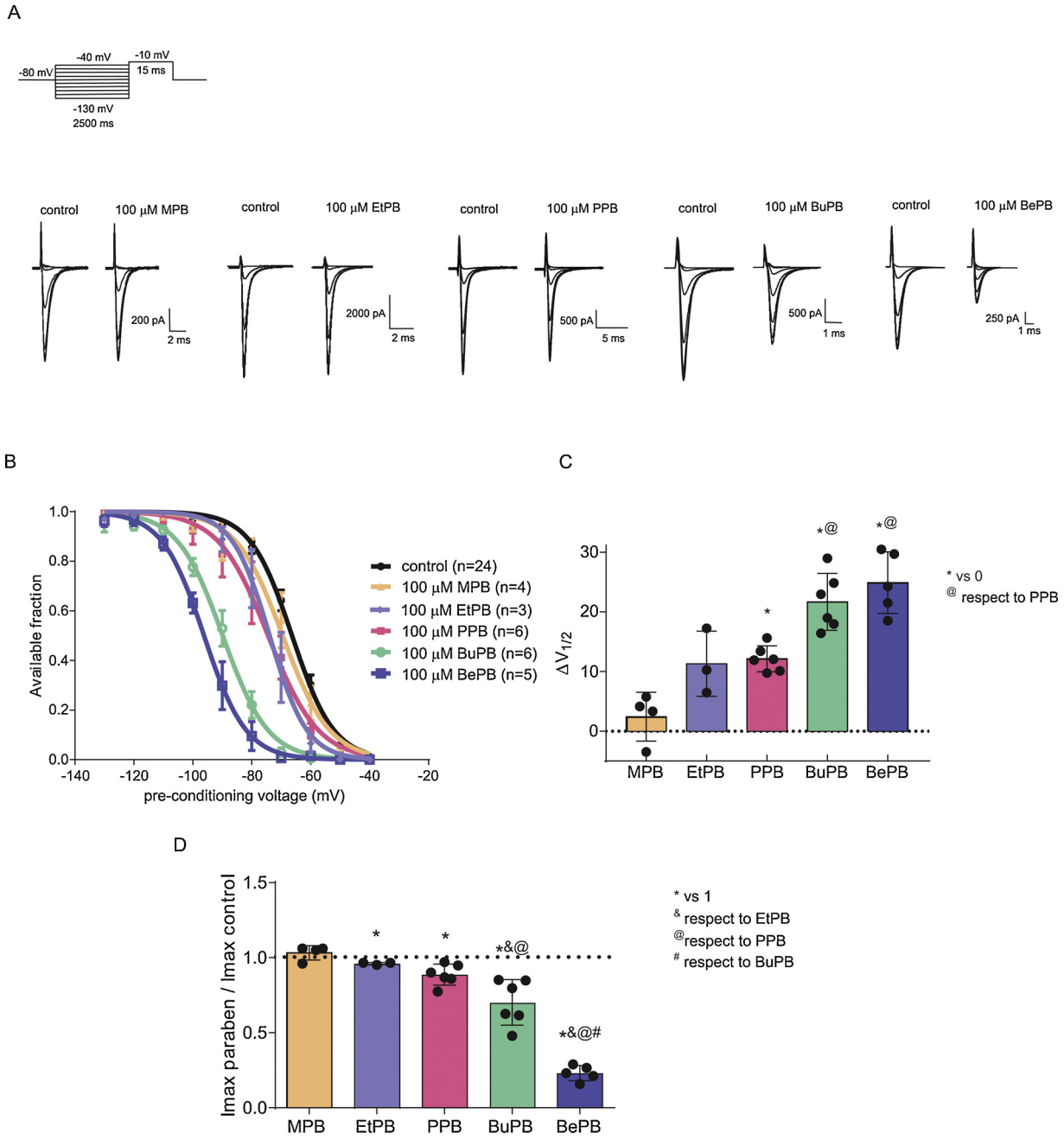

Fig. 5. Parabens inhibitory effect on hNa$_V$1.2 channels is both voltage-dependent and independent. A) Superimposed typical traces of the hNa$_V$1.2 channel mediated currents obtained at the test pulse of the steady-state inactivation protocol before and after 100 µM MPB, EtPB, PPB, BuPB or BePB perfusion. B) Mean steady-state inactivation curves obtained from recordings with the tested parabens. The available fraction was calculated as the peak current observed on each test pulse normalized by the one corresponding to the −130 mV pre-conditioning pulse (I/Imax). Solid lines represent the fit of the points of each condition with a Boltzmann equation. C) Mean $V_{1/2}$ displacement values. The comparison with the control was made using one-sample *t*-test against (vs 0). To compare the effect of BuPB and BePB respect to PPB, one-way ANOVA was used followed by a post hoc Tukey´s multiple comparisons test. D) Mean values of parabens closed state block calculated as the inhibition in the current evoked after a pre-conditioning pulse of −130 mV. The comparison with the control was made using one-sample *t*-test against (vs 1). To compare the effect of different parabens, one-way ANOVA was used followed by a post hoc Tukey´s multiple comparisons test.

high frequency of stimulation.

