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$G\beta_2$ mimics activation kinetic slowing of $Ca_V2.2$ channels by noradrenaline in rat sympathetic neurons

Juan M. Hernández-Castellanos [a], Oscar Vivas [a], Julieta Garduño [a], Lizbeth De la Cruz [a], Isabel Arenas [a], David Elías-Viñas [b], Ken Mackie [c], David E. García [a,*]

[a] Department of Physiology, School of Medicine, Universidad Nacional Autónoma de México (UNAM), Apartado Postal 70250, C.P. 04510 México, D.F., México
[b] Section of Bioelectronics, Department of Electrical Engineering, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Apartado Postal 14740, C.P. 07000 México, D.F., México
[c] Department of Psychological and Brain Sciences, Gill Center for Biomedical Sciences, Indiana University, Bloomington, IN 47405, USA

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ABSTRACT

Several neurotransmitters and hormones acting through G protein-coupled receptors elicit a voltage-dependent regulation of $Ca_V2.2$ channels, having profound effects on cell function and the organism. It has been hypothesized that protein–protein interactions define specificity in signal transduction. Yet it is unknown how the molecular interactions in an intracellular signaling cascade determine the specificity of the voltage-dependent regulation induced by a specific neurotransmitter. It has been suspected that specific effector regions on the Gβ subunits of the G proteins are responsible for voltage-dependent regulation. The present study examines whether a neurotransmitter's specificity can be revealed by simple ion-current kinetic analysis likely resulting from interactions between Gβ subunits and the channel-molecule. Noradrenaline is a neurotransmitter that induces voltage-dependent regulation. By using biochemical and patch-clamp methods in rat sympathetic neurons we examined calcium current modulation induced by each of the five Gβ subunits and found that $G\beta_2$ mimics activation kinetic slowing of $Ca_V2.2$ channels by noradrenaline. Furthermore, overexpression of the $G\beta_2$ isoform reproduces the effect of noradrenaline in the willing–reluctant model. These results advance our understanding on the mechanisms by which signals conveying from a variety of membrane receptors are able to display precise homeostatic responses.

1. Introduction

The Gβγ subunits of heterotrimeric G proteins are major regulators of voltage-dependent ion channels [1,2]. Additionally, Gβγ subunits play a critical role in coupling G protein-coupled receptors (GPCRs) to diverse effectors, including the β isoforms of phospholipase C and phosphatidylinositide 3-kinases [3,4]. Although Gβγ can directly activate many of these effectors through protein–protein interactions *in vitro*, it remains unclear how Gβγ spatially and temporally coordinates the activation of quite a number of effectors *in vivo*. Neurotransmitters and hormones such as noradrenaline (NA), vasoactive intestinal polypeptide, somatostatin and gonadotropine releasing hormone have diverse modulating effects. One common means by which they affect cell function is via voltage-dependent inhibition of $Ca_V2.2$ channels [5–9]. Since $Ca_V2.2$ channels often transduce electrical to chemical signaling in excitable cells, their regulation can modulate intercellular communication. Neurotransmitter-induced calcium channel regulation is often by GPCRs activation [10]. However it is unknown which molecules in the GPCR signaling pathway determine a neurotransmitter's specificity in voltage-dependent regulation. As Gβ subunits directly mediate voltage-dependent inhibition of $Ca_V2.2$ channels [11,12] one possibility is that Gβ subunits mediate this specificity. Previously we showed that $Ca_V2.2$ currents in neurons from rat superior cervical ganglion (SCG) display kinetic slowing and voltage-dependent facilitation after overexpression of $G\beta_1$ or $G\beta_2$ that was similar to that produced by NA [13], suggesting that NA-induced inhibition is mainly via by the $G\beta_1$ and/or $G\beta_2$ subunits. Nevertheless it has not been examined whether some Gβ subunits carry the information of a specific neurotransmitter inducing voltage-dependent regulation and kinetic slowing. The purpose of this paper was to determine the specificity of $Ca_V2.2$ channel inhibition by NA, as a remarkable example of a neurotransmitter inducing voltage-dependent regulation. To this end we compared kinetic slowing and willing–reluctant population

* Corresponding author. Fax: +52 (55) 5623 2241.
E-mail address: erasmo@unam.mx (D.E. García).

