

Short Communication

Voltage-independent inhibition of $\text{Ca}_v2.2$ channels is delimited to a specific region of the membrane potential in rat SCG neurons

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Neurotransmitters and hormones regulate $\text{Ca}_v2.2$ channels through a voltage-independent pathway which is not well understood. It has been suggested that this voltage-independent inhibition is constant at all membrane voltages. However, changes in the percent of voltage-independent inhibition of $\text{Ca}_v2.2$ have not been tested within a physiological voltage range. Here, we used a double-pulse protocol to isolate the voltage-independent inhibition of $\text{Ca}_v2.2$ channels induced by noradrenaline in rat superior cervical ganglion neurons. To assess changes in the percent of the voltage-independent inhibition, the activation voltage of the channels was tested between -40 and $+40$ mV. We found that the percent of voltage-independent inhibition induced by noradrenaline changed with the activation voltage used. In addition, voltage-independent inhibition induced by oxo-M, a muscarinic agonist, exhibited the same dependence on activation voltage, which supports that this pattern is not exclusive for adrenergic activation. Our results suggested that voltage-independent inhibition of $\text{Ca}_v2.2$ channels depends on the activation voltage of the channel in a physiological voltage range. This may have relevant implications in the understanding of the mechanism involved in voltage-independent inhibition.

Keywords voltage-independent inhibition; $\text{Ca}_v2.2$ channel; G-protein; noradrenaline

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Introduction

$\text{Ca}_v2.2$ calcium channels are inhibited by a broad range of neurotransmitters through G-protein-coupled receptors (GPCRs) [1]. This inhibition has been classified into voltage-dependent and voltage-independent [2]. The voltage-dependent inhibition has been extensively studied [3]. The responsible molecules for this inhibition are the $\text{G}\beta\gamma$ subunits [4,5], which diffuse according to the membrane-delimited mechanism [1]. Then, this signaling

pathway is membrane-delimited and requires a direct interaction between $\text{G}\beta\gamma$ and channels. This interaction between the $\text{G}\beta\gamma$ and the channels is sensitive to depolarization, since a strong voltage step removes transiently this inhibition [6,7]. Binding of $\text{G}\beta\gamma$ to the channel leads to characteristic biophysical changes: slowed activation kinetics and voltage-dependence shift [8,9].

On the contrary, voltage-independent inhibition is poorly understood. This inhibition has been called voltage-independent as it cannot be released by depolarization [2]. Therefore, voltage-independent inhibition can be isolated by a double-pulse protocol which removes the voltage-dependent inhibition [6,7]. Nevertheless, the biophysical characteristics and the molecular mechanism are still under study, despite the efficiency of this protocol. Some molecular candidates to explain this inhibition are kinases, phosphatases, and different $\text{G}\beta\gamma$ subunits [10,11].

There are several agonists that exert voltage-independent inhibition on $\text{Ca}_v2.2$ channels, including angiotensin II, substance P, oxotremorine methiodide (oxo-M), and noradrenaline (NA) [12–16]. Voltage-independent inhibition by NA reduces the calcium current amplitude by nearly 30% at both 0 and $+110$ mV in embryonic chick dorsal root ganglion neurons [2]. Voltage-independent inhibition by oxo-M reduces calcium current amplitude to above 10% at both 0 and $+140$ mV in superior cervical ganglion (SCG) neurons from rats [17]. These results first suggested that voltage-independent inhibition is constant at all activation voltages. However, deviations in the percent of voltage-independent inhibition have not been tested within a physiological voltage range. Here, we used the double-pulse protocol to quantify the percent of voltage-independent inhibition induced by NA in SCG neurons. Then, we explored the voltage region in which the voltage-independent inhibition by NA affects $\text{Ca}_v2.2$ channels. Our results showed that the voltage-independent inhibition by NA was not equal at all activation voltages. To further test this idea, we assessed whether or not the voltage-independent inhibition by another agonist, oxo-M,

had a similar pattern. We found that the voltage-independent inhibition by oxo-M was also delimited to a specific voltage range. These results suggested that the voltage-independent inhibition on Ca_v2.2 channels depends on activation voltage.