3.4. Comparison of PPB effects with MPB, EtPB, BuPB, and BePB on hNa$_V$ 1.2 channels

The first screening of the series of parabens showed that all of them, tested at 100 µM, inhibited hNa$_V$ 1.2 channels, which was evidenced by a reduction of peak Na^+ current measured at -20 mV from a holding potential of -80 mV (Table 1). These values show that the inhibitory effect increases with the lipophilicity of the residue in the ester function. So, we explored if all of these parabens share the blocking mechanisms observed for PPB. We probed that BuPB and BePB produced a significant left-shift in the *h* curves (Fig. 5A-C) and a reduction in the Na^+ current evoked after the pre-conditioning pulse of −130 mV (Fig. 5D). The last effect was also observed for EtPB. And again, both properties increased with the lipophilicity of the ester function.

Then, we tested the frequency-dependent inhibition of the complete paraben series. In this case, only the 50 Hz train pulse was used and each paraben was tested at a concentration of 100 µM, except for BePB, which was tested at 30 µM since, at 100 µM, it achieves and immediate

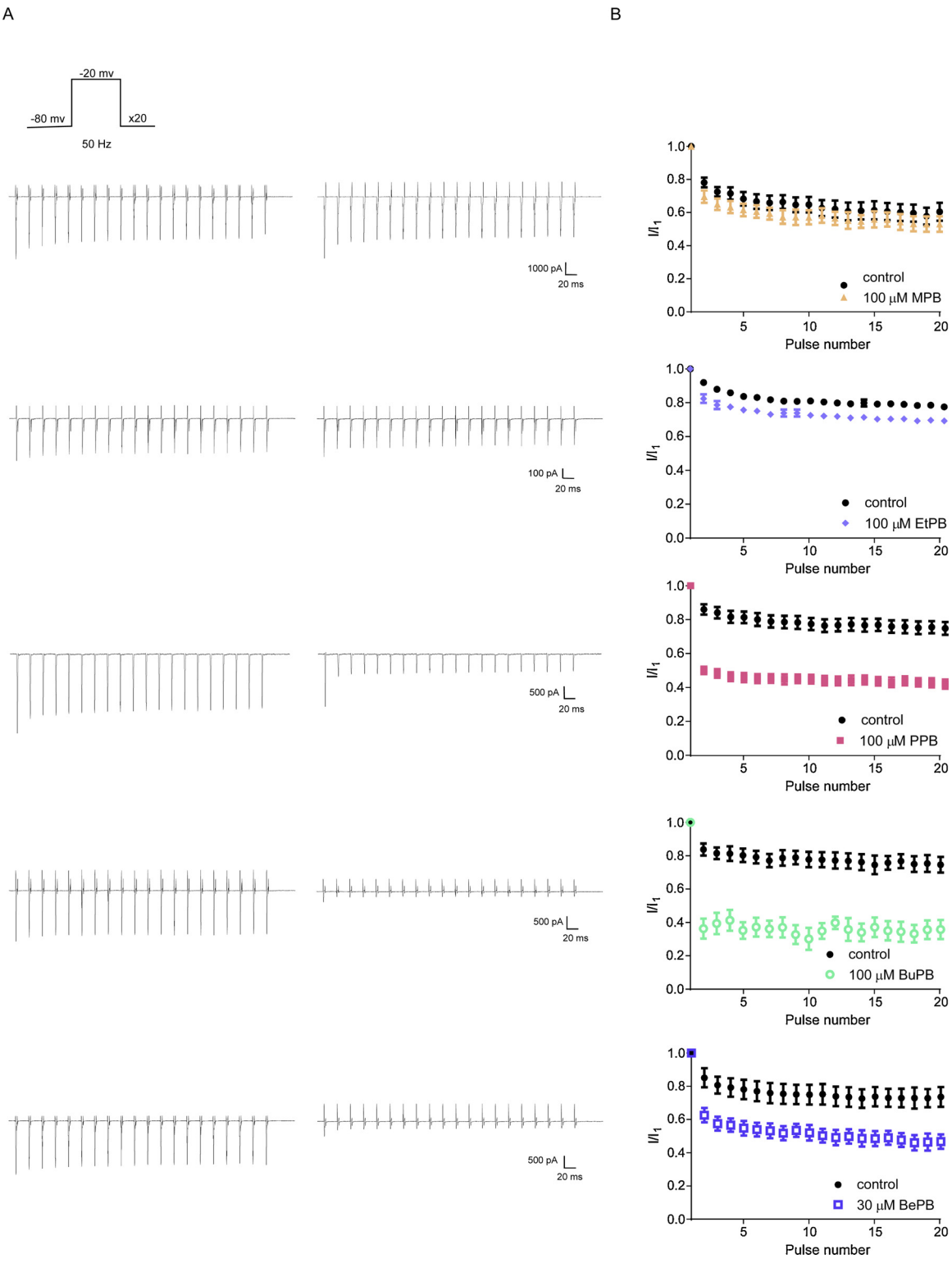

Fig. 6. Frequency-dependent blockage of hNa$_V$1.2 channels by parabens. A) Typical traces of the hNa$_V$1.2 channel mediated currents evoked by a train voltage pulses before and after MPB, EtPB, PPB, BuPB and BePB perfusion. B) Mean current ratio between each pulse and the first pulse vs. pulse number (N = 3-6).

and full inhibition (data not shown). The obtained results presented in Fig. 6 and Table 2, where it can be observed that, except for MPB, all compounds significantly produce a frequency-dependent blockage as PBB do.

3.5. Characterization of BePB inhibition of hNa$_V$1.2 channels

As clearly show our results, the benzyl group (Be) confers the major potency of all parabens tested. Then, we fully characterized the electrophysiological profile of BePB blockage on hNa$_V$1.2 channels.

First, we analyzed the concentration-dependence of BePB hNa$_V$1.2 current inhibition. This paraben, in a concentration-dependent manner,

Table 2
Parabens frequency-dependent block. I_{20}/I_1 represents the fraction of current recorded in 20th pulse respect current recorded in the first pulse. * indicates that differences are statistically significant (Paired *t*-test).

Paraben	Condition	I_{20}/I_1(mean ± SEM)	N
100 μM MPB	control	0.54 ± 0.05	4
	MPB	0.41 ± 0.09	
100 μM EtPB	control	0.77 ± 0.01	3
	EtPB	0.70 ± 0.02*	
