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ION CHANNELS

Importance of voltage-dependent inactivation in N-type calcium channel regulation by G-proteins

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Abstract Direct regulation of N-type calcium channels by G-proteins is essential to control neuronal excitability and neurotransmitter release. Binding of the $G_{\beta\gamma}$ dimer directly onto the channel is characterized by a marked current inhibition ("ON" effect), whereas the pore opening- and time-dependent dissociation of this complex from the channel produce a characteristic set of biophysical modifications ("OFF" effects). Although G-protein dissociation is linked to channel opening, the contribution of channel inactivation to G-protein regulation has been poorly studied. Here, the role of channel inactivation was assessed by examining time-dependent G-protein de-inhibition of $Ca_v2.2$ channels in the presence of various inactivation-altering β subunit constructs. G-protein activation was produced via μ-opioid receptor activation using the DAMGO agonist. Whereas the "ON" effect of G-protein regulation is independent of the type of β subunit, the "OFF" effects were critically affected by channel inactivation. Channel inactivation acts as a synergistic factor to channel activation for the speed of G-protein dissociation. However, fast inactivating channels also reduce the temporal window of opportunity for G-protein dissociation, resulting in a reduced extent of current recovery, whereas slow inactivating channels undergo a far more complete recovery from inhibition. Taken together, these results provide novel insights on the role of channel inactivation in N-type channel regulation by G-proteins and contribute to the understanding of the physiological consequence of channel inactivation in the modulation of synaptic activity by G-protein coupled receptors.

Keywords N-type calcium channel · $Ca_v2.2$ subunit · G-protein · G-protein coupled receptor · μ-opioid receptor · inactivation · β subunit

Abbreviations

GI	G-protein inhibition
GPCR	G-protein coupled receptor
DAMGO	(D-Ala^2,Me-Phe^4,glycinol^5)-enkephalin
rMOR	rat μ-opioid receptor
PCR	polymerase chain reaction
RI	recovery from inhibition
NS	non-statistically significant

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Introduction

Voltage-dependent N-type calcium channels play a crucial role in neurotransmitter release at the central and peripheral synapse [3, 47]. Several subtypes of N-type channels, which differ in their inactivation properties either because of differences in subunit composition [43] or because they represent splice variants [5, 28], are known to exist. N-type

channels are strongly regulated by G-protein coupled receptors (GPCR) [4, 18, 25, 29, 30]. Direct regulation by G-proteins involves the binding of the $G_{\beta\gamma}$ dimer [22, 27] on various structural determinants of $Ca_v2.2$, the pore-forming subunit of N-type channels [1, 11, 15, 23, 34, 38, 44, 53]. This regulation is characterized by typical biophysical modifications of channel properties [16], including (1) a marked current inhibition [7, 51], (2) a slowing of activation kinetics [30], (3) a depolarizing shift of the voltage-dependence of activation [4], (4) a current facilitation after prepulse depolarization [26, 42], and (5) a modification of inactivation kinetics [52]. Current inhibition has been attributed to $G_{\beta\gamma}$ binding onto the channel ("ON" effect), whereas all other channel modifications are a consequence of a variable time-dependent dissociation of $G_{\beta\gamma}$ from the channel ("OFF" effects) [49]. Although the dissociation of $G_{\beta\gamma}$ was previously described as voltage-dependent [17], it was then suggested that channel opening after membrane depolarization was more likely responsible for the removal of $G_{\beta\gamma}$ [35]. More recently, we have shown that the voltage-dependence of the time constant of $G_{\beta\gamma}$ dissociation was directly correlated to the voltage-dependence of channel activation suggesting that $G_{\beta\gamma}$ dissociation is in fact intrinsically voltage-independent [49].

Although $G_{\beta\gamma}$ dissociation, and the resultant characteristic biophysical changes associated with it, has been correlated with channel activation, the contribution of channel inactivation in G-protein regulation has been barely studied. Evidence that such a link may exist has emerged from a pioneering study from the group of Prof. Catterall [23] in which it was demonstrated that mutations of the β-subunit binding domain of $Ca_v2.1$, known to affect inactivation, also modifies G-protein modulation. A slower inactivating channel, in which the Arg residue of the QQIER motif of this domain was substituted by Glu, enhanced the prepulse facilitation suggesting that the extent of G-protein dissociation was enhanced. However, establishing a specific relationship between channel inactivation and G-protein regulation with mutants of such a motif is rendered difficult because this motif is also a $G_{\beta\gamma}$-binding determinant [15, 23, 53]. Indeed, mutations of this motif are expected to decrease the affinity of G-proteins for the channel and, hence, may facilitate G-protein dissociation. Differences in G-protein regulation of $Ca_v2.2$ channels have also been reported if the channel is associated to β subunit that induces different inactivation kinetics [12, 20, 31]. However, a formal link between channel inactivation and G-protein regulation was not established in any of these studies.

In this study, we analyzed how modifying channel inactivation kinetics could affect the parameters of G-protein dissociation (time constant and extent of dissociation). We used a method of analysis that was recently developed on N-type channels for extracting all parameters of G-protein regulation at regular potential values, independently of the use of prepulse depolarizations [48]. The objective was to perform a study in which the structural properties of the pore-forming subunit would remain unaltered to keep the known G-protein binding determinants of the channel functionally intact. Structural analogues of β subunits, known or expected to modify channel inactivation properties, were used [14, 32, 40]. It is concluded that fast inactivation accelerates G-protein dissociation from the channel, whereas slow inactivation slows down the process. However, channel inactivation also reduces the temporal window of opportunity in which G-protein dissociation can be observed. Far less recovery is observed for channels that undergo fast inactivation, whereas slow inactivating channels display almost complete G-protein dissociation. With regard to the landmark effects of G-protein regulation, it is concluded that the "ON" effect (extent of G-protein inhibition) is independent of the type of inactivation provided by β subunits, whereas all "OFF" effects (slowing of activation and inactivation kinetics, shift of the voltage-dependence of activation) are largely influenced by the kinetics of channel inactivation induced by the β constructs. These results better explain the major differences that can be observed in the regulation of functionally distinct N-type channels. Furthermore, they provide an insight of the potential influence of channel inactivation in modulating G-protein regulation of N-type channels at the synaptic level.

Materials and methods

Materials

The cDNAs used in this study were rabbit $Ca_v2.2$ (GenBank accession number D14157), rat β_{1b} (X61394), rat β_{2a} (M80545), rat β_3 (M88751), rat β_4 (L02315), and rat μ-opioid receptor (rMOR, provided by Dr. Charnet). (D-Ala^2,Me-Phe^4,glycinol^5)-enkephalin (DAMGO) was from Bachem (Bubendorf, Germany).

Molecular biology

The CD8–β_{1b} chimera was generated by polymerase chain reaction (PCR) amplification of the full-length β_{1b} using oligonucleotide primers 5′-CGCGGATCCGTCCAGAAGAGCGGCATGTCCCGGGGCCCTTACCCA-3′ (forward) and 5′-ACGTGAATTCGCGGATGTAGACGCCTTGTCCCCAGCCCTCCAG-3′ (reverse), and the PCR product was subcloned into the *Bam*HI and *Eco*RI sites of the pcDNA3-CD8-βARK-myc vector after removing the βARK insert (vector generously provided by D. Lang,

Geneva University, Geneva, Switzerland). The truncated N-terminal β_{1b} construct ($\beta_{1b\,\Delta N}$, coding for amino acid residues 58 to 597) was performed as described above using the primers 5′-CGCGGATCCACCATGGGCTCAGCA GAGTCCTACACGAGCCGGCCGTCAGAC-3′ (forward) and 5′-CGGGGTACCGCGGATGTAGACGCCTTGTCCC CAGCCCTCCAGCTC-3′ (reverse), and the PCR product was subcloned into the *Kpn*I and *Bam*HI sites of the pcDNA3.1(−) vector (Invitrogen). The truncated N-terminal β_3 construct ($\beta_{3\,\Delta N}$, coding for amino acid residues 16 to 484) was performed using the primers 5′-CGCGGATCCAC C ATGGGTTCAGCCGACTCCTACACCAGC CGCCCCTCTCTGGAC-3′ (forward) and 5′-CGGGG TACCGTAGCTGTCTTTAGGCCAAGGCCGG TTACGCTGCCAGTT-3′ (reverse), and the PCR product was subcloned into the *Kpn*I and *Bam*HI sites of the pcDNA3.1(−) vector.

Transient expression in *Xenopus* oocytes

Stage V and VI oocytes were surgically removed from anesthetized adult *Xenopus laevis* and treated for 2–3 h with 2 mg/ml collagenase type 1A (Sigma). Injection into the cytoplasm of cells was performed with 46 nl of various cRNA mixture in vitro transcribed using the SP6 or T7 m Message mMachine Kit (Ambion, Cambridgeshire, UK; 0.3 μg/μl $Ca_v2.2$+0.3 μg/μl μ-opioid receptor+0.1 μg/μl of one of the different calcium channel β constructs). Cells were incubated at 19°C in defined nutrient oocyte medium as described [19].