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changes induced by agonist application and Gβ subunit overexpression. We found that $Ca_V2.2$ channels are characteristically modulated by every Gβ subunit isoform. Furthermore changes in the speed of activation of the current and in the channel population interchange induced by NA are mimicked by overexpression of the $Gβ_2$ isoform. Our results contribute to understand the mechanism by which Gβ subunits specifically mediate neurotransmitter-induced $Ca_V2.2$ current inhibition.

2. Materials and methods

2.1. Cell culture and nuclear microinjection

Single SCG neurons were enzymatically dissociated from 5-week-old male Wistar rats as described previously [14]. Rats were obtained from the animal breeding facility of the School of Medicine at UNAM and were handled according to the Mexican Official Norm for Use, Care and Reproduction of Laboratory Animals (NOM-062-ZOO-1999). After 4 h wait for attachment to the substrate, neurons were intranuclearly microinjected using an Eppendorf (Madison, WI, USA) 5242 microinjector and a 5171 micromanipulator system as described by García et al. [13]. The injection solution contained cDNA constructs encoding for a green fluorescent protein (GFP) mutant fused to the Gβ protein subunit (Gβ-GFP; 100 ng/μl) and the $Gγ_3$ protein subunit (100 ng/μl), i.e., for all the cases described in this paper we performed nuclear co-injections combining each Gβ subunit with $Gγ_3$. Therefore, the description of the result for every Gβ overexpressed, we termed it simply as Gβ. The expression plasmids were mixed with 1 mg/ml 10,000 kDa dextran–fluorescein as an injection marker. Injection pressures of 10–20 kPa for 0.5–0.8 s resulted in no obvious increase in cell volume (<5%). After 18–24 h, successfully injected neurons were identified by their characteristic greenish-blue GFP fluorescence using an inverted microscope equipped with epifluorescence optics.

2.2. Plasmids

DNA encoding $Gβ_1$ was cloned in pCDM8, DNAs encoding $Gβ_2$, $Gβ_4$, and $Gβ_5$ were cloned in pcDNA I, $Gβ_3$ was cloned in pCIS, $Gγ_3$ was cloned in pCI (all from M. Simon, Caltech, Pasadena, CA). GFP was from pEGFP-N1 (Clontech, Palo Alto, CA). Although not in identical vectors, expression of all G proteins was driven by the cytomegalovirus promoter. Additional control experiments with independent $Gβ_1$-GFP plasmids were conducted with same results. Plasmids were purified using commercial kits (Qiagen, Valencia, CA). These procedures have been previously described [13].

2.3. Electrophysiology and data analysis

Membrane current recordings were performed in whole cell voltage clamp configuration of the patch clamp technique [15] using a List EPC-7 amplifier at room temperature (22–24 °C). The recordings were monitored, digitized, recorded and analyzed utilizing BASIC FASTLAB (Indec Systems, Capitola, CA). Pipettes used for recordings were pulled from borosilicate glass with a resistance of 0.8–1.2 MΩ. Series resistance was 1.5 ± 0.44 MΩ (mean ± SEM) and compensated to >70%. Steady-state currents were sampled at 10 kHz and tail currents at 50 kHz using a 'split-clock' protocol. Neurons were constantly superfused locally (1.5 ml/min) with control or NA solutions. Pipettes were filled out with internal solution contained (in mM): 140 CsCl, 20 TEA-Cl, 10 HEPES, 0.1 tetracesium-BAPTA, 5 $MgCl_2$, 5 Na_2ATP, 0.3 Na_2GTP, and 0.1 leupeptin, and were adjusted to pH 7.2 with CsOH. Neurons were superfused during recording (1–2 ml/min) with a solution designed to isolate Ba^{2+} currents flowing through $Ca_V2.2$ channels, external solution contained (in mM): 165 TEA-Cl, 1 $BaCl_2$, 10 HEPES, 8 glucose, 1 $MgCl_2$, 0.0002 TTX, and were adjusted to pH 7.4 with TEA-OH. $Ca_V2.2$ current was defined as the component of the current sensitive to 100 μM $CdCl_2$ in the presence of 5 μM nifedipine. Reagents were obtained from Sigma (St. Louis, MO). To avoid one source of systematic bias, experimental and control measurements were alternated whenever possible, and concurrent controls were always performed. Where appropriate, data are expressed as mean ± SEM.