100 μM PPB	control	0.78 ± 0.05	6
	PPB	0.39 ± 0.03*	
100μM BuPB	control	0.73 ± 0.05	6
	BuPB	0.40 ± 0.05*	
30 μM BePB	control	0.74 ± 0.06	5
	BePB	0.47 ± 0.04*	

significantly changed the $V_{1/2}$ toward a more hyperpolarized membrane potential (Fig. 7). Moreover, and in the same way as PBB, for BePB the IC_{50} is reduced when preconditioning pulses are more depolarized (Fig. 8).

Regarding the recovery time from the fast inactivation state, BePB delayed the recovery kinetic since the time constant value (τ) was significantly higher respect to the control value (Fig. 9). Finally, we demonstrated that 30 μM BePB blocks the channels in a frequency-dependent manner, which was evident at 25 and 50 Hz while it did not affect the Na^+ current decay when it was stimulated at a 10 Hz frequency (Fig. 10).

3.6. p-hydroxybenzoic acid effect on $hNa_V1.2$ channel mediated currents

As a final point, and considering that p-hydroxybenzoic acid (PHBA) is the major metabolite from parabens, resulting from the ester function hydrolysis [20,21], we also tested its effect on $hNa_V1.2$ currents evoked by the steady-state inactivation protocol. Interestingly, 100 μM PHBA produced a significant left-shift of the $hNa_V1.2$ *h* curve ($\Delta V_{1/2}$ = 2.4 ± 0.6) suggesting that it is able to stabilize the inactivated state of $hNa_V1.2$ channels (Fig. 11A-C). However, p-hydroxybenzoic did not produce a significant blockage of closed state channels as the other parabens did (Imax p-hydroxybenzoic acid/ Imax control = 0.9 ± 0.1; one-sample *t*-test vs. 1. *N* = 5) (Fig. 11D). The PHBA effect was mild when compared with the ones observed for PPB and the more potent parabens such as BuPB and BePB.

4. Discussion

In the present study, a preliminary structure-activity relationship of a series of parabens as $hNa_v1.2$ channel inhibitors was established, and characterized the electrophysiological properties of their mechanism of action. First, we show that PBB, a common preservative, and the first paraben described as a Na_V channel inhibitor with anticonvulsant properties [11–13] is able to inhibit the hNav1.2 channel isoform in a state-dependent manner. Then, we demonstrated that hydrophobicity of the residue at the ester group is determinant in the potency for the channel block, being the BePB the most potent among the parabens tested. So, here appeared a new promising compound sharing the blocking profile mechanism of several AEDs, which modulates Nav channels [16,22,23].