Materials and Methods

Cell culture

The SCG neurons were isolated from 5-week-old male Wistar rats as previously described [18]. The rats were obtained from the animal breeding facility of the School of Medicine at the National Autonomous University of Mexico (UNAM). The experimental animals were handled according to the Mexican Official Norm for Use, Care and Reproduction of Laboratory Animals (NOM-062-ZOO-1999). The rats were placed in a container and exposed to CO₂ in an increasing concentration and were sacrificed by decapitation. After dissection, the ganglia were desheathed and sliced into 8–10 identical pieces. The tissue was transferred to a tube containing modified Hank's solution supplemented with 20 U/ml papain and after 20 min at 37°C, it was transferred to a solution containing 1 mg/ml collagenase type I and 10 mg/ml dispase for another 20 min before mechanically disaggregating the tissue. The cell suspension was centrifuged at 180 *g* for 3 min and washed twice in Leibovitz's L-15 medium and once in Dulbecco's modified Eagle's medium (DMEM), both supplemented with 5% (v/v) heat-inactivated fetal bovine serum and 1% penicillin–streptomycin. The cells were then plated on polystyrene culture dishes coated with poly-L-lysine and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were used up to 18 h after plating. L-15 and DMEM were obtained from Invitrogen (Carlsbad, USA), and all other reagents were obtained from Sigma (St Louis, USA).

Current measurement

Currents were recorded in the whole-cell configuration of the patch-clamp technique [19] with an EPC-7 amplifier (List-Electronic, Darmstadt-Eberstadt, Germany) at room temperature. Sylgard (Dow Corning, Midland, USA) coated pipettes of borosilicate glass (Kimble Chase, Vineland, USA) with a resistance of 0.8–1.2 MΩ were used. The series resistance was 1.5 ± 0.14 MΩ [mean \pm SEM (standard error mean)] and compensated to >70%. Steady-state currents were sampled at 10 kHz and tail currents at 50 kHz using a 'split-clock' protocol. Current recordings were filtered at 3 kHz. The cells were continuously bathed with control or test solutions at 1.5 ml/min rate. Ba²⁺ was used as a charge carrier for Ca²⁺ channel current measurements. The control bath solution contained: 162.5 mM tetra-ethylammonium chloride (TEA-Cl), 2 mM

BaCl₂, 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 8 mM glucose, 1 mM MgCl₂, 100 nM tetrodotoxine (TTX), and 5 μM nifedipine, pH adjusted to 7.4 with TEA-OH. The test solutions were control solutions with 10 μM NA or 10 μM oxo-M. The pipette solution contained: 140 mM CsCl, 20 mM TEA-Cl, 10 mM HEPES, 11 mM ethylene glycol tetraacetic acid, 5 mM MgCl₂, 4 mM Na₂ATP, 300 μM Na₂GTP, and 100 μM leupeptin, pH adjusted to 7.4 with CsOH. The Ca_v2.2 channel currents were defined as the component of the current sensitive to 100 μM CdCl₂ in the presence of 5 μM of nifedipine [10]. Steady-state current amplitude was calculated as the mean value of the recorded points between 3 and 4 ms after the onset of the pulse. Tail current was measured as the average current over a 100 μs period starting at 400 μs after repolarization at –40 mV. *I–V* curves were calculated from steady-state current. Plots of percent of inhibition were calculated from tail currents. To avoid systematic bias, the experimental and control measurements were alternated and concurrent controls were performed. The results are expressed as mean \pm SEM.

Results

Measurement of voltage-independent inhibition by NA in SCG neurons with a double-pulse protocol

In order to test whether an agonist with typical voltage-dependent action is able to induce voltage-independent inhibition, we bathed the SCG cells with NA (10 μM) under voltage-clamp conditions. Systematically the cells were challenged with a conditioning pulse in a double-pulse protocol to readily separate voltage-independent from voltage-dependent responses. **Figure 1(A)** shows a representative trace of voltage-clamped calcium currents (*n* = 11). In pulse 1 (P1), NA reduced the current amplitude by ~50% (from 32 ± 3 pA/pF in control to 16 ± 2 pA/pF in NA). Moreover, NA induced a slower current activation (kinetic slowing) [2,9], according to the typical inhibitory effect by voltage-dependent inhibition [4,10]. After a conditioning pulse to +80 mV during 50 ms, recovery of the current was about 80% (27 ± 3 pA/pF in NA from 35 ± 3 pA/pF in control). Also current kinetics recovered the control conditions consistent with the removal of the voltage-dependent inhibition. Thus, the remaining 20% inhibition corresponds to the voltage-independent inhibition induced by NA in the SCG neurons.

