Further characterization of regulation of CaV2.2 by Stargazin

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Stargazin, a transmembrane protein expressed in the nervous system, shares similarities with the γ subunit of skeletal muscle calcium channels. It was thus termed γ1 subunit of neuronal calcium channels. Stargazin downregulates the expression of CaV2.2 channels, however, its functional modulation of these channels remains debated. We have reported that stargazin modulates CaV2.2 channel by a Gβγ-dependent mechanism and suggested that stargazin is not a true subunit of this channel, since all its effects on channel function are dependent on the presence of Gβγ. Moreover, Stargazin also modulated the GIRK channel in a Gβγ-dependent fashion. Here we report that Gβγ-dependent modulation by Stargazin of the biophysical properties of CaV2.2 is unrelated to its negative effect on channel expression and current amplitude. Finally, we suggest that this Gβγ-dependent modulation of Stargazin may have physiological relevance, since it was still present when we used Ca2+ as charge carrier, instead of Ba2+.

Neuronal voltage dependent Ca2+ channels, which give rise to N and P/Q type currents (CaV1.2–1.3), are important regulators of neurotransmitter release from pre-synaptic nerve terminals.1 These channels are negatively regulated by activation of receptors coupled to pertussis toxin sensitive G protein subunits, GPCRs.2 The molecular mechanism of this modulation involves direct binding of the G protein βγ subunit to the main, pore-forming subunit of the calcium channel.3 Ca2+ channels are multi-subunit proteins comprised of a main pore-forming subunit α and 3 ancillary subunits: αδ, β and, in skeletal muscle, also γ4.4 The neuronal protein Stargazin was termed calcium channel γ1 subunit due to structural and functional similarities with the skeletal muscle calcium channel γ1 subunit.5-7 Later studies revealed that Stargazin is a crucial regulator of AMPA receptors.8 Still, Stargazin and other γ proteins were found to inhibit calcium channel currents and expression,9,10 while their effect on channel’s biophysical properties remained debated.7,9,11-14 We have recently discovered that Stargazin modulation of CaV2.2 channels coexpressed in Xenopus oocytes is dependent on the G protein βγ subunit.15 Essentially, Stargazin acts as Gβγ scavenger, “stealing” it away from the channel thus diminishing Gβγ mediated inhibitory effects. In light of our findings we proposed that Stargazin does not modulate the channel directly, as expected from a true subunit, acting instead through a Gβγ-dependent mechanism. Here we report data that further extend our understanding of Stargazin modulation of the CaV2.2 channel expressed in Xenopus oocytes, using the two electrode voltage clamp and confocal imaging methods. One previously characterized role of Stargazin and related proteins in calcium channel modulation is their inhibitory effect on current amplitude and expression of the channels.7,9,10 In accordance with the above reports, our study showed that coexpression of Stargazin with the CaV2.2 channel inhibited current amplitudes, and coexpression of Stargazin and CaV2.2 containing YFP-tagged α1B subunit (YFP-α1B), decreased channel surface expression. This inhibitory effect seemed independent of Gβγ, as Stargazin decreased channel expression in oocytes with or without coexpressed Gβγ.15 To further assess the involvement...
of Gβγ in this modulation by Stargazin, we examined the effects of Stargazin on Gβγ-mediated modulation under similar channel expression levels and/or current amplitudes. Such conditions can be achieved by adjusting channel's RNA doses with or without expression of Stargazin. The results are presented in Figure 1. Gβγ (2.5 and 0.5 ng per oocyte of Gβ1 and Gγ2, respectively) was expressed in both groups. Coexpression of Stargazin with 10 ng (each subunit) of RNA of YFP-α1β, α2/δ and β3 gave rise to similar whole-cell currents as those measured following expression of 0.5 ng of channel RNA without Stargazin (Fig. 1A). Ba²⁺ currents through the expressed calcium channels were measured using the facilitation protocol (Fig. 1A, upper inset) described in ref. 15. We used currents measured after the depolarizing pre-pulse (Fig. 1A -pp) to compare between the test groups, as it indicates true amplitude, eliminating the possible inhibitory effect of Gβγ. Under these conditions, Stargazin still counteracted the Gβγ-mediated modulation of the channel, as judged from the significant acceleration of the kinetics of activation measured without the depolarizing pre-pulse (Fig. 1A -pp and B). We found no significant correlation between current amplitude and the activation kinetics (Fig. 1C). Thus, the acceleration of activation probably did not arise from expression of large amounts of channel RNA, which could lead to an excess of channels over Gβγ and thus to diminished modulation by Gβγ. Stargazin also slightly enhanced the extent of voltage dependent