Electrophysiological recording

After incubation for 2–4 days, macroscopic currents were recorded at room temperature (22–24°C) using a two-electrode voltage-clamp in a bathing medium containing (in millimolar): $Ba(OH)_2$ 40, NaOH 50, KCl 3, HEPES 10, niflumic acid 0.5, pH 7.4 with methanesulfonic acid. Electrodes filled with (in millimolar) KCl 140, EGTA 10, and HEPES 10 (pH 7.2) had resistances between 0.5 and 1 MΩ. Macroscopic currents were recorded using Digidata 1322A and GeneClamp 500B amplifier (Axon Instruments, Union City, CA). Acquisition and analyses were performed using the pClamp 8 software (Axon Instruments). Recordings were filtered at 2 kHz. Leak current subtraction was performed on-line by a P/4 procedure. DAMGO was applied at 10 μM by superfusion of the cells at 1 ml/min. All recordings were performed within 1 min after DAMGO produced maximal current inhibition. We observed that this procedure fully minimized voltage-independent G-protein regulation that took place later, about 5–10 min after DAMGO application (data not shown). Hence, the inhibition by DAMGO was fully reversible as assessed by washout experiments. Also, no rundown was observed during the time course of these experiments. Cells that presented signs of prepulse facilitation before μ-opioid receptor activation (tonic inhibition) were discarded from the analyses.

Analyses of the parameters of G-protein regulation

The method used to extract all biophysical parameters of G-protein regulation (GI_{t_0}, the initial extent of G-protein inhibition before the start of depolarization, τ, the time constant of G-protein unbinding from the channel, and RI, the extent of recovery from inhibition at the end of a 500-ms test pulse, unless specified in the text) were described elsewhere [48]. The key steps required to extract these parameters are briefly summarized in Fig. 1. This method is analogous to the method that relies on the use of prepulses but avoids many of the pitfalls of the latter (use of an interpulse potential that favors G-protein re-association, differences in the rate of channel inactivation between control and G-protein regulated channels, and facilitation that occurs during the control test pulse) [48].

Mathematical and statistical analyses

Current–voltage relationships (I/V) were fitted with the modified Boltzmann equation $I_{(V)} = (G_{max} \times (V - E))/\left(1 + \exp\left(-\left(V - V_{1/2}\right)/k\right)\right)$ where $I_{(V)}$ represents the maximal current amplitude in response to a depolarization at the potential V, G_{max} is the maximal conductance, E is the inversion potential of the Ba^{2+}, and k is a slope factor. All data are given as mean±SEM for n number observations and statistical significance (p) was calculated using Student's t test. Statistical significance for scatter plot analysis was performed using the Spearman rank order correlation test.

Results

N-type current inhibition by G-proteins is independent of the β subunit species

G-protein inhibition is generally studied through the measurement of the peak currents. However, this approach does not take into account the fact that, at the time to peak, a considerable proportion of G-proteins has already dissociated from the channel during depolarization. To better estimate the real extent of N-type current inhibition by G-proteins, we used the technical approach described in Fig. 1 to measure GI_{t_0}, the maximum extent of G-protein inhibition before the start of the G-protein unbinding process. Representative current inhibition and kinetic alterations are shown for $Ca_v2.2$ channels co-expressed with the β_{1b}, β_{2a}, β_3, or β_4 subunit (Fig. 2a, top panel) and

the corresponding GI_{t_0} values were quantified (Fig. 2a, bottom panel). The β subunits did not alter significantly the maximum extents of inhibition that ranged between 59.2±1.4% ($Ca_v2.2/\beta_{2a}$ channels, n=25) and 62.4±1.8% ($Ca_v2.2/\beta_{1b}$ channels, n=25; Fig. 2b). In the following part of this study, three other β subunit constructs have been co-expressed with $Ca_v2.2$, $\beta_{1b\ \Delta N}$, CD8–β_{1b}, and $\beta_{3\ \Delta N}$. As for the wild-type β isoforms, GI_{t_0} varied not significantly (p>0.05) between 58.4±1.8% ($\beta_{1b\ \Delta N}$, n=9) and 63.5±1.3% (CD8–β_{1b}, n=10).

The two parameters that are relevant for the "OFF" effects, τ (the time constant of G-protein unbinding from the channel), and RI (the extent of current recovery from G-protein inhibition after a 500-ms depolarization) will be used to investigate the role of N-type channel inactivation in G-protein regulation. GI_{t_0} is not a time-dependent parameter and cannot be influenced by the time course of inactivation.

Current recovery from G-protein inhibition is altered when the inactivation kinetics of $Ca_v2.2$ channels are modulated by β subunits

Auxiliary β subunits are known to influence the inactivation kinetics of $Ca_v2.2$ channels with a rank order of potency, from the fastest to the slowest, of $\beta_3 \geq \beta_4 > \beta_{1b} >> \beta_{2a}$ [45]. Representative control current traces at 10 mV for $Ca_v2.2$ channels co-expressed with each type of β subunit are shown in Fig. 3a (left panel). As expected from former reports, the β_3 subunit produces the fastest inactivation, whereas β_{2a} induced the slowest inactivation. The β_{1b} and β_4 subunits induce intermediate inactivation kinetics. In agreement with previous reports [12, 20], β subunits markedly affect G-protein regulation. Here, we investigated how channel inactivation affects the kinetics of G-protein departure from the channel and the extent RI. The time constants τ of G-protein dissociation were extracted from the $I_{\text{G-proteins unbinding}}$ traces for each combination of channels (Fig. 3a, middle panel), whereas RI was calculated as the extent of dissociation by comparing the current levels of I_{DAMGO}, $I_{\text{DAMGO wo unbinding}}$, and I_{Control} after 500 ms of depolarization (Fig. 3a, right panel). The data show that both τ and RI values are differentially affected by the kinetics of channel inactivation. Average parameters are reported in Fig. 3b (for τ) and Fig. 3c (for RI). The time constant τ of recovery from G-protein inhibition is 2.9-fold faster for the fastest inactivating channel ($Ca_v2.2/\beta_3$, 37.5±3.3 ms, n=13) than the slowest inactivating channel ($Ca_v2.2/\beta_{2a}$, 107.8±2.7 ms, n=22). Interestingly, the rank order for the speed of recovery from G-protein inhibition ($\beta_3 \geq \beta_4 > \beta_{1b} >> \beta_{2a}$) is similar to that observed for inactivation kinetics. Indeed, Student's t tests demonstrate that differences between β subunits are all highly statistically significant ($p \leq 0.001$) except between β_3 and β_4 were the difference is less pronounced ($p \leq 0.05$; Fig. 3b). Thus, it is concluded that the speed of channel inactivation imposed by each type of β subunit impacts the time constant of recovery from G-protein inhibition. Channel inactivation appears as a "synergistic factor" to channel activation [49] for the speed of G-protein dissociation. Next, the effects of β subunits were investigated on RI values (Fig. 3c). Two of the β subunits (β_3 and β_4) have closely related RI values (56.9±1.8% (n=21) vs 56.8±1.2% (n=34)). In contrast, β_{1b} and β_{2a} statistically decrease (45.0±1.3%, n=24) and increase (96.1±1.4%, n=29) RI values, respectively. From these data, it is clear that faster recovery from inhibition is not necessarily associated with an elevated RI value. Although channel inactivation accelerates the kinetics of G-protein dissociation from the channel, it also reduces the time window in which the process can be completed. In these data, a relationship seems to exist between channel inactivation conferred by β subunits and G-protein dissociation. However, it is unclear whether this link is only due to the kinetics of inactivation conferred by β subunits or also to differences in molecular identities. In order to precisely assess this first observation, we examined how structural modifications of individual β subunits, known to alter channel inactivation, affect the recovery parameters from G-protein inhibition.

Deletion of a β subunit determinant important for fast inactivation alters recovery from G-protein inhibition

Important determinants for the control of inactivation rate have been identified in the past on β subunits [32, 37]. Deletion of the amino terminus of β subunits is known to slow down channel inactivation [14]. According to the data of Fig. 3, slowing of inactivation should increase both the time constant τ of recovery from G-protein inhibition and the extent of RI. Figure 4a,b illustrates the extent of slowing in inactivation kinetics of $Ca_v2.2/\beta_{1b}$ channels when the first 57 amino acids of β_{1b} subunit at the N-terminus are deleted ($\beta_{1b\ \Delta N}$). The amount of inactivation at the end of a 500-ms depolarization at 10 mV shows a 2.2-fold decrease from 58.4±1.6% (n=22) to 26.2±2.3% (n=10; Fig. 4b). Representative traces of DAMGO regulation of $Ca_v2.2/\beta_{1b}$ and $Ca_v2.2/\beta_{1b\ \Delta N}$ currents demonstrate that the deletion of the N terminus of β_{1b} produces a significant modification in G-protein regulation (Fig. 4c, left panel). Notably, DAMGO-inhibited $Ca_v2.2/\beta_{1b\ \Delta N}$ currents display much slower activation kinetics (quantified in Fig. 8c). The analysis of the time course of $I_{\text{G-proteins unbinding}}$ traces in the presence of truncated β_{1b} reveals a slower time course (Fig. 4c, middle panel). Also, the deletion of the N terminus of β_{1b} leads to an increased recovery from G-protein inhibition (Fig. 4c, right panel). Statistical analyses