Characterization of the voltage-independent inhibition by NA

Next, to confirm that this 20% NA-induced inhibition corresponds to the voltage-independent mechanism, we analyzed its effects on the *I–V* relationship and on the activation kinetic of the current in a voltage range between

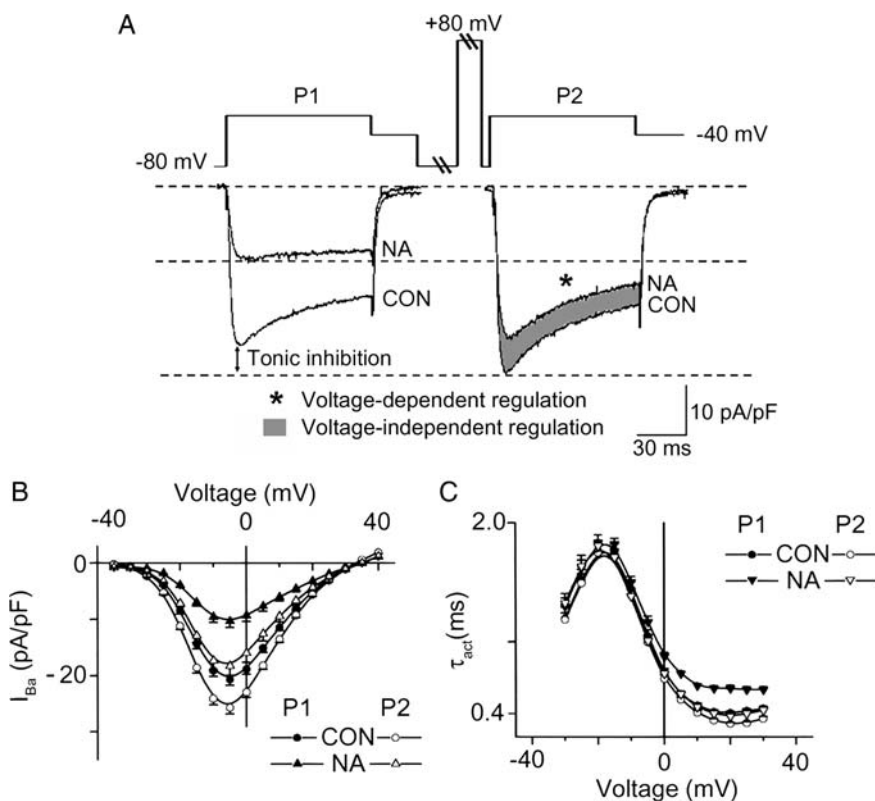


Figure 1 Double-pulse protocol make it possible to isolate voltage-independent inhibition (A) Inhibition of $\text{Ca}_v2.2$ induced by 20 s application of NA. Protocol used is shown above. Below, a representative superimposed calcium currents in control and under NA application. Dashed lines, from top to bottom, represents zero current, total inhibition induced by NA and control current after removal tonic inhibition, respectively. CON, control. (B) $I-V$ relationships for control and NA, in P1 and P2. (C) Time constants of the activation (τ_{act}) obtained fitting with a single-exponential function.