Nav channels blockers are commonly used in the clinic as antiepileptic, antiarrhythmic, and anesthetics. Despite these drugs share a common binding domain in the pore of the channel, they differ in the affinity for the different conformational states that the drugs can stabilize. The ability of Nav channel inhibitors to discriminate between states (closed, open, and inactivated) was early described by B. Hille and known as the "modulated receptor hypothesis" which shows as they promote different blocking profiles well correlate with the clinical utility of Nav channels inhibitors [24,25]. Here, we demonstrated that

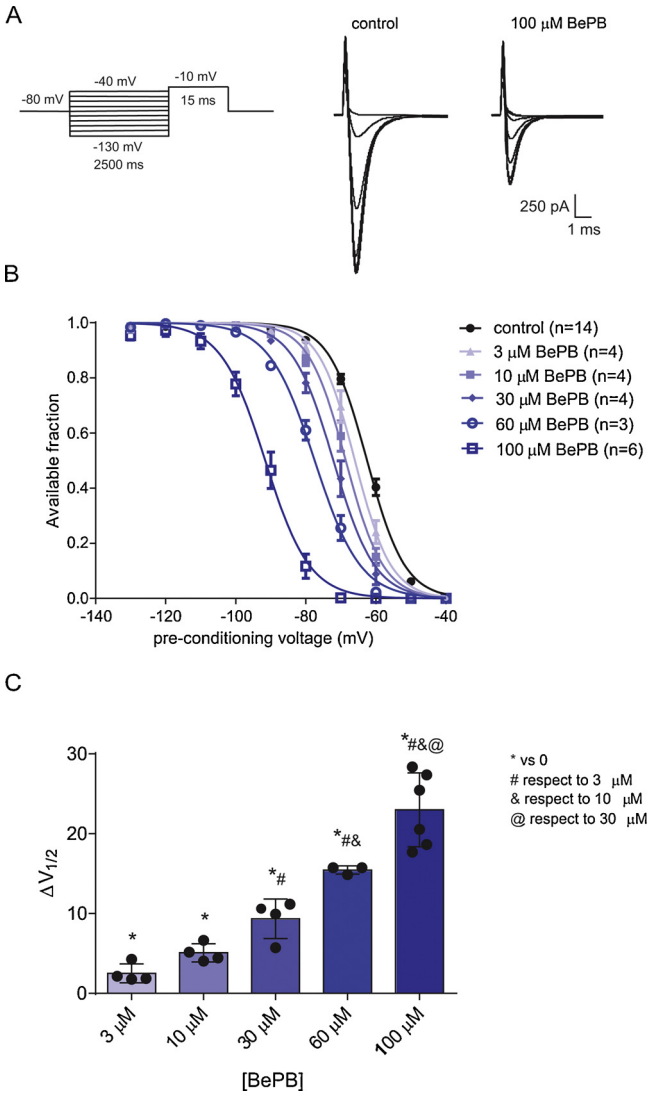

Fig. 7. BePB induces a left-shift in steady-state inactivation curves of $hNa_V1.2$ channels in a concentration-dependent manner. A) Superimposed typical traces of the $hNa_V1.2$ channel mediated currents obtained at the test pulse of the steady-state inactivation curves (protocol given as inset), before and after 100 μM BePB perfusion. B) Mean steady-state inactivation curves obtained from recordings at different BePB concentrations. The available fraction was calculated as the peak current observed on each test pulse normalized by the one corresponding to the −130 mV pre-conditioning pulse (I/Imax). Solid lines represent the fit of the points of each condition with a Boltzmann equation. C) Mean $V_{1/2}$ displacement values at different BePB concentrations. The comparison with the control was made using one-sample *t*-test against a hypothetical value of zero. To compare different concentrations of BePB one-way ANOVA was used followed by a post hoc Tukey´s multiple comparisons test. (*N* = 3-6).

PPB and BePB, as reference compounds of the paraben series, inhibit hNav1.2 channel by a mechanism that presents a major activity on the inactivated state of the channel. We demonstrated that PPB and BePB can stabilize the inactivated state of hNav1.2 channel because of in presence of these compounds, more negative membrane potentials are necessary to have the same available hNav1.2 fraction than in control condition (Figs. 1 and 7), and a longer time is needed to recover the channels from the fast reached inactivated stated (Figs. 3 and 9). However, our data also showed an inhibitory effect on the closed state of the channels, which was visible as the Na^+ current reduction when the cell was preconditioned with very negative membrane potential (−130 mV) where the proportion of channels in the inactivated state is