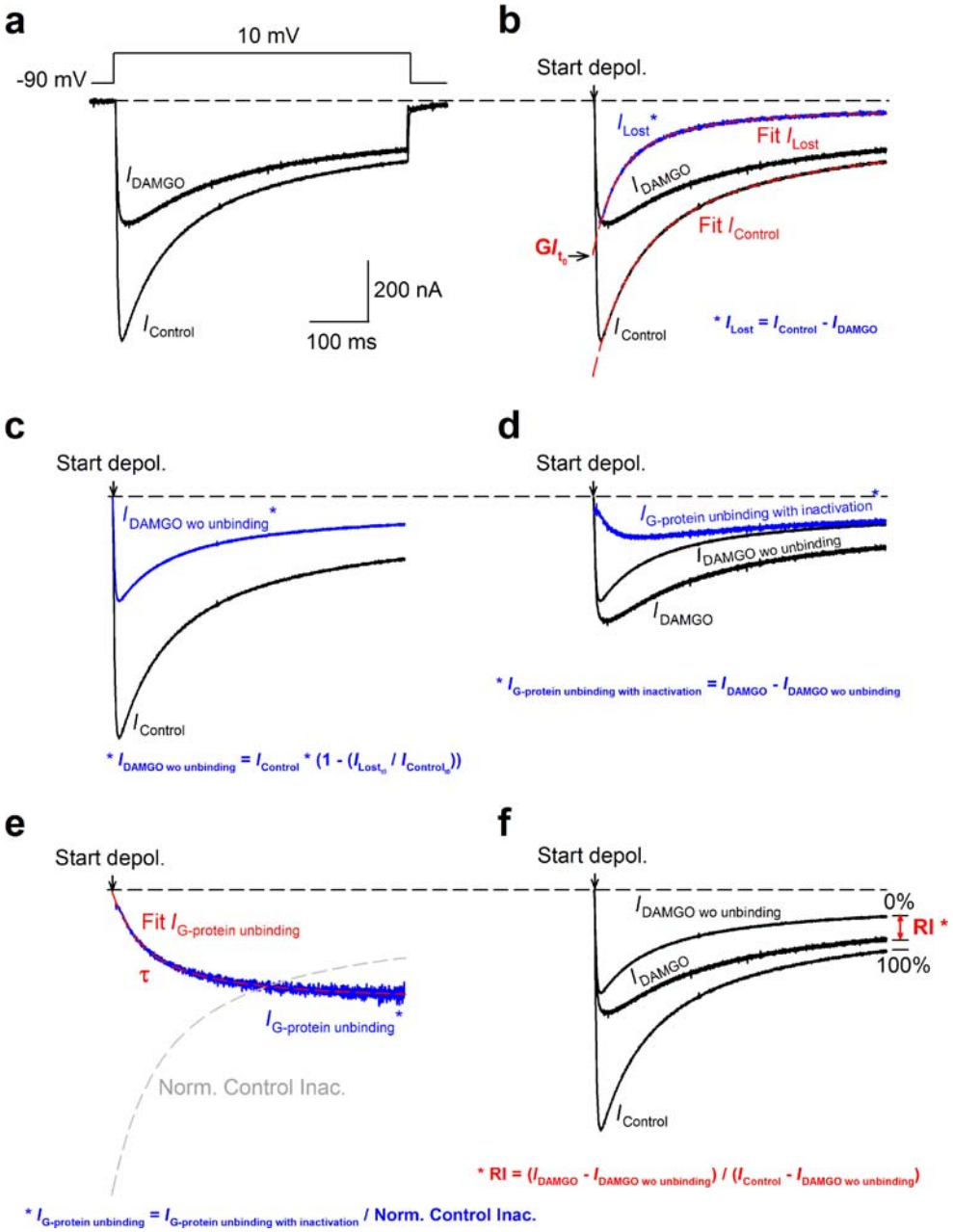

Fig. 1 Illustration of steps leading to the determination of the biophysical parameters of N-type currents regulation by G-proteins according to [48]. **a** Representative $Ca_V2.2/\beta_3$ current traces elicited at 10 mV for control ($I_{Control}$) and DAMGO (I_{DAMGO}) conditions. **b** Subtracting I_{DAMGO} from $I_{Control}$ results in I_{Lost} (*blue trace*), the evolution of the lost current under G-protein activation. $I_{Control}$ and I_{Lost} are then extrapolated to $t=0$ ms (the start of the depolarization) by fitting traces (*red dashed lines*) with a single and double exponential, respectively, to determine GI_{t_0}, the maximal extent of G-protein inhibition. **c** $I_{DAMGO\ without\ unbinding}$ ($I_{DAMGO\ wo\ unbinding}$, *blue trace*) represents an estimate of the amount of control current that is present in I_{DAMGO} and is obtained by the following equation: $I_{DAMGO\,without\,unbinding} = I_{Control} \times \left(1 - \left(I_{Lost_{t_0}} \middle/ I_{Control_{t_0}}\right)\right)$. **d** Subtracting $I_{DAMGO\ wo\ unbinding}$ from I_{DAMGO} results in $I_{G\text{-}protein\ unbinding\ with\ inactivation}$ (*blue trace*), the evolution of inhibited current that recovers from G-protein inhibition after depolarization. **e** $I_{G\text{-}protein\ unbinding\ with\ inactivation}$ is divided by the fit trace (normalized to 1) describing inactivation kinetics of the control current (*gray dashed line*) to reveal the net kinetics of G-protein dissociation ($I_{G\text{-}protein\ unbinding}$, *blue trace*) from the channels. A fit of $I_{G\text{-}protein\ unbinding}$ (*red dashed line*) by a mono-exponential decrease provides the time constant τ of G-protein dissociation from the channel. **f** The percentage of recovery from G-protein inhibition (*RI*, in *red*) at the end of 500 ms pulse is measured as $RI = \left(I_{DAMGO} - I_{DAMGO\,wo\,unbinding}\right) / \left(I_{Control} - I_{DAMGO\,wo\,unbinding}\right) \times 100$. *Arrows* indicate the start of the depolarization

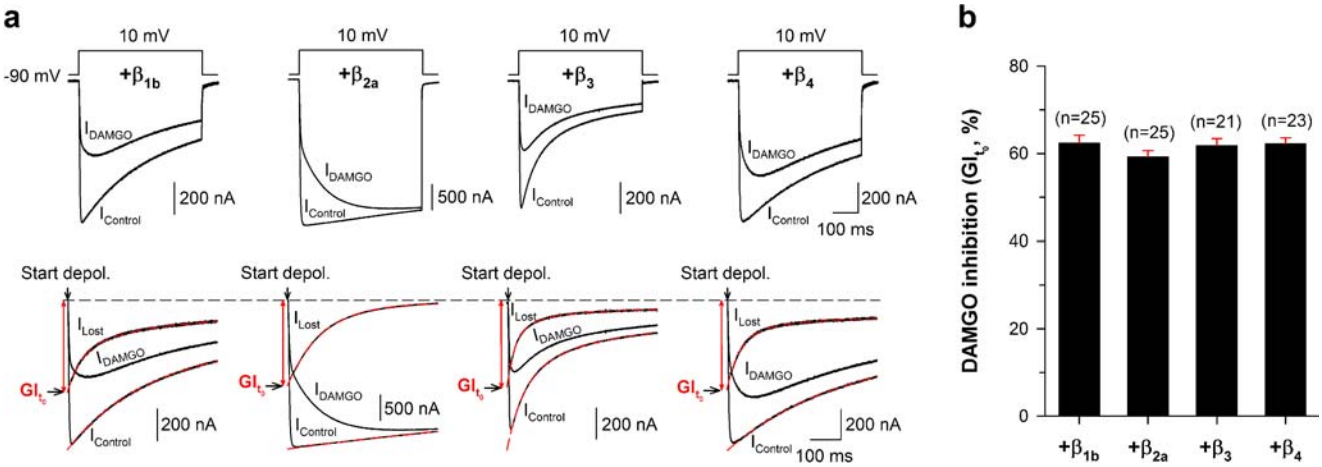

Fig. 2 Maximal G-protein inhibition of N-type currents is independent of the type of β subunits. **a** Representative current traces elicited at 10 mV before ($I_{Control}$) and under 10 μM DAMGO application (I_{DAMGO}) for $Ca_v2.2$ channels co-expressed with the β_{1b}, β_{2a}, β_3, or β_4 subunit (*top panel*). Corresponding traces allowing the measurement of the maximal DAMGO inhibition at the start of the depolarization (GI_{t_0}) are also shown for each experimental condition (*bottom panel*). $I_{Control}$ and I_{Lost} (obtained by subtracting I_{DAMGO} from $I_{Control}$) were fitted by a mono- and a double exponential, respectively (*red dashed lines*), to better estimate the maximal extent of DAMGO-inhibited current before the start of the depolarization (GI_{t_0}). The *red double arrow* indicates the extent the DAMGO-inhibited current at t=0 ms. Traces were normalized at the maximal value of $I_{Control}$ at t=0 ms to easily compare the extent of current inhibition. **b** Block diagram representation of GI_{t_0} for each experimental condition. Data are expressed as mean±SEM (in *red*) for n studied cells

show a significant increase in the time constant τ of recovery (2.0-fold) from 60.0±2.0 ms (n=24) to 118.6±2.5 ms (n=10; Fig. 4d) and an increase in the RI values (1.8-fold) from 45.0±1.3% (n=24) to 79.6±2.5% (n=9) by the deletion of the N terminus of β_{1b} (Fig. 4e).