3. Results

3.1. $Gβ_1$–$Gβ_4$ share high degree of sequence identity

Modulation of ionic channels, particularly the presynaptic calcium channels, by GPCRs, is a key modulator of neurotransmission. Previous work has shown that Gβγ subunits produced voltage-dependent modulation of calcium channels. The extent of the inhibition is dependent on the type of Gβ subunit, and it is antagonized by a strong membrane depolarization (i.e., voltage dependence). Although with different degrees of modulation, a similar inhibition of calcium channels is produced by $Gβ_1$, $Gβ_2$, $Gβ_3$ and $Gβ_4$, whereas $Gβ_5$ produces minimal effects. Considering that the $Gβ_5$ subunit produces no significant modulation, this subunit appears to be unique in the Gβ subunit family [13,16,17]. Building on these observations, we revised the similarities among the five subtypes of Gβ subunits by aligning the amino acid sequences of $Gβ_1$–$Gβ_5$ (Fig. 1A) and using two open access programs (Clustal Omega and FASTA). We observed that $Gβ_1$–$Gβ_4$ share 80–90% of identity, whereas $Gβ_5$ only 55% (Fig. 1B) according to data reported by others [3,18]. In agreement with previously reported results [19–21] it is likely that the $Gβ_1$–$Gβ_4$ subtypes mediate different degrees of ion channel voltage-dependent modulation. This means, the selectivity and potency of inhibition is dependent on the subunit construction, while $Gβ_5$ likely participates in another kind of modulation [4], for instance, voltage-independent inhibition [13,22].

3.2. Different Gβ subtypes mediate different degrees of kinetic slowing of the $Ca_V2.2$ currents

In order to assess the abilities of different Gβ subtypes to modulate calcium channels, we explored the typical $Ca_V2.2$ current kinetic slowing produced by NA in neurons overexpressing different Gβ subunits. Each Gβ subunit was always tested by overexpressing $Gγ_3$ concurrently. Fig. 2A shows superimposed current traces evoked with a double depolarizing pulses protocol separated by application of a strong depolarizing (+80 mV) prepulse (PP). In the absence of Gβ overexpression in SCG neurons, i.e., control condition, calcium current was inhibited by the NA application, and after PP we observed the typical disinhibition of the current (Fig. 2Aa). Fig. 2Ab–f gives examples of the different kinetics produced by different Gβ subunits. $Gβ_2$ produced the major occlusion of the inhibition mediated by NA, whereas $Gβ_5$ did not have any significant effect (Fig. 2Ac, f). The activation time constant obtained in control conditions (Fig. 2B) and that obtained with $Gβ_5$ overexpressing neurons were very similar (1.56 ± 0.47 vs 1.85 ± 0.15 ms, respectively), while the $Gβ_2$ activation time constant was much slower significantly (2.76 ± 0.25 ms). Given these results, we decided to use $Gβ_2$ for subsequent analysis.

3.3. $Gβ_2$ mimics the kinetic slowing of $Ca_V2.2$ channels induced by NA

To date it remains unclear whether the activation of a specific GPCR by a neurotransmitter releases a certain combination of