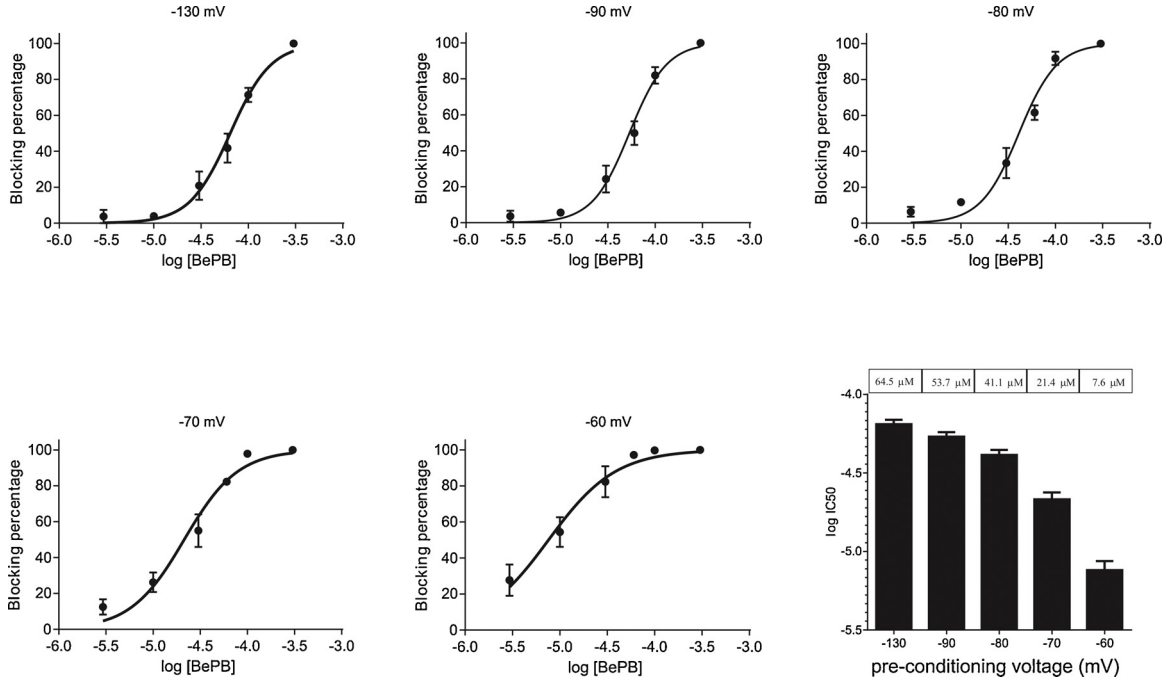

Fig. 8. Voltage-dependence of BePB-induced hNa$_V$1.2 channel inhibition. BePB concentration-response curves of hNa$_V$1.2 current inhibition to pre-conditioning pulses of −130 mV, −90 mV, -80 mV, −70 mV and −60 mV. (N = 3-6). Solid lines represent the fit of the points of each condition with a Hill equation. Bar graph represents the IC50 values obtained from curves adjustments corresponding to mentioned above pre-conditioning potentials.

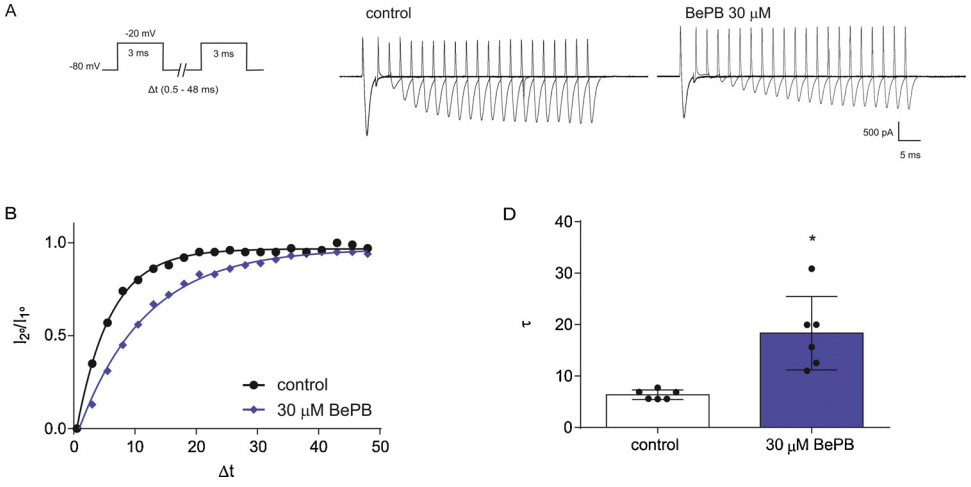

Fig. 9. BePB increases the hNa$_V$1.2 channel time for recovery from inactivation. A) Superimposed typical traces of the hNa$_V$1.2 channel mediated currents obtained from the double-pulse protocol before and after 30 µM BePB perfusion. B) Current ratio between the second and the first pulse as a function of the time elapsed between them for one of the typical cells. Solid lines represent the fit of the points of each condition with an exponential relationship. C) Mean time constant values (τ) obtained for control and 100 µM BePB group. Paired t-test (N = 6).

