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FAST DECAY OF THE $CA_V2.2$ CHANNEL CURRENT IN RAT SYMPATHETIC NEURONS IS PREVENTED BY THE GB_1G_4 DIMER

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As is believed, the voltage-dependent regulation of $Ca_V2.2$ channels by G proteins is carried out by beta-gamma subunits (Gβγ), but little is known whether these subunits regulate the decay of this current. Therefore, we studied $Ca_V2.2$ channel-current decay in cultured $G\beta_1\gamma_4$-injected rat superior cervical ganglion neurons. $Ca_V2.2$ currents were recorded by means of the patch-clamp technique in the whole-cell configuration. We found that the time course of $Ca_V2.2$ current decay in non-$G\beta_1\gamma_4$-injected neurons consisted of two (fast and a slow) components, while $G\beta_1\gamma_4$-injected neurons showed only the slow decay component. The fast decay component is restored by a strong depolarizing pulse according to a voltage-dependent mechanism. A reduction in the macroscopic conductance at 20 msec from starting the depolarizing pulse suggests that the absence of the fast component is due to a substantial fraction of $Ca_V2.2$ channels in a non-conducting state. These results support the statement that $G\beta_1\gamma_4$ subunits regulate $Ca_V2.2$ current decay and prevent the appearance of the fast component.

Keywords: rat superior cervical ganglion (SCG) neurons, $Ca_V2.2$ channels, current decay, G proteins, $G\beta_1\gamma_4$ dimer.

INTRODUCTION

$Ca_V2.2$ channels are regulated by beta-gamma subunits of G proteins (Gβγ) through a voltage-dependent mechanism [1-3]. Many reported studies have been focused on the regulation mediated by Gβγ during the $Ca_V2.2$ current activation phase [4-6]. These studies established that this regulation features are: (i) activation kinetic slowing [7], (ii) a shift in the activation curve toward more positive potentials [8], and (iii) recovery by a strong depolarizing prepulse [9]. However, the decay of the $Ca_V2.2$ channel-current mediated by Gβγ subunits is not well understood. Of particular interest is the $G\beta_1\gamma_4$ dimer, which has been clearly implicated in this type of regulation [10].

The time course of $Ca_V2.2$ current decay consists of a fast component and a slow component [11, 12]. G protein activation by neurotransmitters, such as norepinephrine, is related to voltage-dependent regulation. Norepinephrine slows the current decay [13, 14]. However, whether this slowing is related to Gβγ regulation of one or both decay components has not been determined. Therefore, we investigated in rat superior cervical ganglion (SCG) neurons whether $G\beta_1\gamma_4$ subunits regulate both decay components of the $Ca_V2.2$ channel-current.

We found that $Ca_V2.2$ current decay in $G\beta_1\gamma_4$-injected neurons exhibits only the slow component. In the presence of $G\beta_1\gamma_4$, the fast component is absent, but it is restored by a strong depolarizing prepulse in a voltage-dependent manner. Our findings will lead to further understanding of the detailed mechanism underlying regulation of $Ca_V2.2$ channels.

METHODS

Cell Culture and Nuclear Microinjection. Rat SCG neurons were isolated as previously described [12]. Neurons were placed on polystyrene culture dishes pretreated with poly-l-lysine and incubated at 37°C (5% CO_2) for 6 h before nuclear microinjection with an Eppendorf 5242 microinjector (injection pressure

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10–20 kPa for 0.3 sec) and a 5171 micromanipulator (Eppendorf, Madison, USA). The injection solution contained cDNA constructs encoding a green fluorescent protein (GFP) mutant fused to Gβ1 subunits ($G\beta_1$-GFP; 100 ng/μl) and $G\gamma_4$ subunits ($G\gamma_4$; 100 ng/μl); expression plasmids were mixed with 1 mg/ml 10,000 kDa dextran-fluorescein, which was used as an injection marker. To confirm that the fused $G\beta_1$-GFP did not alter $G\beta_1$ subunit properties, both GFP plasmid (100 ng/μl) and GFP plus $G\beta_1$ subunit plasmids (100 ng/μl) were injected (data not shown). cDNA encoding $G\beta_1$ subunits was cloned in pCDM8, $G\gamma_4$ subunit was cloned in pCI (provided by M. Simon, Caltech, Pasadena, USA), GFP was cloned in pEGFP-N1 (Clontech, Palo Alto, USA), and the $G\beta_1$-GFP fusion plasmid was cloned in pEYFPGb (Clontech). The expression of all vectors was driven by the cytomegalovirus promoter. Plasmids were purified with the use of commercial kits (Qiagen, Valencia, USA). After 18-24 h, successfully injected SCG neurons, referred to as $G\beta_1\gamma_4$-injected neurons, were identified by their distinguishing greenish-blue GFP fluorescence under an inverted microscope (Axiovert 135; Carl Zeiss, Germany) equipped with epifluorescence optics.

