Calcium channel β subunits differentially modulate recovery of the channel from inactivation

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Abstract We examined the effects of calcium channel β subunits upon the recovery from inactivation of α_1 subunits expressed in *Xenopus* oocytes. Recovery of the current carried by the L-type α_1 subunit (*cy*Ca_v1) from the jellyfish *Cyanea capillata* was accelerated by coexpression of any β subunit, but the degree of potentiation differed according to which β isoform was coexpressed. The *Cyanea* β subunit was most effective, followed by the mammalian b_3, b_4, and β_{2a} subtypes. Recovery of the human $Ca_v2.3$ subunit was also modulated by β subunits, but was slowed instead. β_3 was the most potent subunit tested, followed by β_4, then β_{2a}, which had virtually no effect. These results demonstrate that different β subunit isoforms can affect recovery of the channel to varying degrees, and provide an additional mechanism by which β subunits can differentially regulate α_1 subunits.

Key words: Voltage-gated calcium channel; α-1 subunit; β subunit; Inactivation; L-type current; R-type current

1. Introduction

In excitable cells, voltage-gated calcium channels form the link between membrane depolarization and the calcium influx that initiates intracellular processes. The kinetics of channel activation and inactivation are critical in determining the cellular response, and much current heterogeneity stems from the genetic diversity of channel subunits. At least 10 distinct mammalian forms of the α_1 subunit, the pore-forming subunit of the calcium channel, have been identified and categorized as either high voltage-activated (HVA) or low voltage-activated (LVA); α_1 subunits within these families differ considerably in the properties of the currents they gate. Further current variation results from modulation of the α_1 subunit by phosphorylation, G proteins, or auxiliary subunits. In HVA channels, the α_1 subunit is associated with auxiliary β, $\alpha_2\delta$, and γ subunits, of which β subunits have the most profound regulatory effects. β Subunits modulate several parameters of α_1 activity, including level of channel expression, threshold of activation, rate of inactivation, and steady-state inactivation [1–3]. Four mammalian subtypes of the β subunit (β_1–β_4), as well as additional splice variants, have been cloned (for a review see [3]), and regulation of current properties may differ according to the type of β isoform present. For example, the β_{2a} subunit differs from the β_{1b}, β_3, and β_4 isoforms in its effects upon α_1 inactivation [4–6]. This variation is significant in that certain α_1 subunits have been shown to associate with multiple β subunits in vivo [7–9].

We have previously investigated the interactions between an α_1 and a β subunit cloned from the jellyfish *Cyanea capillata* [10]. The L-type calcium channel α_1 subunit (*cy*Ca_v1; previously called CyCaα_1) is modulated by the calcium channel β subunit (*cy*β) in a manner that is consistent with interactions between mammalian calcium channel subunits. When coexpressed with *cy*Ca_v1 in *Xenopus* oocytes, *cy*β increases whole-cell current amplitude, shifts the current–voltage relationship to more hyperpolarized potentials, and alters the kinetics of current inactivation, actions that can be duplicated by coexpression of the mammalian β_{2a} subunit with *cy*Ca_v1. An additional component of the *Cyanea* α_1-β interaction is the ability of *cy*β to accelerate the recovery of the channel from inactivation, a form of modulation that has not been reported for mammalian channels. The present experiments were designed to examine the effects of different β subunits on recovery of both *cy*Ca_v1 and a mammalian α_1 subunit, the human R-type calcium channel $Ca_v2.3$ isoform (h$Ca_v2.3$; previously called α_{1E}).

2. Materials and methods

The cloning and functional expression of the *cy*Ca_v1 [11] and *cy*β [10] subunits have previously been described. All clones were transcribed using the T7 version of the mMessage mMachine kit (Ambion), and the amount and purity of transcribed RNA were determined by denaturation with glyoxal and gel electrophoresis. Oocytes were injected with a constant amount of α_1 RNA, combined either with β subunit RNA or with an equivalent volume of water. RNA was combined in a molar ratio of 1:3 α_1:β RNA in order to ensure that the β subunit was present at saturating levels [6]. Oocytes were incubated for 3–7 days; because levels of channel expression change over time, recordings on any given day were made from oocytes in all treatment groups. Two-electrode voltage clamp recordings were conducted in a bath solution containing 40 mM $Sr(OH)_2$, 40 mM *N*-methylglucamine, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with methanesulfonic acid [12]. Sr^{2+} was used as the charge carrier due to its high permeance of the *cy*Ca_v1 subunit [11]. All oocytes were injected with 40 nl 100 mM BAPTA at least 1 h prior