–40 and +40 mV. An $I-V$ relationship illustrates that NA reduced only the amplitude of the calcium current without affecting the reversal potential either in P1 or in P2 [Fig. 1(B)]. To analyze the effects of voltage-independent inhibition on the activation kinetics, the time course of the rising phase of the current was fitted to an exponential function with a single time constant (τ_{act}) [20]. Our results showed that the τ_{act} of the current regulated by the voltage-independent pathway, in P2, is indistinguishable from the τ_{act} in control, at all activation voltages [Fig. 1(C)]. Thus, the inhibition induced by NA during P2 neither induced kinetic slowing nor changes in the $I-V$ relationship of the channels as expected for a voltage-independent inhibition. On the contrary, NA reduced the τ_{act} at all voltages in P1, accordingly with its fraction of voltage-dependent inhibition [20].

Behavior of voltage-independent inhibition by NA at a wide range of activation voltages

We proceed to characterize the voltage region at which the voltage-independent inhibition modulates $\text{Ca}_v2.2$ channel activity. To this end, a double-pulse protocol with 5 mV increments was elicited [Fig. 2(A)]. Figure 2(B) shows the overlapped average currents in control conditions ($n = 16$) and under NA application ($n = 14$) at –20, –10, 0, and +20 mV.

The portion of voltage-independent inhibition (in gray) was greater when the channels were activated at –10 mV than at more positive or negative test pulses. This result suggests that the portion of voltage-independent inhibition varies with the potential at which the channels are activated.

To illustrate the voltage region in which NA induces the voltage-independent inhibition, we plotted the percent of inhibition calculated from tail currents in P2. To compare it with the total inhibition, we plotted the percent of inhibition in P1 [Fig. 2(C)]. Voltage-independent inhibition by NA, in P2, was maximal (25%) at –15 mV whereas at –30 and +70 mV inhibition was reduced to 10%. On the contrary, in P1, inhibition was 55% at –15 mV test potential whereas at –30 mV NA inhibited calcium current by 40% and by less than 15% at +70 mV. In order to compare the voltage region of the two types of inhibition induced by NA, voltage-dependent inhibition was calculated by subtracting the inhibition in P2 from the total inhibition in P1. The voltage region at which either the voltage-dependent or the voltage-independent inhibition took place is shown in Fig. 2(D), as the percent relative to total inhibition. As expected, the voltage-dependent inhibition was greater than the voltage-independent inhibition for all voltages tested. Interestingly, the two types of inhibition occur at the same voltage region. These results