Electrophysiology. Current recordings were obtained at room temperature (22–24 °C) by the patch-clamp technique in the whole-cell configuration with the use of an EPC-9 amplifier (HEKA Electronik, Lambrecht, Germany). Voltage protocols were generated and current responses were digitized and stored with the use of Patchmaster software (HEKA Electronik). Pipettes were pulled from borosilicate glass capillaries with a horizontal patch electrode puller (Sutter Instruments, USA) and were filled with internal solution containing (mM) 140 CsCl, 20 TEA-Cl, 10 HEPES, 0.1 BAPTA-tetracesium, 5.0 $MgCl_2$, 5.0 Na_2ATP, 0.3 Na_2GTP, and 0.1 leupeptin; pH was adjusted to 7.2 with CsOH. The resistance of the pipettes was 1.8–2.0 MΩ. Neurons were superfused (1–2 ml/min) with external solution designed to isolate Ba^{2+} currents (I_{Ba}) through $Ca_V2.2$ channels. Composition (in mM) of the solution was 165 TEA-Cl, 2 $BaCl_2$, 10 HEPES, 8 glucose, 1 $MgCl_2$, and 0.0002 TTX; pH was adjusted to 7.4 with TEA-OH. The series resistance was compensated to >70% and did not exceed 10 MΩ; the mean cell capacitance was 74.56 ± ± 11.28 pF. $Ca_V2.2$ currents were sampled at 10^4 sec^{-1}. Only neurons with a facilitation index ≥ 3 were included in the analysis, as previously described [2]. Reagents were obtained from Sigma (St. Louis, USA).

Data Analysis. Current decay (%) was calculated by the following equation:

$$CD = 1 - \frac{I_{500}}{I_7}$$

where I_7 and I_{500} are the current amplitudes at times of 7 and 500 msec, respectively. The change in I_{Ba} was taken as the difference in current amplitudes measured every 20 msec during current decay. The $Ca_V2.2$ current decay was fitted to a bi-exponential equation:

$$I(t) = A_{fast}e^{-\frac{t}{\tau_{fast}}} + A_{slow}e^{-\frac{t}{\tau_{slow}}} + Y_0$$

where $I(t)$ is the current amplitude, A is the current amplitude of the fast or slow component, τ is the time constant of decay, and Y_0 is the residual current. The steady-state current voltage curve was obtained by measuring the current amplitude at 6 msec of the test pulse. The conductances were calculated according to the following equation:

$$G(V) = \frac{I}{V_a - V_r}$$

where $G(V)$ is the total conductance at each voltage, I is the current amplitude, Va is the membrane potential applied during the test pulse, and Vr is the reversal potential obtained from the current-voltage relationship. The conductance-voltage relationship data points were fitted to the following Boltzmann equation:

$$G(V) = G_{max}(1 + e^{\frac{V_h - V}{k}} - 1)$$

where G_{max} is the maximal conductance, V_h is the half-activation voltage, and k is the slope factor.

Data are shown as means ± s.e.m.. The statistical significance of differences was determined by the t-test; diferenses with $P < 0.05$ were considered significant.

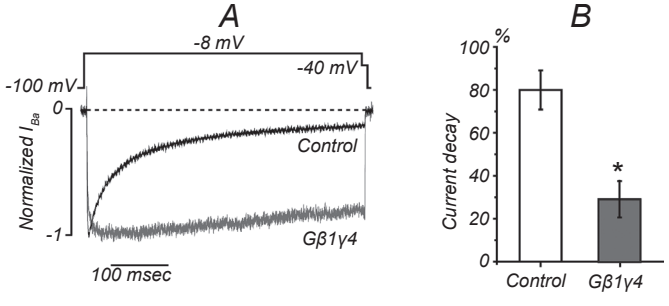
RESULTS

$Ca_V2.2$ Channel Current Decay was Diminished by $G\beta_1\gamma_4$ Subunits. Previous reports have shown that G-protein activation diminishes the $Ca_V2.2$ current decay [3, 12, 15]. We investigated whether $G\beta_1\gamma_4$ subunits, dimers that mimic the voltage-dependent regulation, diminish the $Ca_V2.2$ channel-current decay over a 500-msec pulse. This depolarizing pulse was used to observe the current decay in the steady state.