null (Fig. 1 and 7). However, a higher affinity of these parabens for the hNav1.2 channel inactivated state, respect to the closed state, was observed as a lower IC_{50} of channel inhibition obtained when depolarized membrane potential promotes an increase in the numbers of channels in the inactivated state.

On the other hand, when the activity of Nav channels is induced by a repetitive stimulation, most of Nav channels blockers can promote a Na^+ current reduction, by different mechanisms. The open channel blockers require the opening of channels to allow the drug binding to a channel domain accessible only is the channel reaches the open state. Thus, under repetitive stimulation, there is an additive channel blockage at each pulse of the train stimulation, since more channels are passing through the open conformation. This blocking mechanism, called use-dependent block, depends on the number of channels that adopt the open conformation and it is low influenced by the time occurred between each pulse, in other words, the frequency of the pulse train. In contrast, if the time between two stimuli is short (higher stimulation frequencies), drugs that delay the recovery from the inactivated state reduces the available fraction of Nav channels to be opened at the next pulse. This reduction in the available fraction is additive in subsequent pulses and increases whit the frequency of stimulation [24,25]. Here, we demonstrate that the hNav1.2 channel block by PPB and BePB is frequency-dependent (Figs. 4 and 10) confirming their preference for the inactivated state of the channel.

These properties of the state-dependent block directed to the inactivated state is a common feature of AEDs that target Nav channels because it confers some advantages as achieving a selective effect on depolarized neurons, and therefore hyperexcitable, that participate in the epileptic focus [4,23–25]and became important to diminish the activity in neurons with higher frequencies of firing. Therefore, we can hypothesize that neurons involved in seizure propagation presenting higher firing frequencies[4] would be more susceptible to the Na$_V$ channel inhibition induced by parabens, and neurons that are not participating in seizure initiation and/or propagation would be less affected. This fact makes parabens interesting candidates also in terms of reducing possible adverse effects related to their effects on well-