To confirm that these effects are independent of the nature of the β subunit involved, similar experiments were conducted with a 15-amino acid N-terminal truncated β_3 subunit $\beta_{3\ \Delta N}$. As for $\beta_{1b\ \Delta N}$, $\beta_{3\ \Delta N}$ produces a slowing of channel inactivation kinetics. After 500 ms at 10 mV, $Ca_v2.2/\beta_3$ channels inactivate by 68.9±1.7% (n=21) compared to 41.1±1.1% (n=10) for $Ca_v2.2/\beta_{3\ \Delta N}$ channels (Fig. 5a,b). As expected, DAMGO inhibition of $Ca_v2.2/\beta_{3\ \Delta N}$ channels produces currents with slower activation and inactivation kinetics than $Ca_v2.2/\beta_3$ channels (shift of the time to peak of the current from 20.7±2.5 ms with β_3 (n=21) to 77.0±7.6 ms with $\beta_{3\ \Delta N}$ (n=10); Fig. 5c, left panel). Moreover, the time course of $I_{G\text{-proteins unbinding}}$ was slowed down with the N-terminal truncation of β_3 (Fig. 5c, middle panel), and the recovery from inhibition was enhanced (Fig. 5c, right panel). Quantification of these effects reveals a statistically significant slowing (1.8-fold) of the time constant of recovery τ from G-protein inhibition from 37.5±3.3 ms (n=13) to 67.4±4.5 ms (n=10; see Fig. 5d) and an increase of RI values (1.2-fold) from 56.9±1.8% (n=21) to 66.9±2.1% (n=10; see Fig. 5e). However, the time constant of recovery in the presence of $\beta_{3\ \Delta N}$ remains fast compared to the inactivation kinetics, which may explain the lower increase in RI values compared to what has been measured with $\beta_{1b\ \Delta N}$. Also, the starting value of RI is high for β_3 (56.9%) compared to β_{1b} (45.0%), which limits the possibility of increase.

Slowing of channel inactivation by membrane anchoring of β subunit also alters the properties of recovery from G-protein inhibition

Another approach to modulate channel inactivation is to modify the docking of the β subunits to the plasma membrane [13, 40]. For that purpose, we expressed a membrane-inserted CD8 linked to the β_{1b} subunit (CD8–β_{1b}) along with $Ca_v2.2$. As shown in earlier studies using the same strategy but with a different β subunit [2, 40], membrane anchoring of the β_{1b} subunit significantly slows down the inactivation kinetics (Fig. 6a). Indeed, inactivation was reduced by 1.5-fold from 58.4±1.6% (n=22) to 38.1±1.8% (n=10; see Fig. 6b). Membrane anchoring of β_{1b} via CD8 slowed down the DAMGO-inhibited current activation kinetics (Fig. 6c, left panel). Under DAMGO inhibition, a greater shift of the time to peak of the current was observed for CD8–β_{1b} than for β_{1b} (from 57.0±4.1 ms with β_{1b} (n=12) to 168.8±7.0 ms with CD8–β_{1b} (n=10)). Also, recovery from inhibition was slowed 1.9-fold from 60.0±2.0 ms (n=24) to 112.3±5.4 ms (n=8; Fig. 6d), whereas RI increased 1.3-fold from 45.0±1.3% (n=24) to 58.0±1.9% (n=9; see Fig. 6e).

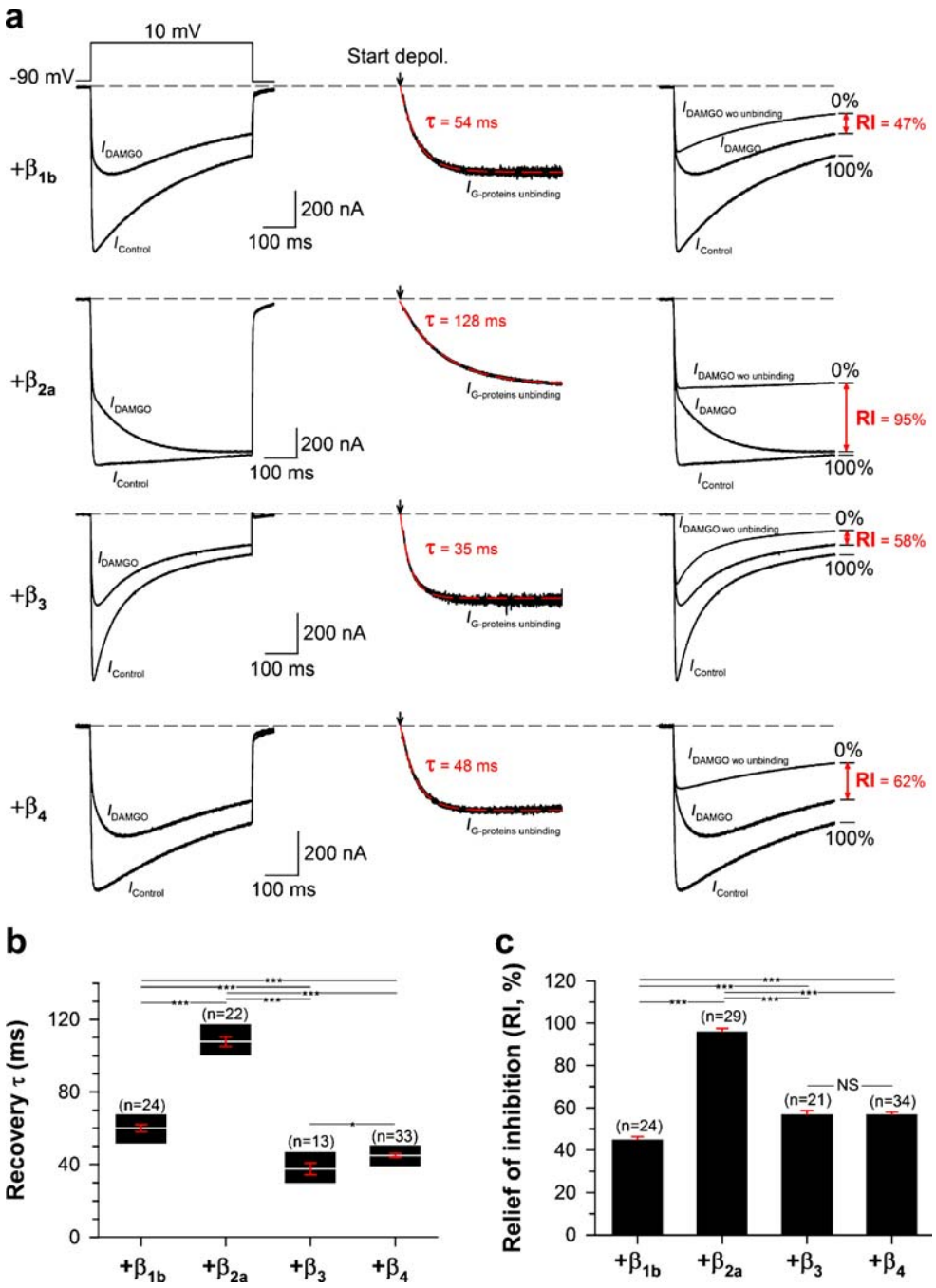

Fig. 3 Influence of β subunits on the recovery of N-type channel inhibition by G-proteins. **a** Representative current traces before ($I_{Control}$) and during application of 10 μM DAMGO (I_{DAMGO}) are shown at 10 mV for $Ca_v2.2$ channels expressed with β_{1a}, β_{2a}, β_3, or β_4 subunit (*left panel*). Corresponding $I_{G\text{-protein unbinding}}$ traces are shown for each condition (*middle panel*) and were fitted by a mono-exponential decrease (*red dashed line*) to determine the time constant τ of G-protein unbinding from the channel. The *arrow* indicates the start of the depolarization. Traces were normalized to better compare kinetics. Traces that allowed the measurement of RI values (in *red*) are also shown for each condition (*right panel*). **b** *Box plot* representation of the time constant τ of G-protein unbinding as a function of the type of β subunit co-expressed with $Ca_v2.2$ channels. The number of cells studied is indicated in *parentheses*. **c** Block diagram representation of RI values measured after 500 ms depolarization as a function of the type of the β subunit expressed with $Ca_v2.2$ channels. Data are expressed as mean±SEM (in *red*) for *n* studied cells. Statistical *t* test: *NS* not statistically significant, *single asterisk* $p \leq 0.05$, *two asterisks* $p \leq 0.01$, *three asterisks* $p \leq 0.001$

Inactivation limits the maximum observable recovery from G-protein inhibition

As demonstrated above, inactivation influences both the time constant of recovery and the maximal observable recovery from inhibition. To study the effect of channel inactivation on the maximum recovery from inhibition independently of the time constant of recovery, we compared RI values and inactivation at a fixed time constant of recovery. The time constant of recovery from inhibition shows a voltage dependence similar to that of channel opening [49]. An example of this voltage dependence is illustrated in Fig. 7a (left panel) for $Ca_v2.2/\beta_{1b}$ channels. A plot of the time constant of recovery as a function of membrane depolarization indicates a great extent of variation in τ values (Fig. 7a, middle panel). This voltage dependency of τ values was observed for all channel combinations (data not shown). We then chose to impose the τ value to 50±5 ms for all expressed channel combinations by selecting the appropriate recordings from the set of traces obtained at various test