Figure 1. For figure legend, see p. 353.
Figure 1 (See opposite page). Effect of stargazin on current amplitude, surface expression and activation kinetics. (A) Left part: exemplary traces obtained by the facilitation protocol (upper inset) from oocytes expressing 0.5 ng RNA of YFP-α1B/α2δ/β3 channels with 2.5/0.5 ng RNA of Gβγ (Gβγ - upper trace) or 10 ng RNA of YFP-α1B/α2δ/β3 channels with 2.5/0.5 ng RNA of Gβγ and 1 ng RNA of stargazin (stg + Gβγ - lower trace). -pp and +pp indicate currents measured before and after the depolarizing pre-pulse respectively. Numbers on the protocol inset are the voltage steps used in the protocol. Capacitance transients and the outward current elicited by the pre-pulse to 100 mV are trimmed for simplicity. Traces shown here represent net Ina after the subtraction of Cd²⁺-resistant currents. When subtraction with Cd²⁺ was not available, we used the step from -80 to -90 mV to calculate and subtract the leak current. Data summary of current amplitudes recorded after the depolarizing pre-pulse are presented on the right. No statistical significance was found between the test groups by student’s t-test. (B) data summary of activation kinetics in oocytes expressing 0.5 ng RNA of YFP-α1B/α2δ/β3 channels with Gβγ (Gβγ) or 10 ng RNA of YFP-α1B/α2δ/β3 channels with Gβγ and stargazin (stg + Gβγ). Kinetics of activation was assessed by calculating the time from the beginning of the depolarizing pulse, without pre-pulse, to 90% of peak amplitude (t₉₀%). Stargazin significantly (p < 0.01) accelerated kinetics of activation. (C) Correlation between current amplitude and the kinetics of activation in oocytes expressing the non tagged α1B/α2δ/β3 channels with 5/1 ng RNA of Gβγ. No significant correlation was found between the magnitude of current after pre-pulse and the kinetics of activation by Pearson correlation test (correlation coefficient = 0.389, p > 0.05, n = 21). (D) Left part: confocal images from representative oocytes expressing 0.5 ng RNA of YFP-α1B/α2δ/β3 channels with 2.5/0.5 ng RNA of Gβγ (Gβγ) or 10 ng RNA of YFP-α1B/α2δ/β3 channels with 2.5/0.5 ng RNA of Gβγ and stargazin (stg + Gβγ). Data summary of YFP emission intensity is presented on the right. Significantly higher intensity was measured in stargazin expressing oocytes (p < 0.01). (E) Summary of current amplitudes (left) and YFP emission intensities (right) from oocytes expressing 0.5, 2 or 5 ng RNA of YFP-α1B/α2δ/β3 channels. Similar currents were recorded from the 2 and 5 ng RNA expressing groups, yet YFP emission intensity was significantly higher in 5 ng expressing group than in the 2 ng RNA expressing group (p < 0.01). (F) Summary of normalized current amplitudes (left) and YFP emission intensities (right) from oocytes expressing 5 ng RNA of YFP-α1B/α2δ/β3 channels with 2.5/0.5 ng RNA of Gβγ (Gβγ) or with 2.5/0.5 ng RNA of Gβγ and stargazin (stg + Gβγ). Data from each oocyte was normalized to mean value of the Gβγ group. Stargazin significantly reduced both current amplitudes after pre pulse (p < 0.01) and YFP emission intensity (p < 0.01). Number of oocytes in each group is shown in the respective bars. In (A, B, D and F) statistical comparison was performed with Student’s t-test **p < 0.01; in (E) statistical significance is shown in each RNA dose relative to the group expressing 5 ng of channel RNA. *p < 0.05; **p < 0.01 by one-way ANOVA, with post hoc—Student-Newman-Keuls test.