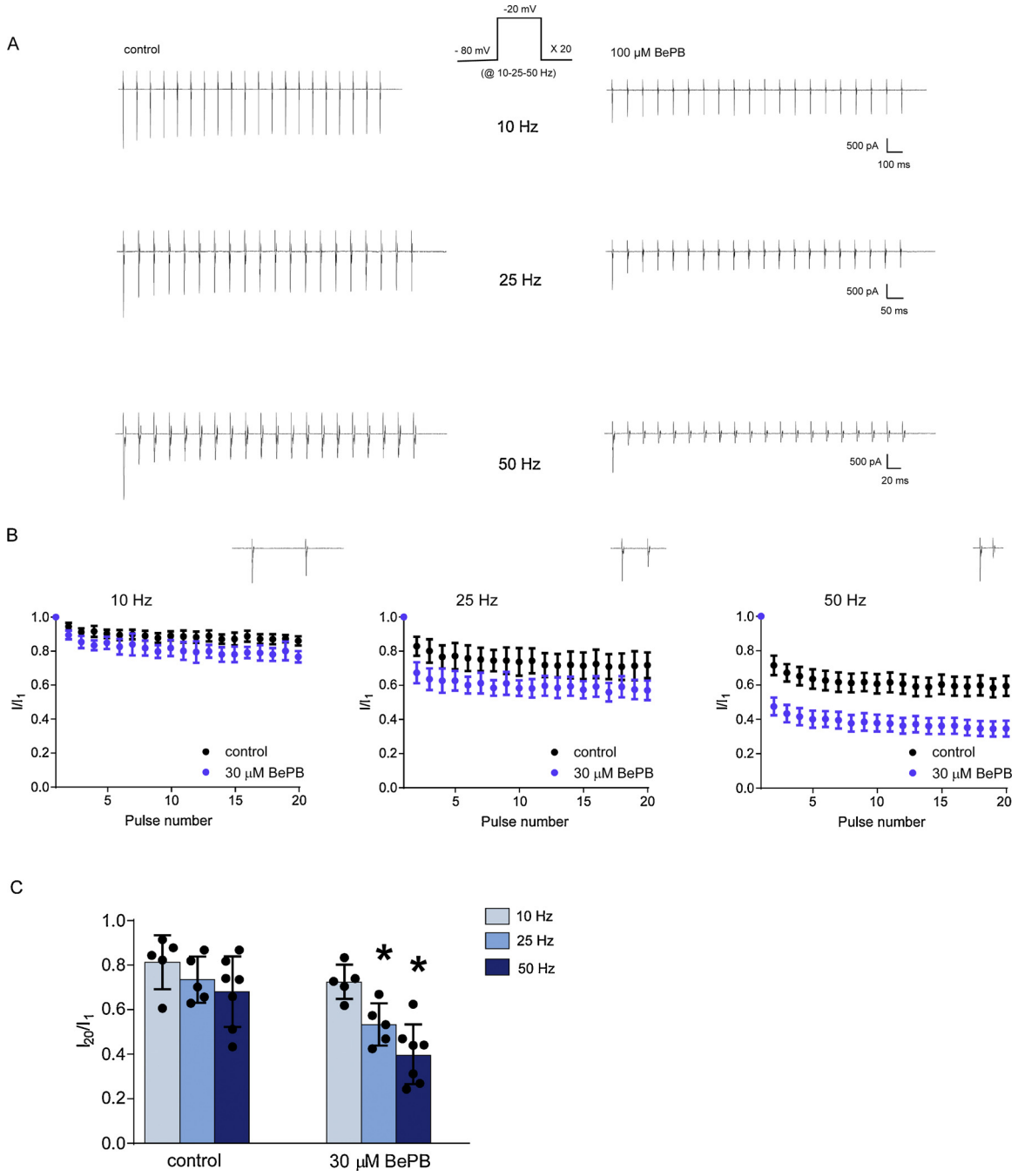

Fig. 10. BePB promoted a use-dependent inhibition on hNa$_V$1.2 channels A) Typical traces of the hNa$_V$1.2 channel mediated currents evoked by a train voltage pulses before and after 30 µM BePB perfusion. B) Mean current ratio between each pulse and the first pulse vs. pulse number (N = 5-7). The inset graphs represent the recordings of the first and the last pulses obtained in the presence of 30 µM BePB. C) Mean current ratio between the last and the first pulse obtained at frequency of 10 Hz, 25 Hz and 50 Hz. The asterisk means that currents are significantly affected stimulatory frequency and BePB (two-way ANOVA, N = 5-7, Bonferroni post-test).

functioning neurons.

The ability of Nav channels blockers to discriminate between states is related to the facility to access to their common binding site. Open channel blockers are mostly hydrophilic and require an aqueous pathway that appears with the opening of the intracellular activation gate, which is tightly closed. In contrast, small hydrophobic drugs can access the binding site from the lipid membrane using fenestrations sideways into the drug receptor site without requiring channel opening [24,26]. The latter mechanism could be an explanation for the increased parabens activity with the hydrophobicity of the residue at the ester function (Figs. 5 and 6). Particularly, the potency in state-dependent inhibition of BePB was three-times higher, than PPB, so they are expected to present a higher in vivo activity. Knowing the protective effects of PPB in mice [11] and rats [13], it is expected that BePB will present a higher anticonvulsant effect *in vivo*.

We also demonstrated that the main metabolite of parabens, the p-hydroxybenzoic acid shared the state-dependent inhibitory effect with their esterified analogous. Although its potency was much lower compared with parabens, it could contribute to their anticonvulsant activity, exerting a residual effect after the metabolism of parabens, and so extending the time of the anticonvulsant in vivo effect.

Parabens safety and pharmacokinetics have been extensively studied in a diversity of animal species and clinical studies, including acute, sub-chronic and chronic toxicity assessment, carcinogenesis,