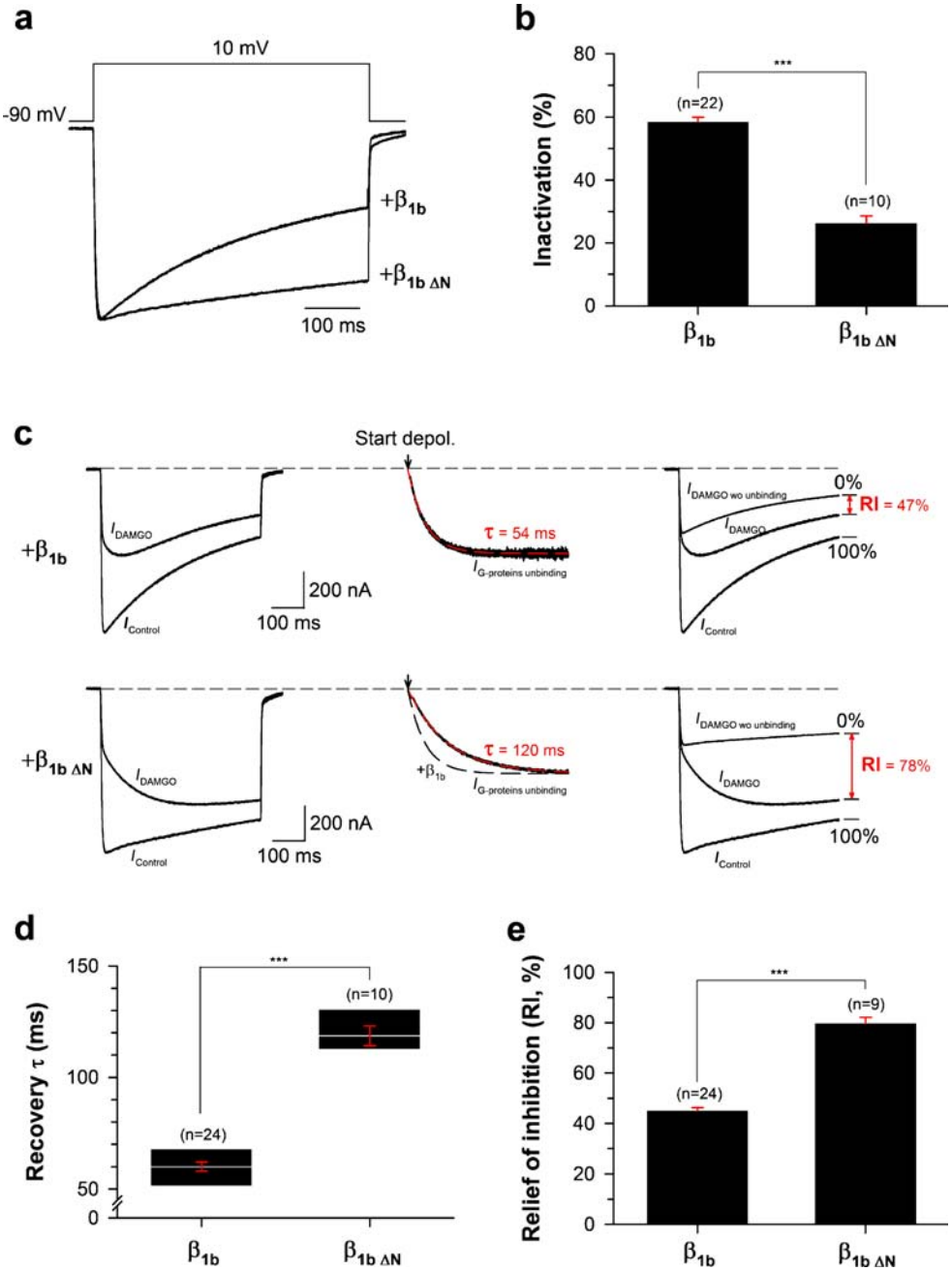

Fig. 4 Slowing of inactivation kinetics by N-terminal truncated β_{1b} subunit modifies the recovery of N-type current inhibition by G-proteins. **a** Representative current elicited by a step depolarization at 10 mV for $Ca_v2.2$ channels co-expressed with the wild-type β_{1b} subunit or with the N-terminal truncated subunit. Current traces were normalized to facilitate comparison of the kinetics and extent of inactivation. **b** Block diagram representation of the extent of inactivated current after 500 ms depolarization. **c** Representative current traces before ($I_{Control}$) and during application of 10 μM DAMGO (I_{DAMGO}) are shown at 10 mV for $Ca_v2.2$ channels co-expressed with the wild-type β_{1b} subunit or with the truncated subunit (*left panel*). Corresponding normalized $I_{G\text{-protein unbinding}}$ traces fitted by a mono-exponential decrease (*red dashed line*) are shown for each condition (*middle panel*). The *arrow* indicates the start of the depolarization. The *black dotted line* represents the $Ca_v2.2/\beta_{1b}$ channel condition shown for comparison. Corresponding traces, which allowed the measure of RI values (in *red*), are also shown for each experimental condition (*right panel*). **d** Box plot representation of time constants τ of recovery from G-protein inhibition at 10 mV for each experimental condition. **e** Block diagram representation of RI values after 500 ms depolarization at 10 mV for each experimental condition. Data are expressed as mean±SEM (in *red*) for *n* studied cells. Statistical *t* test: *three asterisks* denote $p \leq 0.001$

potentials (Fig. 7a, right panel). This τ value was chosen because it allows the incorporation of a large number of recordings in the analysis. Also, with a τ of 50 ms, the RI value at 500 ms after depolarization has reached saturation (95% of recovery after 150 ms of depolarization). For traces that underwent a recovery from inhibition with a τ value of 50±5 ms, we measured the extent of RI and of inactivation, both at 500 ms. Representative examples for different channel combinations ($Ca_v2.2$ along with either β_{2a}, β_4, or β_{1b} from the slowest to the fastest inactivation) are shown in Fig. 7b (left panel) where the RI values and the extent of inactivation (right panel) are measured in each experimental condition. Figure 7c shows the negative correlation existing between the extent of maximum recovery from inhibition and the extent of inactivation (statistically significant at $p<0.001$, $n=62$). These results demonstrate that the only restriction to observe a complete current recovery from G-protein inhibition is the inactivation process. Indeed, channels that have almost no inactivation ($Ca_v2.2$ / β_{2a}) show a complete recovery from inhibition. The curve predicts that, for completely non-inactivating channels, 100% of the current would recover from inhibition. These results confirm that the experimental protocol used herein to minimize voltage-independent

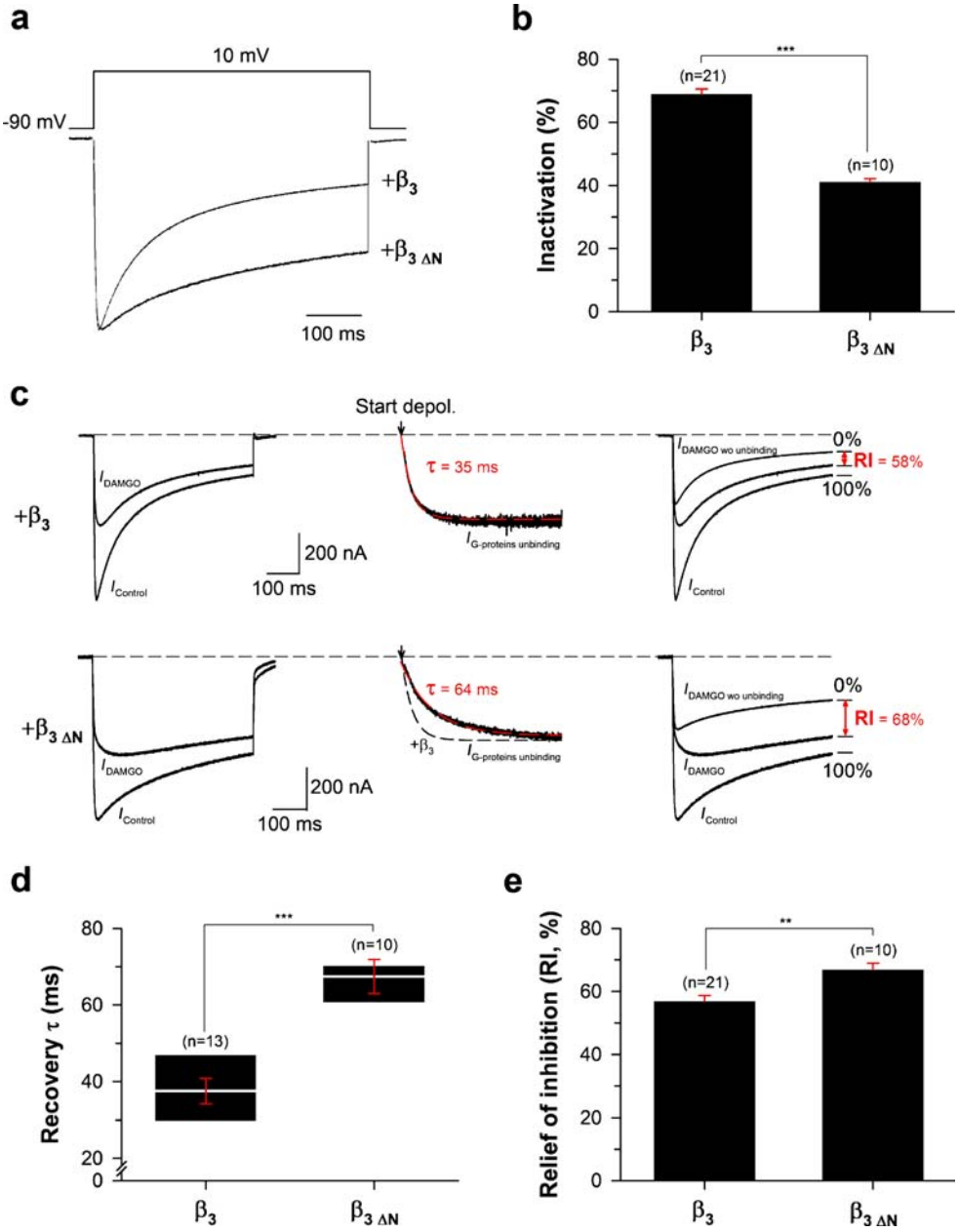

Fig. 5 Slower inactivation kinetics induced by N-terminal truncated β_3 subunit also modifies recovery of N-type current inhibition by G-proteins. Legends as in Fig. 4 but for cells expressing $Ca_v2.2$ channels in combination with the wild-type β_3 subunit or with the N-terminal truncated subunit. Data are expressed as mean±SEM (in *red*) for *n* studied cells. Statistical *t* test: *two asterisks* denote $p \leq 0.01$, while *three asterisks* denote $p \leq 0.001$

inhibition was fully functional. Conversely, channels that present the most inactivation present the smallest amount of recovery from inhibition.