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Abbreviations: *cy*Ca_v1, *Cyanea capillata* L-type calcium channel α_1 subunit; h$Ca_v2.3$, human R-type calcium channel (previously called α_{1E}); *cy*β, *Cyanea capillata* calcium channel β subunit; HVA, high voltage-activated; LVA, low voltage-activated

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to recording to block the endogenous calcium-activated Cl^- current [13]. In experiments involving *cy*Ca_v1, complete inactivation of the current was produced by a 1.5 s voltage prepulse from −110 mV to +10 mV, and recovery was determined by a 50 ms test pulse to +10 mV after a hyperpolarizing step of varying duration or voltage. The human $Ca_v2.3$ subunit was studied in the same manner, except that the length of the prepulse was 9.6 s (16.2 s when $Ca_v2.3$ was coexpressed with β_{2a}, due to the slower rate of inactivation). In most instances, the extended prepulse inactivated the channel completely (< 2% of peak current remaining by the end of the prepulse), but when $hCa_v2.3$ was coexpressed with β_{2a}, approximately 7% of the current remained at the end of the prepulse. For this reason, recovery was measured as (peak current elicited by test pulse−residual current at end of prepulse)/(peak current elicited by prepulse−residual current at end of prepulse). Successive episodes during a series of tests were separated by 30 s (*cy*Ca_v1) or 90 s ($hCa_v2.3$); the amplitude of the current elicited by the prepulse after this delay demonstrated that the channel had completely recovered from inactivation.

3. Results

Because the purpose of the present experiments was to examine the effects of calcium channel β subunits upon the recovery from inactivation of α_1 subunits, we studied two α_1 subunits, *cy*Ca_v1 [11], and the human $Ca_v2.3$ subunit [14], that produce robust, rapidly inactivating currents in oocytes in the absence of coexpressed β subunits. A consistent effect of β subunit modulation is a leftward shift in the current–voltage relationship of the α_1 subunit. Such a shift was seen for the *cy*Ca_v1 subunit in every instance in which a β subunit was coexpressed (Fig. 1A), demonstrating that each mammalian β subunit studied can regulate *cy*Ca_v1. A similar, though smaller, shift in the $hCa_v2.3$ curve was produced by coexpression of either *cy*β or the mammalian β subunits (Fig. 1B). The magnitude of the effect is comparable to that previously reported for the $hCa_v2.3$ subunit [14].

Recovery from inactivation was measured by administering an extended prepulse to fully inactivate the current, then repolarizing the cell and measuring the current elicited by a test pulse after a certain delay. In this manner, recovery could be distinguished from phenomena such as facilitation or inactivation from intermediate closed states. The initial experiments investigated the time course of recovery after repolarization to −110 mV. As other studies have shown [15,16], recovery of the calcium current is best fit by a biexponential function. The parameters of curves fit to individual experiments varied significantly, so curves were fit to mean values to provide an estimate of the fast and slow time constants of recovery. The faster component accounted for 40% of the recovery of the current carried by *cy*Ca_v1 (Fig. 2A and Table 1). Coexpression of any β subunit accelerated the recovery of *cy*Ca_v1, though the degree of potentiation differed for each subunit. *cy*β had the greatest effect, followed by β_4 and β_3, each of which decreased the time constants of both components of recovery. The *cy*β subunit also increased the proportion of recovery accounted for by the fast component, as did the

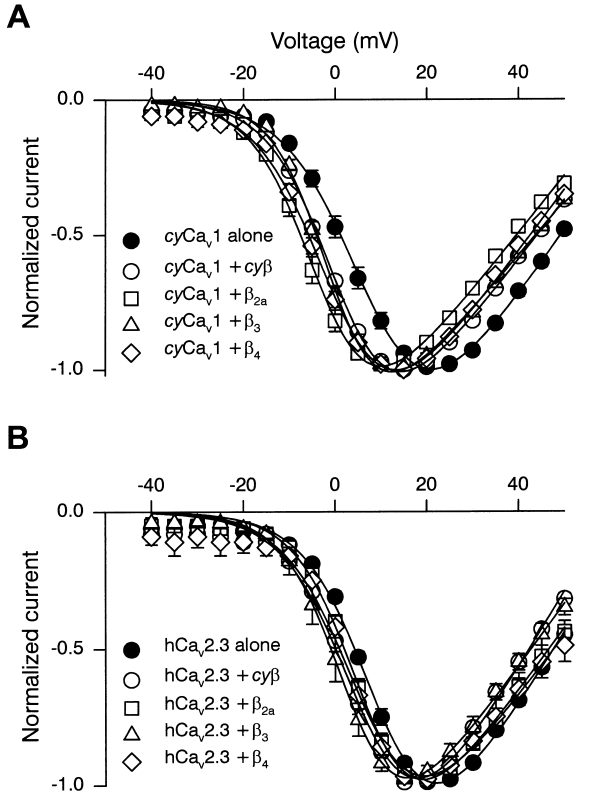

Fig. 1. A: Current–voltage relationships for *cy*Ca_v1 expressed alone or in the presence of the *cy*β, β_{2a}, β_3, or β_4 subunits. Currents were measured in 40 mM Sr^{2+}. B: Current–voltage relationships for the human $Ca_v2.3$ subunit in the absence and presence of β subunits. $n = 7$–10 cells.

