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Gating charges per channel of $Ca_V2.2$ channels are modified by G protein activation in rat sympathetic neurons

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ABSTRACT

It has been suggested that voltage-dependent G protein modulation of $Ca_V2.2$ channels is carried out at closed states of the channel. Our purpose was to estimate the number of gating charges of $Ca_V2.2$ channel in control and G protein-modulated conditions. By using a Cole–Moore protocol we observed a significant delay in $Ca_V2.2$ channel activation according to a transit of the channel through a series of closed states before channel opening. If G protein voltage-dependent modulation were carried out at these closed states, then we would have expected a greater Cole–Moore lag in the presence of a neurotransmitter. This prediction was confirmed for noradrenaline, while no change was observed in the presence of angiotensin II, a voltage-insensitive G protein modulator. We used the limiting slope method for calculation of the gating charge per channel. Effective charge z was 6.32 ± 0.65 for $Ca_V2.2$ channels in unregulated conditions, while GTPγS reduced elementary charge by ~4 e_0. Accordingly, increased concentration of noradrenaline induced a gradual decrease on z, indicating that this decrement was due to a G protein voltage-sensitive modulation. This paper shows for the first time a significant and reversible decrease in charge transfer of $Ca_V2.2$ channels under G protein modulation, which might depend on the activated G protein inhibitory pathway.

Introduction

$Ca_V2.2$ (N-type) calcium channels are modulated by a wide range of neurotransmitters through G protein coupled receptors [1]. It has been suggested that this G protein voltage-dependent modulation is carried out at the closed states transiting the channel toward its opening [2]. The transition between these states requires charge transfer of the voltage sensor [3]. Therefore, a way to study the molecular mechanism of modulation in these sub-states, is determining the number of elementary charge transferring per channel in the electric field during its activation.

$Ca_V2.2$ is the best known calcium channel in terms of voltage-sensitive regulation by G proteins, however, its effective charge of activation has not yet been reported. This task would be relatively trivial for a channel with exactly two states: closed and open. However, the problem is that most of the voltage-activated channels present more than one transitional closed state. In the case of the simplest model of two states, the relationship between the channel open probability and voltage, exhibits the shape of a Boltzmann distribution of two states [4]. Thus, the charge that moves during the transition is obtained from the slope of the curve by fitting the experimental data. By contrast, for channels with more than two states, this approach results in an underestimation of the transferred charge, due to the increasing occupancy of intermediary states. We utilized dissociated superior cervical ganglion (SCG) neurons, as G protein coupled receptors are available in this preparation to demonstrate that noradrenaline (NA) decreases gating charge transfer, slowing activation of channels. G protein modulation of $Ca_V2.2$ channels in these cells is well documented [5,6]. In a previous work we demonstrated that G protein activation decreases the total intramembrane charge movement in this preparation [7]. G proteins also decreased the amplitude of gating currents and produced depolarizing shifts in the voltage-dependent activation of gating charge movement from $Ca_V2.2$ channels in heterologous systems [8]. Therefore, the purpose of the present study was to estimate the number of elementary charges that are transferred through the electric field during activation of the $Ca_V2.2$ channel in control and G protein-modulated conditions. To this end we proposed the use of the classical limiting slope method for effective charge calculation. This method postulates that if the kinetic model is a linear sequence involving a few closed and a single open states, at very negative potentials, i.e., when the open probability is extremely small, the slope of the logarithm of this probability versus the membrane potential reaches a threshold value proportional to the effective charge of activation per channel [9]. Our results show that elementary charge transfer per channel in $Ca_V2.2$ decreases in the presence

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of NA in a reversible manner, whereas with the voltage-resistant G protein modulator angiotensin II (angioII) there is no a significant change, suggesting that the reduction is exclusively due to the G protein voltage-sensitive modulation.

Materials and methods

Cell culture

SCG neurons were dissected, dissociated and placed in a primary culture. Cells were taken from 5-week-old male Wistar rats, as described previously [10]. Rats were placed in a container and exposed to CO_2 in a rising concentration, then were killed by decapitation, performed according to authorized procedures of the Institutional Animal Care and Use Committee at the Universidad Nacional Autónoma de México (UNAM). After dissection, ganglia were dissociated using papain, collagenase and dispase in Hank's solution. Then, neurons were washed in Leibovitz's L-15 medium, and finally were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated bovine serum and 1% penicillin–streptomycin. Cells were placed on Polystyrene Tissue Culture Dishes coated with poly-L-lysine, incubated at 37 °C (5% CO_2) overnight, and used within 24 h.