Differences in calcium channel inactivation generate drastic differences in the biophysical characteristics of G-protein regulation

Since recovery from G-protein inhibition induces an apparent slowing of activation and inactivation kinetics, and shifts the voltage dependence of activation towards depolarized values [49], differences in channel inactivation that affect the recovery process should also affect the biophysical effects of G-proteins on N-type channels. Calcium currents are generally measured at peak amplitudes. The consequences of this protocol are shown for $Ca_v2.2/\beta_{1b}$ and $Ca_v2.2/\beta_{1b\ \Delta N}$ channels that present different inactivation kinetics (Fig. 8a,b). Several observations can be raised. First, it is observed that the slowing of the $Ca_v2.2$ inactivation induced by truncating the N terminus of β_{1b} is responsible for a drastic slowing of activation kinetics under DAMGO application. This effect is most pronounced at low potential values and is significantly reduced at high potential values. These effects are quantified in Fig. 8c. For instance, at 0 mV, the average shift of the time to peak for $Ca_v2.2/\beta_{1b\ \Delta N}$ channels (307.7±

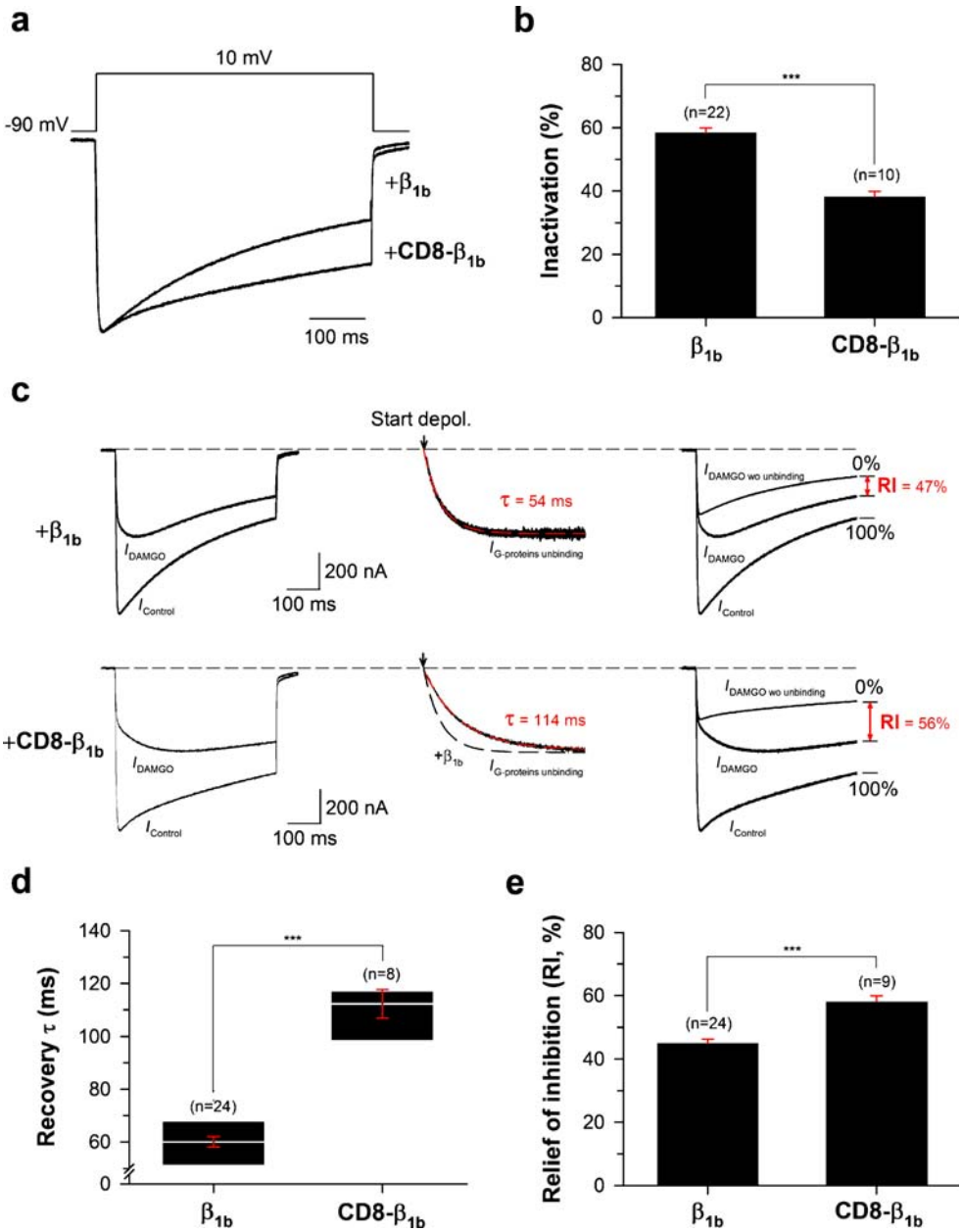

Fig. 6 Slowing of inactivation kinetics by membrane anchoring of β_{1b} subunit modifies recovery of N-type current inhibition by G-proteins. Legends as in Fig. 4 but for cells expressing $Ca_v2.2$ channels in combination with the wild-type β_{1b} subunit or with the membrane-linked CD8 $-\beta_{1b}$ subunit. Data are expressed as mean±SEM (in *red*) for *n* studied cells. Statistical *t* test: *three asterisks* denote $p \leq 0.001$

9.0 ms, $n=10$) is, on average, 9.2-fold greater than that observed for $Ca_v2.2/\beta_{1b}$ channels (33.4±5.2 ms, $n=19$; Fig. 8c). Differences in slowing of activation kinetics, triggered by the two β subunits, remain statistically significant for potential values up to 30 mV. Above 30 mV, the convergence of both curves can be explained by the fact that recovery from G-protein inhibition becomes too rapid to be influenced by changes in inactivation kinetics. Second, at the time points of the peak of the current, slowing of inactivation by the N-terminal truncation of β_{1b} induces (1) a hyperpolarizing shift of the voltage dependence of RI_{peak} values, and (2) an increase in RI_{peak} values for potentials equal or below 30 mV (Fig. 8d). Since RI_{peak} values represent a voltage-dependent gain of current that is added to the unblocked fraction of control currents under G-protein regulation, they apparently modify the voltage dependence of channel activation (*I*/*V* curves) and reduce the level of DAMGO inhibition [49]. For the $Ca_v2.2/\beta_{1b}$ channels, the average half-activation potential values were significantly shifted by 6.4 ± 0.9 mV ($n=13$) under DAMGO inhibition, whereas for the $Ca_v2.2/\beta_{1b\,\Delta N}$ channels, a nonsignificant shift by 1.9±0.5 mV ($n=10$) was determined (Fig. 8e,f). This difference in behavior can readily be explained by the voltage dependence of RI_{peak} values. In the case of $Ca_v2.2/\beta_{1b}$, the maximal RI_{peak} occurs at 30 mV (Fig. 8d), a depolarizing shift of 20 mV