An overlapping of normalized I_{Ba} in the non-Gβ1γ4-injected (control) neurons and $G\beta_1\gamma_4$-injected neurons showed that the current decay decreases under this condition (Fig. 1A). This observation was confirmed when we measured the percentage of current decay at 500 msec (Fig. 1B). $G\beta_1\gamma_4$ subunits diminished 62% of the $Ca_V2.2$ current decay (control neurons, 80 ± 0.9%; $G\beta_1\gamma_4$-injected neurons, 30 ± 0.8%). Notably, as is seen in Fig. 1A, the time course of the current in control neurons was characterized by a biphasic decay, but in $G\beta_1\gamma_4$-injected neurons only the monophasic decay was observed. Our data indicate that $G\beta_1\gamma_4$ subunits regulate the time course of $Ca_V2.2$ current decay, suggesting the presence of an underlying mechanism at the start of the channel-current decay.

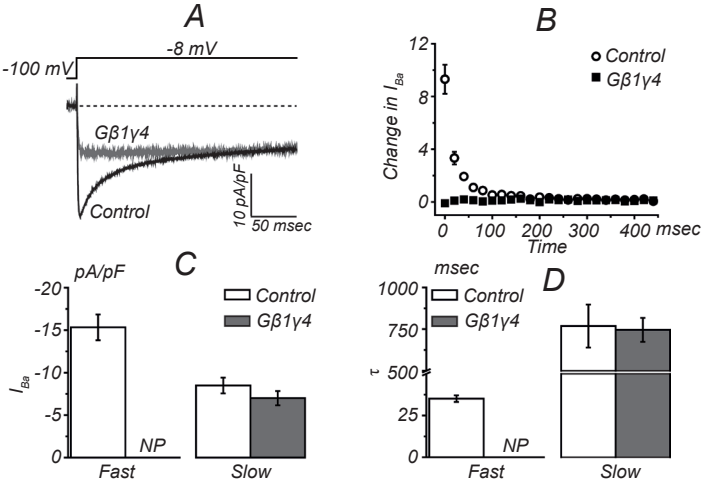
$G\beta_1\gamma_4$-Injected Neurons Exhibited Only the Slow Component of $Ca_V2.2$ Channel-Current Decay. In the absence of G protein regulation, the $Ca_V2.2$ current decay has both a fast component and a slow component [12, 16]. At the same time, whether the $G\beta_1\gamma_4$ subunits regulate both components, remained unclear. The I_{Ba} traces from a control neuron and a $G\beta_1\gamma_4$-injected neuron during a 250-msec depolarizing pulse are superimposed on the graph in Fig 2A. The courses of the two traces are nearly the same after 100 msec, confirming the absence of the first decay component in the $G\beta_1\gamma_4$-injected neuron. To support this observation, we measured changes in the I_{Ba} decay every 20 msec during a 500-msec-long depolarizing pulse (Fig. 2B). In control neurons, the initial I_{Ba} change rate was fast; the change slowed significantly thereafter. In $G\beta_1\gamma_4$-injected neurons, a consistently lower rate of change was observed. This result prompted us to examine the kinetic parameters of the current decay components. In control neurons, current decay traces were fitted to a bi-exponential function. The *A* value (current amplitude) was −15.3 ± 1.5 pA/pF for the fast component and −8.5 ± 0.9 pA/pF for the slow component (Fig. 2C). The fast τ was 35 ± 1.9 msec, whereas the slow τ was 768.16 ± 128.6 msec (Fig. 2D). The current decay in $G\beta_1\gamma_4$-injected neurons was also fitted to a bi-exponential function; however, the best fit was to a mono-exponential function. The *A* value was −7.0 ± 0.8 pA/pF, and *τ* was 745.8 ± 71.5 msec, values that did not differ statistically from those of the slow component in control cells (Fig. 2C, D). Thus, the $G\beta_1\gamma_4$-injected neurons showed only the slow component of the $Ca_V2.2$ current decay; the fast component was absent under those conditions.