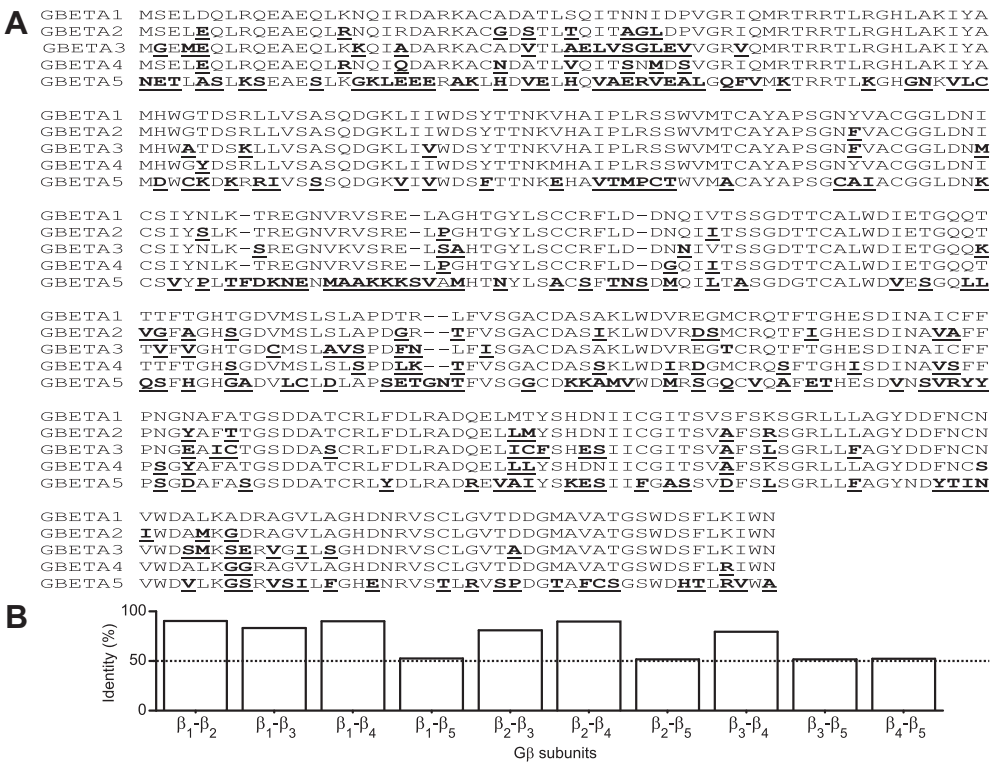

Fig. 1. Gβ subunits alignment shows high percentage of identity between $G\beta_1$–$G\beta_4$. (A) Alignment of amino acids sequences of $G\beta_1$–$G\beta_5$ in *Rattus norvegicus*. Amino acids sequences were obtained from GenBank database. Sequence alignment and percentage of identity were generated by Clustal Omega and FASTA programs with similar results. The first eight amino acids (MATDGLHE) of $G\beta_5$ were not aligned. Bold and underline letters show differences related to $G\beta_1$. (B) Graph of percentage of identity. Dotted line depicts 50% of identity, closer to $G\beta_5$ and conversely to $G\beta_1$–$G\beta_4$ subunits.