Figure 2. Effect of stargazin using Ca²⁺ as charge carrier. (A) exemplary traces obtained using the facilitation protocol (Fig. 1A) from oocytes expressing α1B/α2δ/β3 channels (control), channels with 2.5/0.5 ng RNA of Gβγ (Gβγ) or channels with 2.5/0.5 ng Gβγ and stargazin 2 ng (stg + Gβγ). (B) Data summary of current amplitudes (left), voltage-dependent facilitation ratio (middle) and kinetics of activation (right) recorded in 20 mM Ca²⁺ containing solution. Stargazin significantly reduced current amplitudes with or without pre-pulse (p < 0.01), reduced facilitation ratio (p < 0.05) and significantly accelerated activation kinetics (p < 0.01) compared to the channel and Gβγ expressing group (Gβγ). Number of oocytes in each group is shown in the respective bar of facilitation ratio graph. Statistical significance is shown in each treatment relative to the group expressing the channel and Gβγ. *p < 0.05; **p < 0.01 by one-way ANOVA, with post hoc—Student-Newman-Keuls test.
facilitation (Fig. 1A and data not shown). This is expected owing to the bell shaped dependency of the effect of Gβγ on facilitation, when at high expression levels of Gβγ the facilitation is reduced compared with lower Gβγ doses, while a scavenger can increase it by reducing the concentration of free Gβγ. Surprisingly, the YFP fluorescence, which reports plasma membrane levels of YFP-α1βδγ was much higher in oocytes expressing the high dose of channel RNA and Stargazin than with the low RNA dose without Stargazin (Fig. 1D). To understand this phenomenon, we injected increasing amounts of RNA of YFP-α11βδγ and βδγ. The maximal current amplitude was achieved at 2 ng RNA, while the maximal YFP emission intensity was detected at the higher, 5 ng RNA dose (Fig. 1E). Therefore, it is possible that, at high expression levels of CaV2.2, a fraction of the YFP emission that we measured came from non-functional channels expressed in the plasma membrane or in sub-membrane compartments, which do not contribute to the whole-cell current. When Stargazin was expressed while keeping the amount of channel RNA (5 ng per oocyte) constant, Stargazin caused a similar reduction (~80–90%) both in current amplitudes and channel surface expression (Fig. 1F). These results support previous findings regarding Stargazin effects on channel synthesis and trafficking. They also call for caution in interpreting the imaging data to assess plasma membrane levels of expressed proteins, emphasizing the need to use more than one independent method (reviewed in ref. 16). In conclusion, the inhibitory effect of Stargazin on Gβγ-mediated modulation of CaV2.2 is independent from, and additional to, it’s effect on channel expression and trafficking.

Our standard measurements of currents through the CaV2.2 channels were conducted using Ba2+ as charge carrier.13 However, this is not the situation in native cells, where Ca2+ ions flow through the channel. We thus tested the effects of Stargazin on the Gβγ modulation of CaV2.2 with Ca2+ as charge carrier. We used the facilitation protocol as described15 to assess Stargazin effect on Gβγ mediated modulation of CaV2.2 recorded in the standard solution15 but with 20 mM Ca2+ substituting the Ba2+. Exemplary traces from representative oocytes are presented in Figure 2A. Stargazin caused similar inhibitory effects regardless of the charge carrier, significantly reducing current amplitude (Fig. 2B left bars), voltage dependent facilitation ratio (middle bars,) and accelerating the kinetics of activation (Fig. 2B right bars). In conclusion, these results show that charge carrier does not alter the effect of Stargazin on current amplitude, voltage dependent facilitation or activation kinetics. These results show that Stargazin’s effect on Gβγ mediated modulation of CaV2.2 channels may exist under physiological conditions.

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