The Fast Component was Restored After Application of a Strong Depolarizing Prepulse in $G\beta_1\gamma_4$-Injected Neurons. Voltage-dependent inhibition by $G\beta_1\gamma_4$ subunits is relieved by a strong depolarizing prepulse [5, 17]. We tested whether this prepulse



F i g. 1. $Ca_V2.2$ current decay in control neurons and $G\beta_1\gamma_4$-injected units over a long depolarizing pulse. A) Superimposed I_{Ba} traces obtained in the control and in a $G\beta_1\gamma_4$-injected neuron under the pulse protocol indicated at the top. B) Diagram showing normalized current decay (%) in the control and in $G\beta_1\gamma_4$-injected neurons (n = 7) at 500 msec, *P < 0.05.

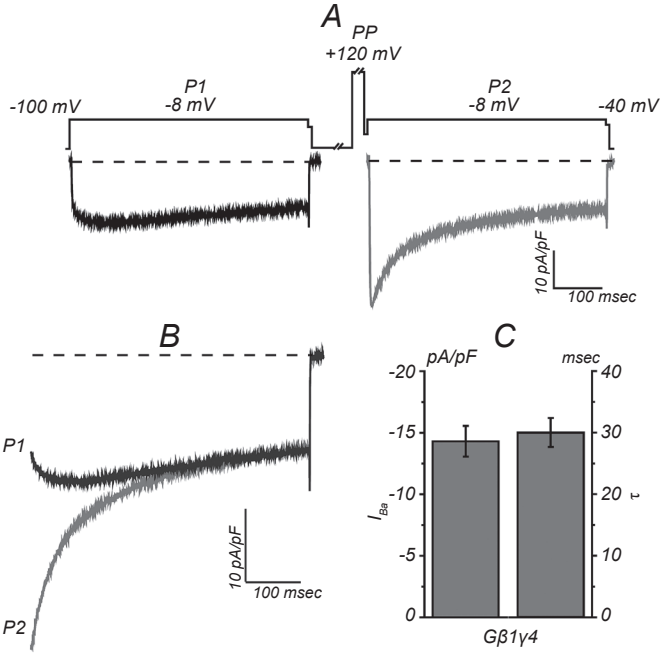
Р и с. 1. Спад струму через $Ca_V2.2$-канали в контрольних нейронах і в клітинах, в які ін'єкували $G\beta_1\gamma_4$, при дії тривалого деполяризаційного імпульсу.



F i g. 2. $Ca_V2.2$ current decay in $G\beta_1\gamma_4$-injected neurons; the presence of only the slow component. A) Superimposed I_{Ba} traces in the control and a $G\beta_1\gamma_4$-injected neuron evoked by the protocol indicated at the top. B) Time course of changes in the averaged I_{Ba} amplitude obtained at 20-msec intervals in the control and in $G\beta_1\gamma_4$-injected neurons. C) Diagram of the *A* values (maximal amplitude) of the fast and slow decay components. D) Diagram of the *τ* components under both conditions (n = 7); NP, not present. Values in C and D were obtained from bi-exponential equation fitted to the $Ca_V2.2$ current decay.

Р и с. 2. Спад $Ca_V2.2$-струму в нейронах, у котрі ін'єкували $G\beta_1\gamma_4$ (наявність лише «повільного» компонента).

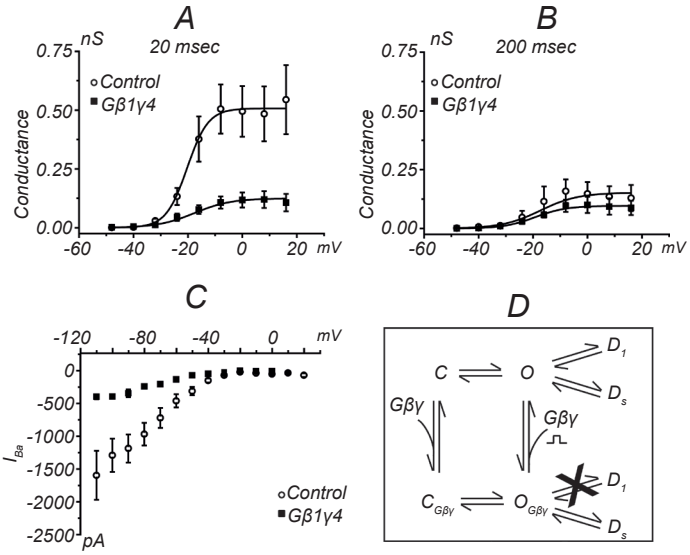
restores the fast component of $Ca_V2.2$ current decay. I_{Ba} traces from a $G\beta_1\gamma_4$-injected neuron evoked under a prepulse protocol are shown in Fig. 3A. In pulse 2 (panel P2), the fast component was observed after a strong depolarizing pulse (PP). P1 and P2 overlapped; the activation phase of the current in control neurons was absent (Fig. 3B). The current amplitude and τ of the fast component obtained from the best fit to the bi-exponential function in P2 (*A* value was −14.3 ± ± 1.24 pA/pF; τ was 30.02 ± 2.4 msec) are shown in Fig. 3C. This fast component exhibited the same amplitude and τ that were seen in control neurons, indicating that they obey the same mechanism (Fig. 2C, D). These results support the statement that $G\beta_1\gamma_4$ dimer activity underlies inhibition of the fast decay.