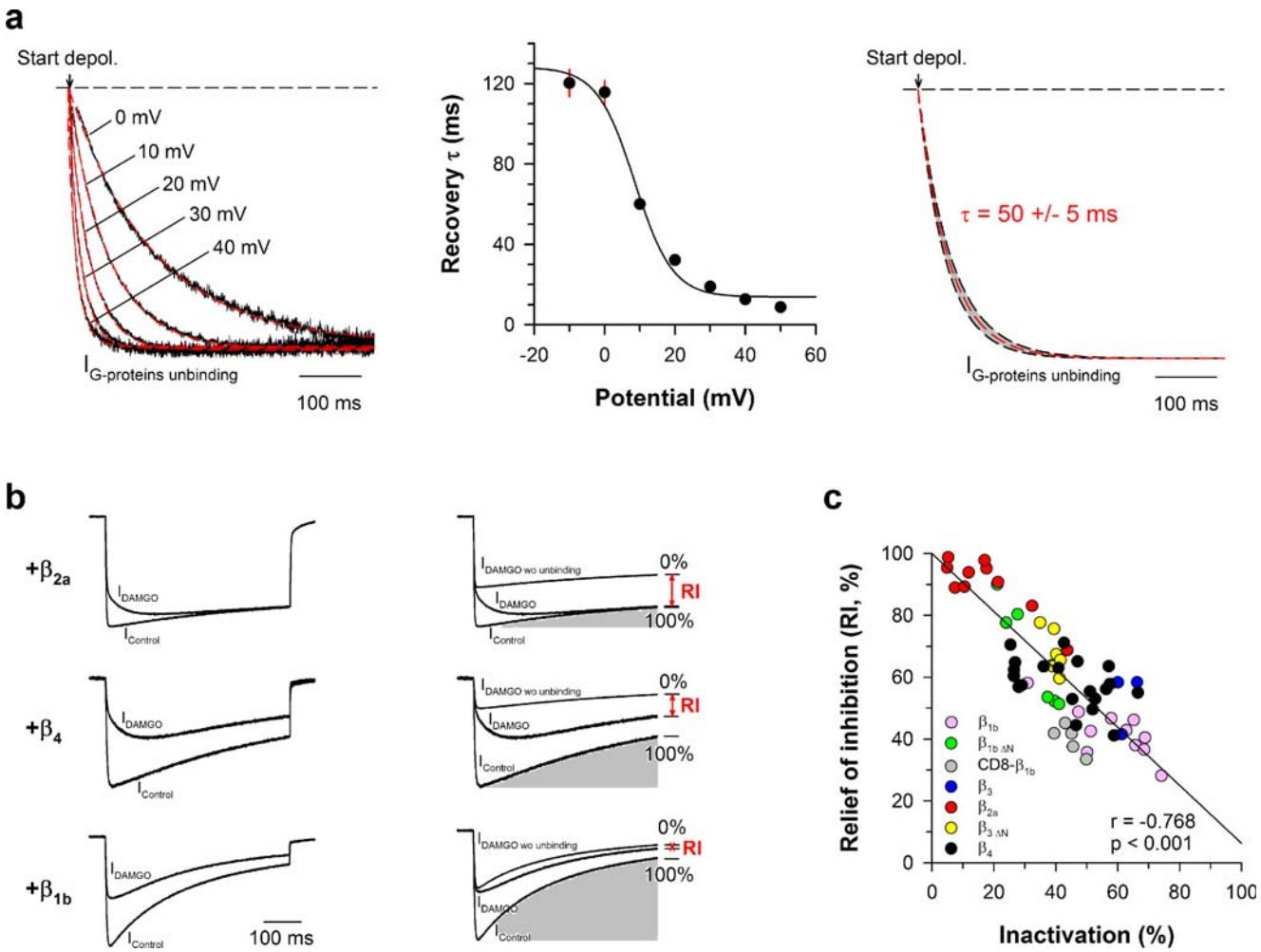

Fig. 7 The extent of N-type channel inactivation correlates with the extent of current recovery from G-protein inhibition. **a** An example of the influence of membrane potential values on the time constant τ of current recovery from G-protein inhibition is shown for $Ca_v2.2/\beta_{1b}$ channels. Normalized $I_{G\text{-protein unbinding}}$ traces fitted by a mono-exponential decrease (*red dashed line*) are shown for a range of potentials from 0 to +40 mV (*left panel*). The *arrow* indicates the start of the depolarization. Traces were superimposed to facilitate kinetic comparisons. Corresponding voltage-dependence of the time constant τ of current recovery from G-protein inhibition (n=13) is shown (*middle panel*). Data are expressed as mean±SEM (in *red*) and were fitted with a sigmoid function. Scheme illustrating normalized $I_{G\text{-protein unbinding trace}}$ for a defined time constant τ of 50±5 ms (*red* and *black lines*, respectively; right panel). The *gray area* represents the accepted variation in τ values (±10%) for the incorporation of current traces in our subsequent analyses. The *arrow* indicates the virtual start of the depolarization. **b** Representative normalized current traces before ($I_{Control}$) and under 10 μM DAMGO application (I_{DAMGO}) for $Ca_v2.2$ expressed in combination with β_{2a}, β_4, or β_{1b} subunit at +20, +10, and +10 mV, respectively (*left panel*). Traces were selected on the basis of the measured recovery G-protein inhibition time constant τ (between 45 and 55 ms). Corresponding traces allowing the measurement of RI values (in *red*) after a 500-ms depolarization (*right panel*). The *gray area* represents the extent of current inactivation during a 500-ms depolarization. **c** Scattered plot representation of RI values after a 500-ms depolarization as a function of the extent of inactivation. Values are shown for various $Ca_v2.2/\beta$ combinations (n=62) showing a time constant τ of recovery from G-protein inhibition of 50±5 ms independently of the test potential. Fitting these values by a linear curve provided a linear regression coefficient of −0.768, which is statistically significant at p <0.001 (Spearman rank order correlation test)

compared to control $Ca_v2.2/\beta_{1b}$ currents, which is responsible for the depolarizing shift of the *I*/*V* curve under DAMGO inhibition (Fig. 8e). Conversely, for $Ca_v2.2/\beta_{1b\ \Delta N}$, the maximal RI_{peak} value is observed at 10 mV (Fig. 8d), which is −5 mV hyperpolarized to the control $Ca_v2.2/\beta_{1b\ \Delta N}$ peak currents and, therefore, influences far less the *I*/*V* curve under DAMGO inhibition (Fig. 8f). Finally, it should be noted that with a slowing of inactivation kinetics, the resultant increase in RI_{peak} values (Fig. 8d, for potentials below 40 mV) produces an apparent reduction in DAMGO inhibition that is clearly evident when one compares the effect of DAMGO on *I*/*V* curves of $Ca_v2.2/\beta_{1b}$ and $Ca_v2.2/\beta_{1b\ \Delta N}$ (Fig. 8e,f).

In conclusion, these data indicate that slowing of channel inactivation kinetics increases the slowing of the time to peak by DAMGO, whereas it reduces both the peak current inhibition and the depolarizing shift of the voltage dependence of activation.

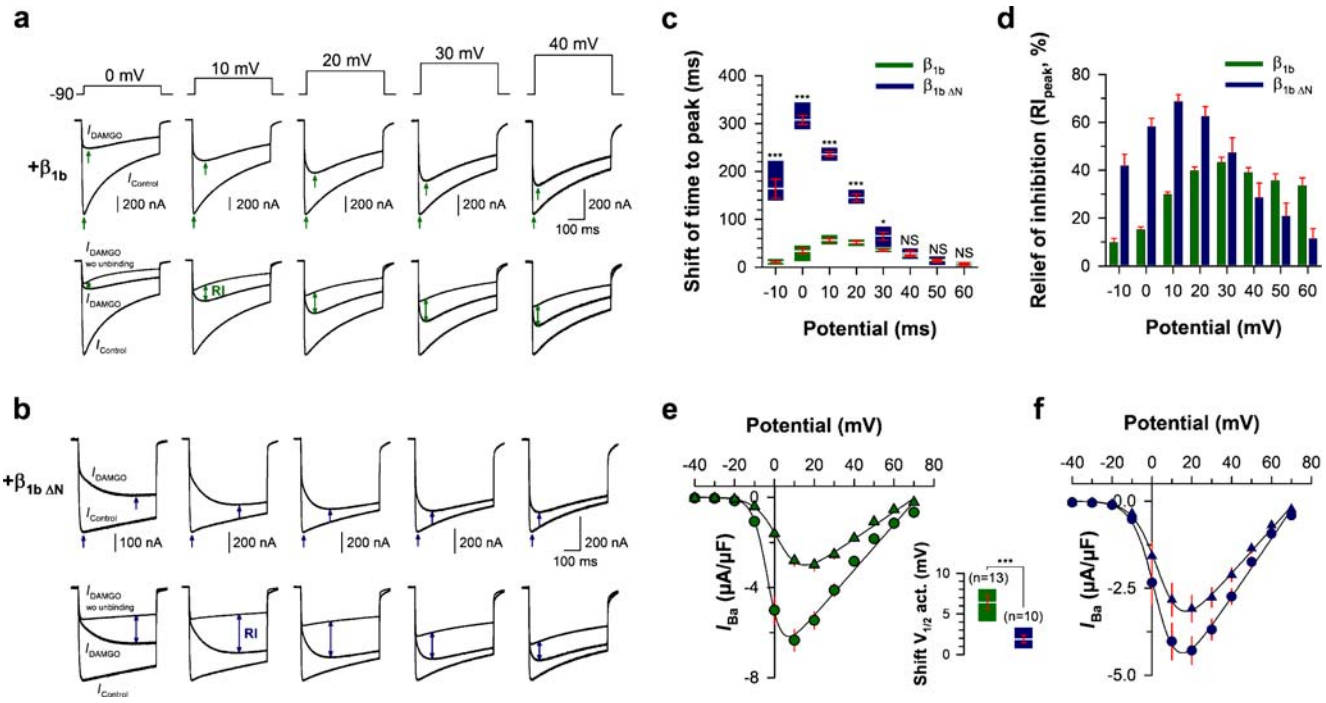

Fig. 8 Effect of channel inactivation on characteristic biophysical changes induced by G-protein activation. Representative current traces before ($I_{Control}$) and under 10 μM DAMGO application (I_{DAMGO}) and corresponding traces allowing the measurement of RI values are shown for $Ca_v2.2/\beta_{1b}$ (**a**) and $Ca_v2.2/\beta_{1b\,\Delta N}$ (**b**) at various membrane potentials illustrating DAMGO effects on channel activation kinetics and current recovery from G-protein inhibition in two conditions of channel inactivation. *Arrows* indicate the time to peak of the currents for control and DAMGO conditions (top panels). The time to peak of DAMGO-inhibited currents (I_{DAMGO}) was also indicated on RI traces (*arrows* in *lower panels*). *Double arrows* indicate the extent of current recovery from G-protein inhibition at these time points (RI_{peak}). **c** Box plot representation of the shift of the current time to peak induced by DAMGO application for $Ca_v2.2/\beta_{1b}$ channels (*green boxes*, $n=14$) and $Ca_v2.2/\beta_{1b\,\Delta N}$ channels (*blue boxes*, $n=10$) as a function of membrane potential. **d** Histogram representation of RI_{peak} values at the peak of DAMGO currents (I_{DAMGO}) for $Ca_v2.2/\beta_{1b}$ channels (*green bars*, $n=14$) and $Ca_v2.2/\beta_{1b\,\Delta N}$ channels (*blue bars*, $n=10$) as a function of membrane potential. Current–voltage relationship (I/V) were performed for $Ca_v2.2/\beta_{1b}$ channels (*green plots*, $n=13$; **e**) and $Ca_v2.2/\beta_{1b\,\Delta N}$ channels (*blue plots*, $n=10$; **f**) for control (*circle symbol*) and DAMGO-inhibited (*triangle symbols*) currents measured at their peak. Data were fitted with a modified Boltzmann equation as described in the "Materials and methods" section. The *insert* represents the shift of the half-maximum current activation potential ($V_{1/2}$) induced by DAMGO application for $Ca_v2.2/\beta_{1b}$ (*green box*, $n=13$) and $Ca_v2.2/\beta_{1b\,\Delta N}$ channels (*blue box*, $n=10$). Data are expressed as mean±SEM (in *red*) for *n* studied cells. Statistical *t* test: *NS* not statistically significant, *single asterisk* $p\leq0.05$, *two asterisks* $p\leq0.01$, *three asterisks* $p\leq0.001$