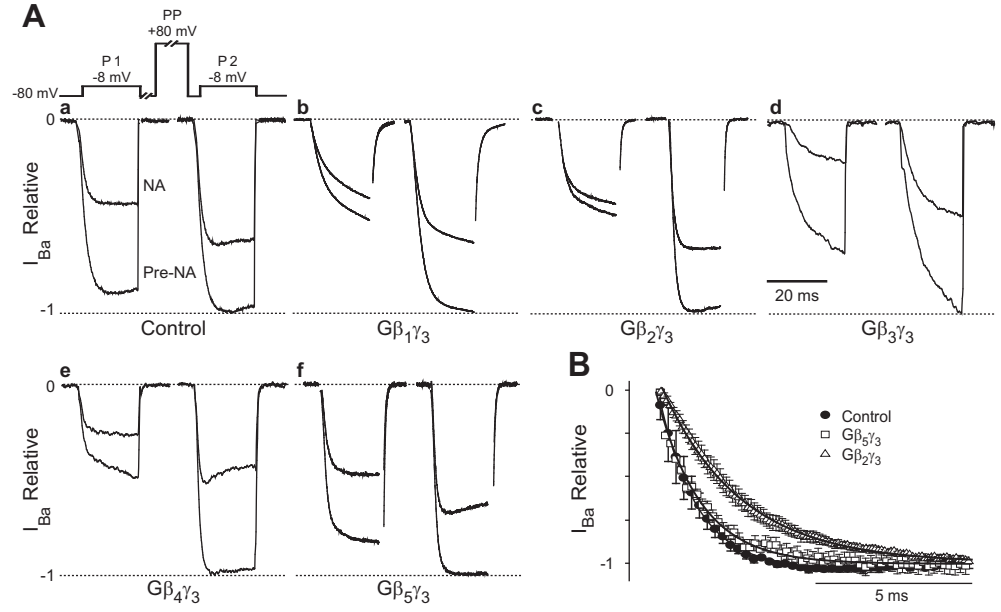

Fig. 2. Gβ subunits produce differential kinetics on $Ca_V2.2$ activation. (A) Superimposed current traces were evoked with a pair of 20 ms depolarizing pulses to −8 mV (P1, P2) from a holding potential of −80 mV, separated by a prepulse (PP) of +80 mV during 50 ms (double-pulse protocol, above of the current traces in Aa). The Ba^{2+} currents were recorded in the absence (bottom traces, Pre-NA) and presence (top traces, NA) of 10 μM NA for control (Aa), $G\beta_1\gamma_3$ (Ab), $G\beta_2\gamma_3$ (Ac), $G\beta_3\gamma_3$ (Ad), $G\beta_4\gamma_3$ (Ae) and $G\beta_5\gamma_3$ (Af) overexpressing neurons. (B) Activation kinetics of Ba^{2+} currents graph. Averaged current traces were recorded during the first 10 ms (P1) for control (black circles), $G\beta_5\gamma_3$ overexpressing cells (white squares), and $G\beta_2\gamma_3$ overexpressing neurons (white triangles) (mean ± SEM, $n = 4$). Data were fitted to an exponential decay function; the solid lines indicate the best fitting values.

Gβγ subunits to stimulate a particular signaling pathway [13,17,20]. In order to address this topic, we investigated which subunit best mimics the kinetic slowing of calcium channels induced by NA. We compared the values of $G\beta_1$–$G\beta_4$ activation time curse with those obtained by NA application. Fig. 3A illustrates the slowing of activation kinetics of $G\beta_2$ compared with

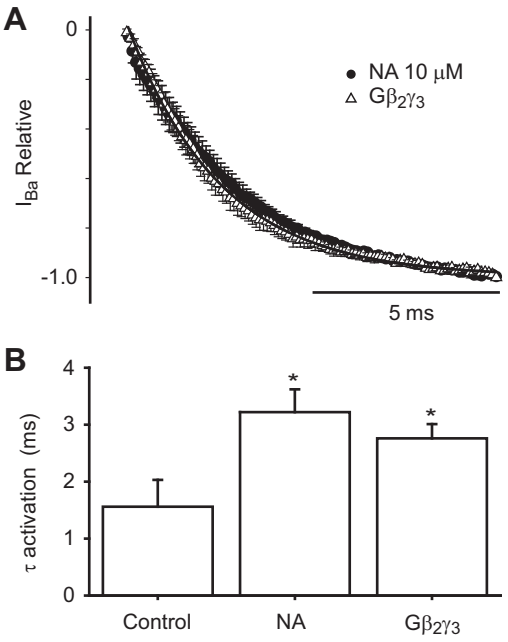

Fig. 3. Kinetic slowing of Ba^{2+} current induced by NA is mimicked by $G\beta_2\gamma_3$. (A) Activation kinetics of Ba^{2+} currents for control neurons in the presence of NA or $G\beta_2\gamma_3$ overexpressing neurons. The current values were obtained as explained in Fig. 2B for control (uninjected) cells in the presence of NA (black circles), and $G\beta_2\gamma_3$ overexpressing neurons (white triangles). Data were fitted to an exponential decay function, and the solid lines indicate the best fitting. (B) Summary of effects on τ activation of control neurons, NA (uninjected cells) and $G\beta_2\gamma_3$ expressing cells. Data are presented as mean ± SEM ($n = 3$), $^*p < 0.05$ with respect to control.

activation time curse obtained in the presence of 10 μM NA. As seen, overexpression of $G\beta_2$ subunits closely mimics the kinetic slowing observed in calcium channels by NA. The monoexponential fits of the activation time course gave tau activation values for control: 1.56 ± 0.47, NA: 3.22 ± 0.4 and $G\beta_2$: 2.76 ± 0.25 (Fig. 3B). This result supports the mimicking and occlusion of NA responses by $G\beta_2$ overexpression.