F i g. 3. Restoration of the fast component of the $Ca_V2.2$ current decay by a strong depolarizing prepulse. (A) Representative I_{Ba} trace evoked by a depolarizing pulse to −8 mV (P1, P2) from a holding potential of −100 mV over 500 msec, separated by a strong depolarizing prepulse (PP) to ±120 mV over 90 msec in a $G\beta_1\gamma_4$-injected neuron. (B) Superimposed P1 and P2 current traces are shown after the 5th millisecond of the depolarizing pulse. (C) Bar graph of the *A* values (maximal amplitude) and τ of the fast component obtained from bi-exponential equation fitted to the $Ca_V2.2$ current decay in P2 current traces (n = 6).

Р и с. 3. Поновлення «швидкого» компонента спаду $Ca_V2.2$-струму після потужного попереднього деполяризаційного імпульсу.

$Ca_V2.2$ Channel Conductance was Reduced by $G\beta_1\gamma_4$ Subunits. Activation of G proteins by norepinephrine decreases the Ca_V conductance [18], suggesting that $G\beta_1\gamma_4$ subunits account for the reduction in the $Ca_V2.2$ channel conductance. Thus, the absence of the fast decay component could be explained by this reduction. Therefore, we calculated the voltage-conductance relationship, and these data were fitted to a Boltzmann function. At 20 msec, the mean maximal conductance was 0.5 ± 0.024 nS in control neurons but only 0.11 ± 0.006 nS in $G\beta_1\gamma_4$-injected neurons (Fig. 4A). Notably, significant differences were observed between the control and $G\beta_1\gamma_4$-injected neurons. However, at 200 msec, the maximal conductance was 0.12 ± 0.018 nS in control neurons and 0.09 ± 0.006 nS in $G\beta_1\gamma_4$-injected neurons (Fig. 4B); no statistically significant difference was observed. The slope calculated at 20 msec was 3.8 ± 0.26 mV in control neurons but 6.7 ± 0.4 mV in $G\beta_1\gamma_4$-injected neurons, supporting the notion that $G\beta_1\gamma_4$ subunits reduce the voltage dependence of the



F i g. 4. Reduction in the conductance and voltage dependence of $Ca_V2.2$ currents under the action of $G\beta_1\gamma_4$ subunits. A and B) Voltage-conductance curves in the control (n = 7) and in $G\beta_1\gamma_4$-injected neurons (n=7) at 20 and 200 msec respectively. Solid lines show the fit to a Boltzmann function. C) Steady-state current-voltage curve for $Ca_V2.2$ channels in the control (n = 7) and in $G\beta_1\gamma_4$-injected neurons (n=5). D) Diagram summarizing Gβγ-related regulation of the $Ca_V2.2$ current decay. C, closed state; O, open state; Df, fast decay state; Ds, slow decay state; CGβγ, closed regulated state, and OGβγ, open regulated state.

Р и с. 4. Зменшення провідності та потенціалзалежність $Ca_V2.2$-струмів в умовах дії субодиниць $G\beta_1\gamma_4$.

fast component. We then obtained the current-voltage relationship in the steady state. The $G\beta_1\gamma_4$ subunits reduced I_{Ba} from −110 mV to −40 mV, after which no changes were observed (Fig. 4C). $Ca_V2.2$ activation potential is –40 mV [12]. Thus, the reduction in the current after this potential has been reached suggests that the absence of the fast component produced by the $G\beta_1\gamma_4$ subunits is due to a substantial fraction of $Ca_V2.2$ channels in a non-conducting state.