Discussion

Relevant parameters to study the influence of inactivation on N-type channel regulation by G-proteins

N-type channel regulation by G-proteins can be described accurately by three parameters: the G-protein inhibition level at the onset of depolarization (GI_{t_0}), the time constant of recovery from inhibition (τ), and the maximal extent of recovery from inhibition (RI). GI_{t_0} is indicative of the "ON" effect, whereas τ and RI are the quantitative parameters leading to all "OFF" effects of the G-protein regulation [49]. Since GI_{t_0} is a quantitative index of the extent of G-protein inhibition at the start of the depolarization, i.e., at a time point where no inactivation has yet occurred, inactivation cannot influence this parameter. On the other hand, G-protein dissociation is a time-dependent process at any given membrane potential and can thus be affected by channel inactivation since both processes occur within a similar timescale. In this study, we aimed at investigating this issue and came up with two novel conclusions. First, channel inactivation kinetics influences the speed of G-protein dissociation, and second, removal of G-proteins occurs within a time window that is closely controlled by inactivation. Hence, the speed of G-protein dissociation and the time window during which this process may occur control the extent of current recovery from G-protein inhibition at any given time. These conclusions were derived from the use of a recent biophysical method of analysis of N-type calcium channel regulation by G-proteins, which is independent of potential changes in channel inactivation behavior while G-proteins are bound onto the channels [48].

G-protein inhibition is completely reversible during depolarization provided that the channel has slow inactivation

There are two physiological ways to terminate direct G-protein regulation on N-type calcium channels: (1) the end of GPCR stimulation by recapture or degradation of the agonist (experimentally mimicked by washout of the bath medium), and (2) membrane depolarization by trains of action potentials (experimentally simulated by a prepulse application). Whereas the first one always leads to a complete recovery from G-protein inhibition, the second one produces a transient and variable recovery. Interestingly, a very slowly inactivating channel, such as the one produced by the combination of $Ca_v2.2$ and β_{2a} subunits, can lead to a complete recovery from G-protein inhibition after membrane depolarization, whereas a fast inactivating channel, such as the one produced by the co-expression of the β_{1b} subunit, leads only to a partial recovery. For slow inactivating channels, the time window for G-protein dissociation is large since channel inactivation does not interfere with the process. Conversely, for fast inactivating channels, the time window for G-proteins to unbind from the channel is considerably reduced since inactivation prevents the observation of a complete recovery from inhibition. For these channels, the extent of recovery from inhibition is controlled by both the speed of G-protein dissociation and the time window of opportunity. Hence, the speed of current recovery from G-protein inhibition is controlled by channel inactivation and by channel opening as previously shown [49], whereas the time window opportunity of this process is only controlled by channel inactivation. It is likely that both parameters (the time constant of recovery τ and the time window of opportunity) are under the control of additional molecular players or channel-modifying agents such as phosphorylation that may act on one or the other parameters in an independent manner, and could contribute to a fine control of the direct G-protein regulation.

There is an unexpected relationship between the channel inactivation kinetics and the kinetics of current recovery from G-protein inhibition

One surprising observation from this study is that fast inactivation accelerates the speed of current recovery from G-protein inhibition, whereas slower inactivation slows down G-protein dissociation from the channel. This was first demonstrated through the use of different β subunit isoforms (see also [12, 20]), and then confirmed with β subunit constructs known to modify channel inactivation kinetics. Besides this functional correlation, there might be a structural basis that underlies a mechanistic link between channel inactivation and G-protein dissociation. Indeed, Herlitze et al. [23] illustrated that an R to A mutation of the QXXER motif (one of the $G_{\beta\gamma}$-binding determinant within the I–II linker of $Ca_v2.x$ channels [15]) slows both the inactivation kinetics and the recovery from G-protein inhibition. The I–II loop of $Ca_v2.2$ appears as a particularly interesting structural determinant for supporting G-protein dissociation. First, it contains several $G_{\beta\gamma}$-binding determinants whose functional role remain unclear [11, 15, 23, 33, 53, 54]. Second, this loop is known to contribute to fast inactivation [21, 23, 46]) possibly through a hinged lid mechanism that would impede the ion pore [46]. Third, some of the residues of the QXXER motif have been found to contribute to inactivation in a voltage-sensitive manner [41]. A possible working hypothesis for the contribution of the I–II loop to G-protein regulation can be proposed: (1) the channel openings provide an initial destabilizing event favoring G-protein dissociation, and (2) the hinged lid movement of the I–II loop triggered by the inactivation process further accelerates G-protein dissociation through an additional decrease in affinity between $G_{\beta\gamma}$ and the channel.

However, there is an alternative possibility based on the expected relationship between channel opening probability and rate of G-protein dissociation [49]. At the potential at which we performed this study (10 mV), all channel combinations are at their maximal activation (data not shown) and should produce maximal opening probabilities. Nevertheless, we cannot rule out that the various β subunits and structural analogues introduce differences in the maximal opening probabilities of the channel thereby explaining differences in the rate of G protein dissociation, e.g., β_{2a} with a lower opening probability and, thus, slower recovery from inhibition. However, this would imply that anything that leads to a slowing of inactivation kinetics, through a modification of β subunit structure, produces a reduced opening probability. The likelihood of this hypothesis is probably low, but cannot be dismissed.

Inactivation differentially affects each characteristic biophysical channel modification induced during G-protein regulation

Since time-dependent G-protein dissociation is responsible for the characteristic biophysical modifications of the channel [49], inactivation, by altering the parameters of the recovery from inhibition, plays a crucial role in the phenotype of G-protein regulation. Two extreme case scenarios were observed. G-protein regulation of slowly inactivating channels, such as $Ca_v2.2/\beta_{1b\,\Delta N}$, induces an important slowing of the activation kinetics, but no or little depolarizing shift of the voltage dependence of activation and less peak current inhibition. Conversely, faster inacti-

vating channels, such as $Ca_v2.2/\beta_{1b}$, present reduced slowing of activation kinetics, but a greater peak current inhibition and a marked depolarizing shift of the voltage dependence of activation. These data point to the fact that characteristic biophysical changes of the channel under G-protein regulation should not be correlated with each other. Indeed, an important shift of the time to peak is not necessarily associated with an important depolarizing shift of the voltage dependence of activation or a greater peak current reduction. Thus, it seems important to be cautious on the absence of a particular phenotype of G-protein regulation that does not necessarily reflect the lack of direct G-protein inhibition.

Physiological implications of channel inactivation in G-protein regulation

N-type channels are rather heterogeneous by their inactivation properties because of differences in subunit composition [43] or in alternative splicing [5, 28]. Very little information is available on the targeting determinants that lead to N-type channel insertion at the synapse. However, a contribution of the β subunits and of specific C-terminal sequences of $Ca_v2.2$ is thought to be involved in the sorting of mature channels [24]. An epileptic lethargic phenotype in mouse is known to arise from the loss of expression of the β_4 subunit, which is accompanied by a β-subunit reshuffling in the N-type channels [9]. These animals present an altered excitatory synaptic transmission suggesting the occurrence of a modification in channel composition and/or regulation at the synapse [10]. Synaptic terminals that arise from single axons present a surprising heterogeneity in calcium channel composition and in processing capabilities [39]. One of the synaptic properties most influenced by calcium channel subtypes is presynaptic inhibition by G-proteins. Evidence has been provided that the extent of N-type current facilitation (hence, current recovery from G-protein inhibition) is dependent on both the duration [8] and the frequency of action potentials (AP) [36, 50]. Low frequencies of AP produce no or little recovery, whereas high-frequency action potentials more dramatically enhance recovery. Hence, slowly inactivating channels should allow much better recovery from G-protein inhibition than fastly inactivating channels, thereby further enhancing the processing abilities of synaptic terminals. In that sense, a model of synaptic integration has been proposed by the group of Dr. Zamponi [6] that would be implicated in short-term synaptic facilitation or depression. It should be noted that the inactivation of calcium channels does not only rely on a voltage-dependent component and that other modulatory signals (calcium-dependent inactivation, phosphorylation) need to find a place in the integration pathway.

Conclusion

These data permit a better understanding of the role of inactivation in N-type calcium channel regulation by G-proteins and will call attention to the contribution of the different β subunits in physiological responses at the synapse.

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