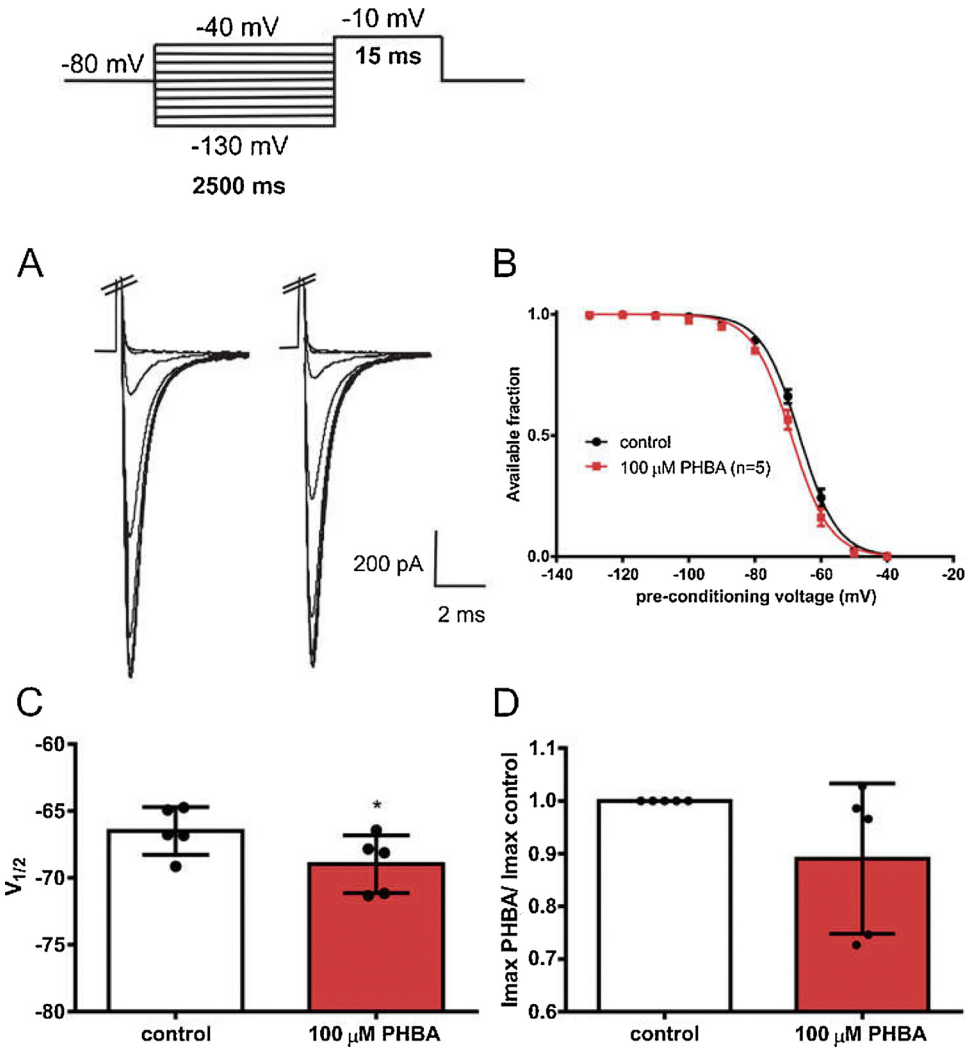

Fig. 11. p-hydroxybenzoic acid induces a left-shift in steady-state inactivation curves of hNa$_V$1.2 channels. A) Superimposed typical traces of the hNa$_V$1.2 channel mediated currents obtained at the test pulse of the steady-state inactivation protocol before (left) and after (right) 100 μM p-hydroxybenzoic (PHBA) perfusion. B) Mean steady-state inactivation curves obtained from recordings showed in A. The available fraction was calculated as the peak current observed on each test pulse normalized by the one corresponding to the −130 mV pre-conditioning pulse (I/Imax). Solid lines represent the fit of the points of each condition with a Boltzmann equation. C) Mean $V_{1/2}$ values. The comparison with the control was made using paired *t*-test against ($p < 0.05$, $N = 5$). D) Mean values of PHBA closed-state block calculated as the inhibition in the current evoked after a pre-conditioning pulse of −130 mV. The comparison with the control was made using one-sample *t*-test against (vs 1).

teratogenesis, and genotoxicity studies, among others [27,28]. Unfortunately, most of the studies were done *in vivo*, which difficult a direct comparison with the concentrations needed to obtain hNav 1.2 block *in vitro* and with a human protein. However, the PPB dose needed for its anticonvulsant activity in mice (30, 100, and 300 mg/kg) is below the toxic doses reported in this species (> 8 g/kg)[11,27]. In contrast, considerable controversy has risen in the last decades around the endocrine-disrupting/estrogenic and androgenic potential of parabens [29], which led different regulatory agencies around the world to limit concentrations of PPB and BuPB in cosmetic and other products. In vitro studies in human cells are needed to compare with those reported here to inhibit hNaV1.2 channels, future studies should focus on the development of parabens analogs/derivatives with no endocrine disruption potential.

5. Conclusions

Our results describe the molecular mechanism of action of the PPB as a hNa$_V$1.2 channel blocker, and show that, with a higher potency, other parabens including butyl and benzyl esters share this desirable characteristic for AEDs. These facts, together with the anticonvulsant and neuroprotective activities of PPB reported in previous studies, allow us to postulate parabens repositioning for the treatment of epilepsy. Moreover, our findings put in evidence like the lipophilicity of the ester group is a relevant structural requirement to increase parabens ability to inhibit the hNa$_V$1.2 channel, representing a promising starting point for the design of more potent paraben analogs.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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