3.4. $G\beta_2$ also mimics the willing–reluctant model with NA in a $Ca_V2.2$ current population

The model proposed by Bean [6] remains the best model to explain how neurotransmitters regulate calcium channels. This model posits that there are two functional states of the calcium channels: unregulated willing channels, and G protein-regulated reluctant channels, with the latter releasing in response to strongly depolarized potentials. The hallmark of this regulation is the termed kinetic slowing [2,9,23]. However, changes in voltage-dependence by Gβs also need to be considered. To this end, we did a population analysis from activation curves. Amplitude of tail currents, obtained at a potential of −40 mV following the termination of 20 ms test pulses from −80 to +80 mV, were measured isochronally from 1 to 2 ms average data points after the pulse step. The relationships between tail currents amplitudes and the test pulse potentials were fitted to a two-component Boltzmann equation (Fig. 4A, black continuous lines). The decomposition of the double in a single Boltzmann is shown in gray continuous and black discontinuous lines, representing the willing and reluctant populations respectively (Fig. 4). The population analysis from tail currents is shown in pulses 1 and 2 for neurons in control

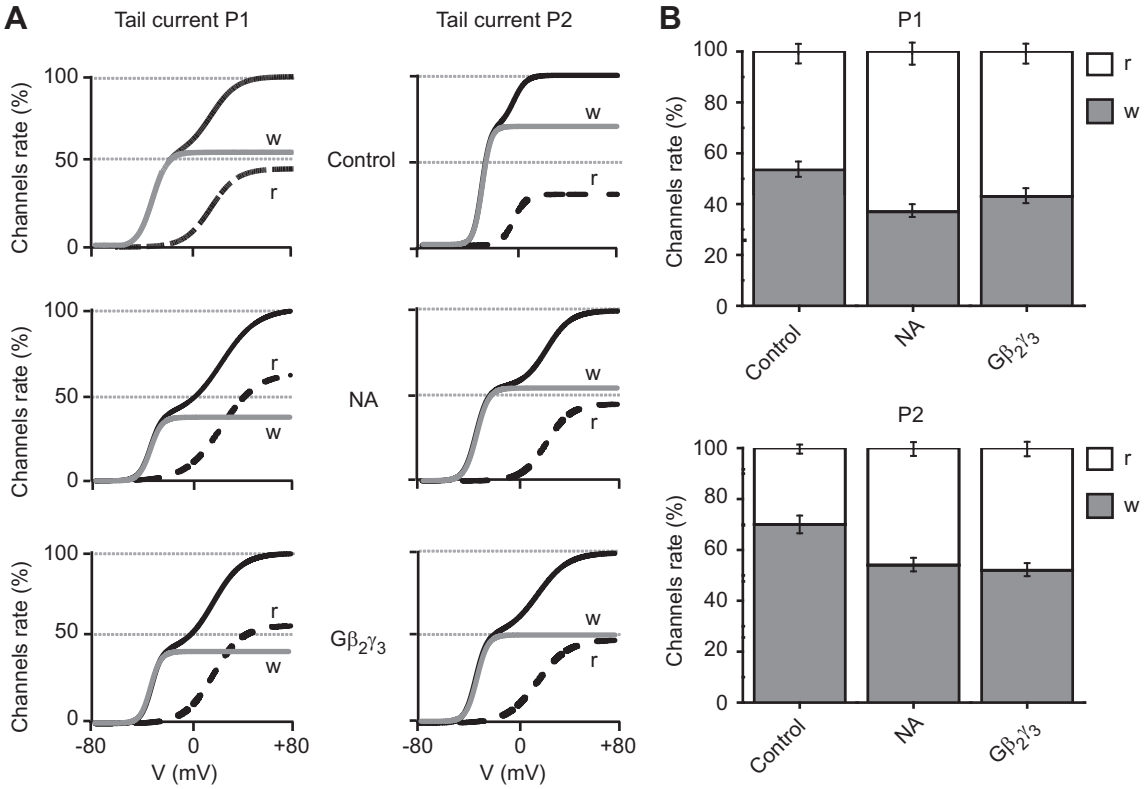

Fig. 4. NA produces interchange between willing and reluctant mode similar to $G\beta_2\gamma_3$ overexpression cells. (A) Channels rate (%) at different voltage membrane potentials in Control, NA and $G\beta_2\gamma_3$ overexpression neurons during a double-pulse protocol (Tail current P1 and P2). The black continuous lines represent fitting to a double Boltzmann function to the mean values from 1 to 2 ms of the tail currents. The decomposition of double Boltzmann in single Boltzmann are showed like gray continuous and black discontinuous lines, representing the willing (w) and reluctant (r) modes respectively. (B) Summary of channels rate (%) of willing and reluctant modes. Data are presented as mean ± SEM, ($n = 4$).