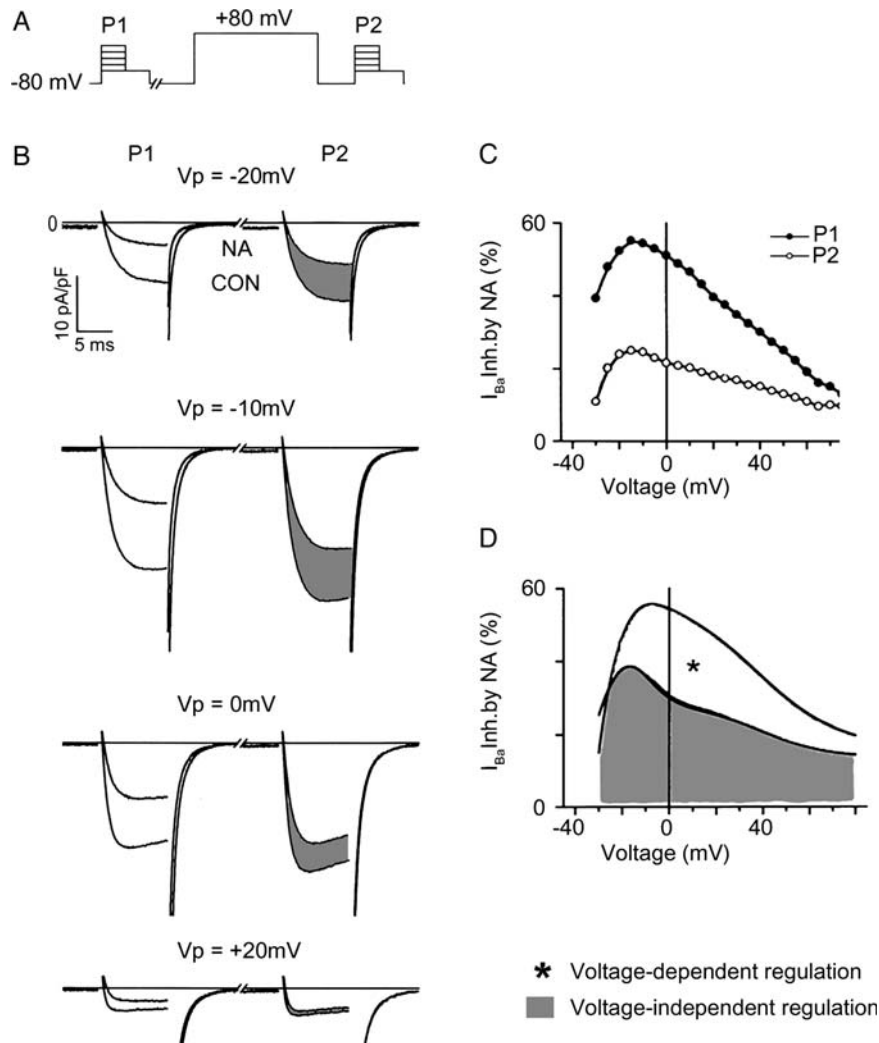


Figure 2 Percent of voltage-independent inhibition changes with the voltage used to activate $Ca_v2.2$ channels (A) A double-pulse protocol with voltage steps from -40 to $+40$ mV, every 5 mV, was applied to change the activation voltage of $Ca_v2.2$ channels. (B) Average superimposed currents in control ($n = 16$) and under NA application ($n = 14$) at different test potentials, V_p . Voltage-independent inhibition is marked in gray. (C) Percent of inhibition induced by NA in pulse 1 (P1, filled circles) and pulse 2 (P2, open circles). (D) Percent of voltage-dependent (*) and voltage-independent (in gray) inhibition at different voltages.

showed that the voltage-independent inhibition occurs at a specific voltage region between -30 and 0 mV. This finding answers the initial fundamental question of these experiments.

Voltage-independent inhibition by oxo-M also depends on activation voltage

To assess whether the confinement of the inhibition to a voltage region is a common feature for GPCRs agonists, we analyzed the voltage-independent inhibition induced by oxo-M. Application of oxo-M inhibited $Ca_v2.2$ channels nearly equals in P1 and P2 according to the idea that this agonist induced mainly voltage-independent inhibition [$n = 11$, Fig. 3(B)]. The magnitude of the inhibition in P2 was different for each activation voltage, similar to that observed in currents regulated by NA [Fig. 3(A)]. Then we plotted the percent of voltage-independent inhibition

against activation voltage. It can be seen that the voltage-independent inhibition was bigger at 0 mV than at other activation voltage [Fig. 3(C)]. These results supported the idea that the voltage-independent inhibition exerts its effects in a delimited range of activation voltage. Taking into account that: (i) this inhibition has been called independent because it is not released by a depolarizing voltage and (ii) there is a strong relationship of voltage-independent inhibition with the activation voltage, we proposed to call this kind of regulation as voltage-resistant inhibition.

Discussion

Neurotransmitters and hormones regulate $Ca_v2.2$ calcium channels through GPCR [1]. In 1986, Marchetti *et al.* [9] first showed evidence that this inhibition is voltage-