Solutions

External solution was applied in the bath by using a focal perfusion system that exchanges the solution locally within 5–10 s. Solutions were designed to isolate Ba^{2+} currents flowing through N-type Ca^{2+} channels. The Ca^{2+} current of rat SCG neurons is carried ~85–90% in N-type channels and the remainder in L-type channels, with no detectable P/Q component [10–12]. Therefore, the N-type Ca^{2+} current was defined as the component of the current sensitive to 100 μM $CdCl_2$ in the presence of 5 μM of Nifedipine. Bath solution contained (in mM): 180 TEA-Cl, 10 Hepes, 8 glucose, 2 $BaCl_2$, 1 $MgCl_2$, 0.0001 TTX, 0.005 Nifedipine, and pH adjusted to 7.4 with TEA-OH. The pipette solution (internal solution) contained (in mM): 140 CsCl, 20 TEA-Cl, 10 Hepes, 5 $MgCl_2$, 6 MgATP, 0.3 Na_2GTP, 14 Phosphocreatine, 0.1 Leupeptin, 0.1 BAPTA-4Cs, and pH adjusted to 7.2 with CsOH. All experiments were performed at room temperature (22–25 °C). Reagents were obtained as follows: BAPTA (Molecular Probes, Eugene, OR, USA), NA (Calbiochem) and DMEM (Invitrogen Corp., Carlsbad, CA, USA). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Electrophysiology and data analysis

Membrane current measurements were performed in the whole-cell configuration of the patch–clamp technique [13] using a EPC-9 (HEKA Instruments, Inc.) amplifier, and pipettes of borosilicate glass with resistance of 1–2 MΩ when filled with the internal solution. Capacity transients were canceled using the EPC-9 circuitry, and series resistance was compensated to >70%. Steady-state currents were sampled at 10 kHz and ramp currents at 200 Hz. Voltage-pulse command protocols were generated, and data were digitized, recorded, and analyzed using MatLab version 7.4 (The MathWorks Inc.) and OriginPro 7 (OriginLab Corporation, Northampton, MA, USA). Differences were evaluated using unpaired *t*-test. Statistical significance was set at $p < 0.05$.

In experiments with the perforated whole-cell recording configuration, the pipette tip was filled with amphotericin-free solution and then backfilled with solution that contained 1.2 μg/ml amphotericin-B [14,15]. After forming a cell attached seal, the membrane resistance and capacitance were monitored to asses the progress of perforation.

Limiting slope analysis

The barium current recorded without leak subtraction during a voltage ramp was converted into conductance by dividing the ionic current by its driving force. Current data points recorded at potentials more negative than −75 mV were fitted for the linear leakage. The conductance versus potential (G–V) plot was fitted to a mono-exponential approximation of the Boltzmann function in the limit for very negative potentials (until −40 mV),

$$G(V) = A\exp(ze_oV/kT) \quad (1)$$

where $G(V)$ is the conductance, z the number of effective charges per channel, e_o the electronic charge (1.6×10^{-19} C), k the Boltzmann constant (1.38×10^{-23} J/K), T the absolute temperature, V the membrane potential, and A the amplitude of the exponential. This approximation was resolved for a linear sequential model with one open state [9].

Results

We have used the classical approach of the limiting slope analysis to calculate the transference of charge per channel by fitting the initial phase of the conductance–voltage curve to a single exponential (see Methods). Fig. 1A shows typical barium currents flowing through $Ca_V2.2$ channels in response to a slow voltage ramp protocol from −90 to +70 mV with a speed of 4 μV per millisecond. This ramp is adequate to obtain a quasi-steady state conductance versus voltage relationship at negative potentials. The steady state of the G–V curve was confirmed by varying the speed of the ramp and by testing the symmetry of the current response to a symmetric triangle wave (data not shown). Ionic current was almost completely inhibited by 100 μM Cd^{2+} (Fig. 1A). Fig. 1B is the corresponding conductance versus voltage curve at hyperpolarizing potentials, which was fitted to a mono-exponential function. Interestingly, under the standard whole-cell technique, the evaluated effective charge z of the fit was 3.78 ± 0.24, which is about one-half of the value obtained for other voltage dependent calcium channels [16]. $Ca_V2.2$ currents present a significant rundown in sympathetic neurons [17]. To verify whether this rundown of channel activity could be responsible of an underestimation of the effective charge transfer, we wanted to know the rundown amount in our experimental conditions, and how much this can be minimized by the alternative perforated patch technique. Inset in Fig. 1C shows an overlap of typical $Ca_V2.2$ currents in response to the same voltage pulse at different times after breaking the patch under control conditions, where there is clearly a reduction of the amplitude of the current over time (one pulse every 50 s), with no apparent change on its kinetic behavior. In order to eliminate the effect of this depletion of the current on the determination of the number of charges transferred during the activation of the channel, we decided to use the perforated patch technique. As shown in the graph in Fig. 1C, the rundown decreases markedly using this strategy (open symbols; time constant = 1940.43 s), compared with his later measurement after breaking through the patch (closed symbols; time constant = 141.72 s) in the same cell. Patch perforated technique with amphotericin-B modestly enhanced the calculated effective charge suggesting that underestimation of e_0 can result from the standard whole-cell technique. In Fig. 1D we present a summary of data. Thus, we found an increase, although non-significant enhancement ($p = 0.092$), in the