DISCUSSION

Our experiments revealed that the $Ca_V2.2$ current decay in $G\beta_1\gamma_4$-injected neurons consists only of the slow component (Fig. 2), supporting the theory that Gβγ subunits prevent the appearance of the fast component. Interestingly, the fast component is restored by a strong depolarizing prepulse, in agreement with a previously reported voltage-dependent mechanism [19]. It has been documented that this mechanism comes from direct binding of Gβγ subunits to the α subunit of $Ca_V2.2$ channels [20, 21] with a greater affinity to closed states [22]. It has also been reported that a strong depolarizing pulse unbinds Gβγ subunits from the channel, releasing the channel from the voltage-dependent regulation [9]. Therefore, the recovery of the fast component can be explained by the release of $G\beta_1\gamma_4$ subunits from $Ca_V2.2$ channels (Fig. 3).

Current decay that occurs during a depolarizing pulse is defined as inactivation [23]. Thus, the components of $Ca_V2.2$ current decay could correspond to fast and slow inactivation [12, 16]. This suggests that, in $G\beta_1\gamma_4$-injected neurons, the absence of the fast component is due to an uncoupling mechanism belonging to the fast inactivation component. However, it has been reported that G protein activation by norepinephrine lowers the prevalence of the fast open probability channels [24] and decreases the conductance [25]. In accordance with these reported findings, we observed a reduction in the macroscopic conductance, which can be accounted for by a decreased prevalence of $Ca_V2.2$ channels with a fast open probability. The effects on the $Ca_V2.2$ current decay mediated by Gβγ subunits are illustrated in Fig. 4D. Unregulated channels transit from a closed to an open state. Afterward, these channels can be converted into a fast or slow decay state. As G proteins are activated, Gβγ subunits bind to the closed-state channels. Channels in the open regulated state shift to the slow decay state only. Under experimental conditions, direct interaction between the Gβγ subunits and the channels was released by a strong depolarizing prepulse [9]. Thus, the open regulated state can transit to the open state after a strong depolarizing prepulse; thereafter, channels may transition to the fast or slow decay states, as under control conditions. According to a reported model in which the voltage sensor of the channel is trapped by Gβγ subunit charges [26] and to the proposal that voltage-dependent inactivation of $Ca_V2.2$ is coupled to the voltage sensor [27], we suggest that the absence of the fast component in $G\beta_1\gamma_4$-injected neurons is due to uncoupling of inactivation. Therefore, trapping of the voltage sensor by the Gβγ subunits may impede transition of the channels to the inactivated state, thereby preventing the fast decay. Under physiological conditions, regulation of the fast component decay by $G\beta_1\gamma_4$ subunits could impact the Ca^{2+} influx, which in turn acts as a modulator of the neuronal firing rate [28]. Thus, this type of regulation may govern spatial and temporal physiological processes underlying the flow of information in the respective neuronal networks.

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All procedures performed in studies involving animals were in accordance with the international ethic norms, in particular the Mexican Official Norm for Use, Care and Reproduction of Laboratory Animals (NOM-062-ZOO-1999).

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ВПЛИВ ДИМЕРА GB_1g_4 ПРОТИДІЄ ШВИДКОМУ СПАДУ СТРУМУ ЧЕРЕЗ $CA_V2.2$-КАНАЛИ В СИМПАТИЧНИХ НЕЙРОНАХ ЩУРА

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Р е з ю м е

Як вважають, потенціалзалежна регуляція активності каналів $Ca_V2.2$ G-протеїнами реалізується субодиницями бета-гамма (Gβγ). Відомості про те, чи контролюють дані субодиниці фазу спаду відповідних струмів, є обмеженими. З урахуванням цього ми вивчали процес затухання струму через згадані канали в культивованих нейронах верхнього шийного ганглія щура, в які ін'єкували димер $Gβ_1γ_4$. Струми через канали $Ca_V2.2$ відводили з використанням методики петч-клемп у конфігурації «ціла клітина». Було виявлено, що спад цих струмів у нейронах, не ін'єкованих вказаним димером, складався із двох компонентів («швидкого» та «повільного»), тоді як в аналогічних нейронах після такої ін'єкції спостерігався лише «повільний» компонент. «Швидкий» компонент спаду поновлювався після прикладання потужного деполяризуючого імпульсу, і механізм такої дії був потенціалзалежним. Зменшення макроскопічної провідності через 20 мс після початку деполяризаційного поштовху вказує на те, що відсутність «швидкого» компонента зумовлена непровідним станом істотної частки $Ca_V2.2$-каналів. Такі результати узгоджуються з твердженням, що субодиниці $Gβ_1γ_4$ контролюють процес спаду струму через ці канали та протидіють розвитку «швидкого» компонента даного процесу.

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