conditions, during NA application and in neurons overexpressing $G\beta_2$. Application of 10 μM NA increased the reluctant channel population and concomitantly decreased the willing channel population in the first pulse. After the conditioning pulse to relieve voltage-dependent regulation, the willing component increased and the reluctant fraction decreased in the second pulse. Constitutive voltage-dependent inhibition by G proteins and its relief by strong depolarizations in SCG neurons are well documented [8,13]. In the first pulse the reluctant channel population increased and in the second pulse the willing component increased, concomitantly decreasing the reluctant component (Fig. 4). Notably, overexpression of $G\beta_2$ mimics NA in pulse 2 (Fig. 4B). These results suggest that each isoform of Gβ exerts differential effects on channel populations which can be reflected as differences not only in association constants but also in voltage-sensitive binding.

4. Discussion

Thousands of presynaptic terminal buttons converge onto cell dendrites and soma, releasing neurotransmitters to elicit a variety of intracellular signaling responses. Remarkably, protein–protein based interactions and specificity are closely related phenomena [24]. As a result, actions upon effectors are largely preserved so that proper homeostatic responses are timely and spatially coordinated. However, it remains unclear how a neurotransmitter promotes a certain combination of Gβγ subunits to generate a particular response by a signaling pathway. Several reports agree that NA primarily produces a voltage-dependent inhibition of $Ca_V2.2$ channels [9]. Here we found that overexpressed $G\beta_2$ mimics the biophysical actions produced by NA, such as kinetic slowing [13]. By contrast, overexpression of $G\beta_5$ elicits a Ba^{2+} current phenotype that is very similar to that obtained in control conditions. Together our observations reinforce the hypothesis that $G\beta_1$–$G\beta_4$ lead to a voltage-dependent signaling, while $G\beta_5$ does not. Apparently this Gβ subunit belongs to a distinct system of ion channel regulation [4,13]. This indicates that the voltage-dependent inhibition produced by a natural agonist, such a NA, through the activation of GPCRs on the voltage gated calcium channels in rat SCG neurons, may be mediated by a particular Gβ subunit. Certainly, it is also expected that a range of Gβs, instead of a particular one, could account for by this regulation. Previously, we reported for the first time that overexpression of $G\beta_2$ subunit inhibits $Ca_V2.2$ currents, increases the facilitation ratio, and partially occludes the actions of NA; in addition, that Gαs themselves do not reproduce any voltage-dependent regulation on $Ca_V2.2$ channels [11]. In general, overexpression of $G\beta_1$–$G\beta_4$ produces qualitatively similar effects; in contrast, $G\beta_5$ only weak effects [17]. This observation correlates well with the percent of identity among different Gβs (Fig. 1B) [3,18]. It is also cut clear from slowed activation in current recordings that overexpression of each Gβ subunit specifically changes current kinetics (Fig. 2A). In good agreement with our current hypothesis, every Gβ subunit isoform elicits a differential voltage-dependent inhibition of calcium currents, elucidated by characteristic kinetic slowing, facilitation ratio and agonist occlusion, except for $G\beta_5$. It also expands the possibility that voltage-dependent regulation induced by different neurotransmitters, may be mediated by different Gβ subunit combinations carrying the specificity of the voltage-dependent modulation for every ion channel type, enzyme or transporter. Therefore, future experiments should be extended to other GPCRs with a variety of neurotransmitters. Furthermore, Gβ subunit isoforms binding with different dissociation constants to GPCRs should be considered. Conclusively, we propose that specificity of voltage-dependent regulation of $Ca_V2.2$ is mediated by a Gβ subunit or a Gβ subunits combination. In addition, every type of GPCR may bind with different ratios to all of the five known of Gβ isoforms making possible the variety of intracellular signaling for such a wide range of neurotransmitters eliciting voltage-dependent modulation [9].

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