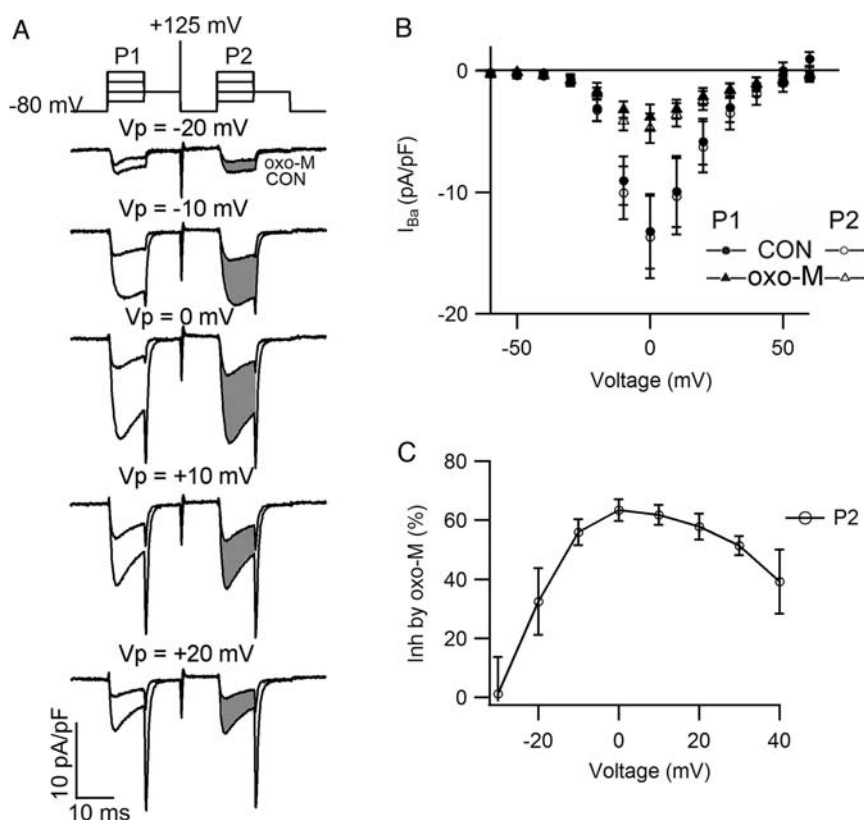


Figure 3 Voltage-independent inhibition by oxo-M depends on activation voltage (A) Superimposed currents in control conditions ($n = 11$) and under oxo-M ($n = 11$) obtained with the protocol shown above at different activation potentials. Voltage-independent inhibition is marked in gray. (B) $I-V$ relationship in P1 and P2 under control or test conditions ($10 \mu\text{M}$ oxo-M). (C) Percent of inhibition induced by oxo-M in pulse 2 (P2, open circles) against different activation voltages.

dependent; calcium current is less inhibited when activated at $+10$ mV than at 0 mV. They also proposed that depolarization at a large potential would release the inhibition, which was effectively confirmed years later [6,7]. This type of inhibition was called voltage-dependent. Then, a protocol to release the inhibition induced by G-proteins was established and was called facilitation or double-pulse protocol. However, experiments using the double-pulse protocol gave evidence of the existence of a remaining inhibition after the depolarization [2,15]. This inhibition was proven to be dependent also on G-proteins [21]. Therefore, the inhibition by GPCRs was classified into voltage-dependent and voltage-independent [2]. The molecular mechanism and biophysical properties of voltage-independent inhibition are still controversial [3].

It has been proposed that calcium current is inhibited equally whatever voltage is used to activate channels then this inhibition would not depend on activation voltage. In this study, we measured the percent of inhibition at different voltages to activate $Ca_v2.2$ channels, before and during the application of NA and oxo-M. NA is an agonist that mainly induces voltage-dependent inhibition whereas oxo-M mainly induces voltage-independent inhibition. We found that the relation between voltage-independent

inhibition and activation voltage of $Ca_v2.2$ channels is not linear. On the contrary, the percent of voltage-independent inhibition induced by both agonists changes with the activation voltage. We analyzed changes in the inhibition at a voltage range between -40 and $+40$ mV as this is the physiological range of voltage at which neurotransmitters and neuromodulators act. Therefore, voltages beyond $+40$ mV were not explored.

Major physiological processes governing action potential firing and neurotransmitter release also occur in the same voltage region. Thus, voltage-independent inhibition could be related to these physiological processes. Concerning the molecular mechanism by which voltage-independent inhibition occurs, dependence on the activation voltage suggests that the binding of the molecule involved changes with the activation voltage of $Ca_v2.2$ channel. Whatever the mechanism turns out to be, it may change the state of the channel without altering its voltage sensor and therefore the biophysical features of the current. Further studies are needed to address the molecular mechanisms underlying the specificity of the voltage range involved in the voltage-independent inhibition. An interesting possibility to be further examined is that the specificity of the voltage region is somehow related to the voltage sensor of channels [22,23] or of GPCRs [24–26].

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