Table 1
Parameters of curves fitted to data in Figs. 2 and 3

	Recovery after −110 mV interpulse			Recovery after −60 mV interpulse			Voltage dependence of recovery			
	τ_1 (ms)	τ_2 (ms)	p_1	τ_1 (ms)	τ_2 (ms)	p_1	max rec (%)	k	$V_{1/2}$ (mV)	min rec (%)
cyCa_v1	312.4 ± 30.5	2177 ± 189	0.40 ± 0.04	50.2 ± 48.6	3850 ± 257	0.06 ± 0.02	0.42 ± 0.02	18.1 ± 1.4	−75.4 ± 1.9	0.07 ± 0.0
cyCa_v1+ *cy*β	135.2 ± 13.1	1188 ± 136	0.58 ± 0.04	136.9 ± 11.9	2277 ± 221	0.49 ± 0.02	0.68 ± 0.03	12.8 ± 1.6	−35.2 ± 1.7	−0.01 ± 0.03
cyCa_v1+ β_{2a}	382.6 ± 78.4	2558 ± 1372	0.63 ± 0.14	98.8 ± 20.3	3444 ± 136	0.12 ± 0.01	0.66 ± 0.10	25.4 ± 4.6	−77.9 ± 6.5	0.03 ± 0.02
cyCa_v1+β_3	162.4 ± 20.4	1398 ± 120	0.43 ± 0.04	142.4 ± 19.4	2822 ± 200	0.28 ± 0.02	0.64 ± 0.06	20.9 ± 3.1	−38.7 ± 2.9	−0.07 ± 0.04
cyCa_v1+β_4	141.0 ± 21.1	1304 ± 150	0.47 ± 0.05	123.4 ± 4.2	2636 ± 57	0.33 ± 0.01	0.65 ± 0.05	19.6 ± 3.0	−40.4 ± 2.8	−0.03 ± 0.04
$hCa_v2.3$	62.9 ± 7.2	877 ± 91	0.63 ± 0.04	167.2 ± 20.4	2270 ± 85	0.21 ± 0.02	0.78 ± 0.06	17.9 ± 2.3	−66.0 ± 2.5	0.05 ± 0.02
$hCa_v2.3$+*cy*β	110.2 ± 6.5	1190 ± 103	0.64 ± 0.02	256.7 ± 84.3	6193 ± 2031	0.27 ± 0.06	0.80 ± 0.04	17.1 ± 1.2	−77.5 ± 1.6	0.04 ± 0.01
$hCa_v2.3$+β_{2a}	86.7 ± 8.5	1102 ± 140	0.64 ± 0.04	161.0 ± 16.0	2707 ± 110	0.24 ± 0.01	ND	ND	ND	ND
$hCa_v2.3$+β_3	115.0 ± 6.1	1683 ± 135	0.61 ± 0.02	311.6 ± 24.4	16290 ± 2510	0.25 ± 0.01	0.77 ± 0.03	17.0 ± 0.8	−82.7 ± 1.3	0.00 ± 0.00
$hCa_v2.3$+β_4	90.3 ± 12.5	1133 ± 211	0.64 ± 0.05	304.6 ± 53.0	9458 ± 3594	0.34 ± 0.04	0.80 ± 0.04	16.6 ± 1.4	−71.4 ± 1.6	0.02 ± 0.01

Recovery curves were fitted using a biexponential function: percent recovery = $1-p_1\exp(-t/\tau_1)-(1-p_1)\exp(-t/\tau_2)$, where p_1 and $(1-p_1)$ represent the proportion of recovery accounted for by the time constants τ_1 and τ_2, respectively. The voltage dependence of recovery curves were fit by the Boltzmann function: percent recovery = min rec+max rec/$[1+\exp(V-V_{1/2})/k]$, where min rec and max rec represent the minimum and maximum recovery values, $V_{1/2}$ represents the voltage corresponding to half-maximal recovery, and k is a slope factor. Parameters were generated from mean values by curve-fitting algorithms of SigmaPlot, which also provided the asymptotic standard error of each estimate. ND = not determined.

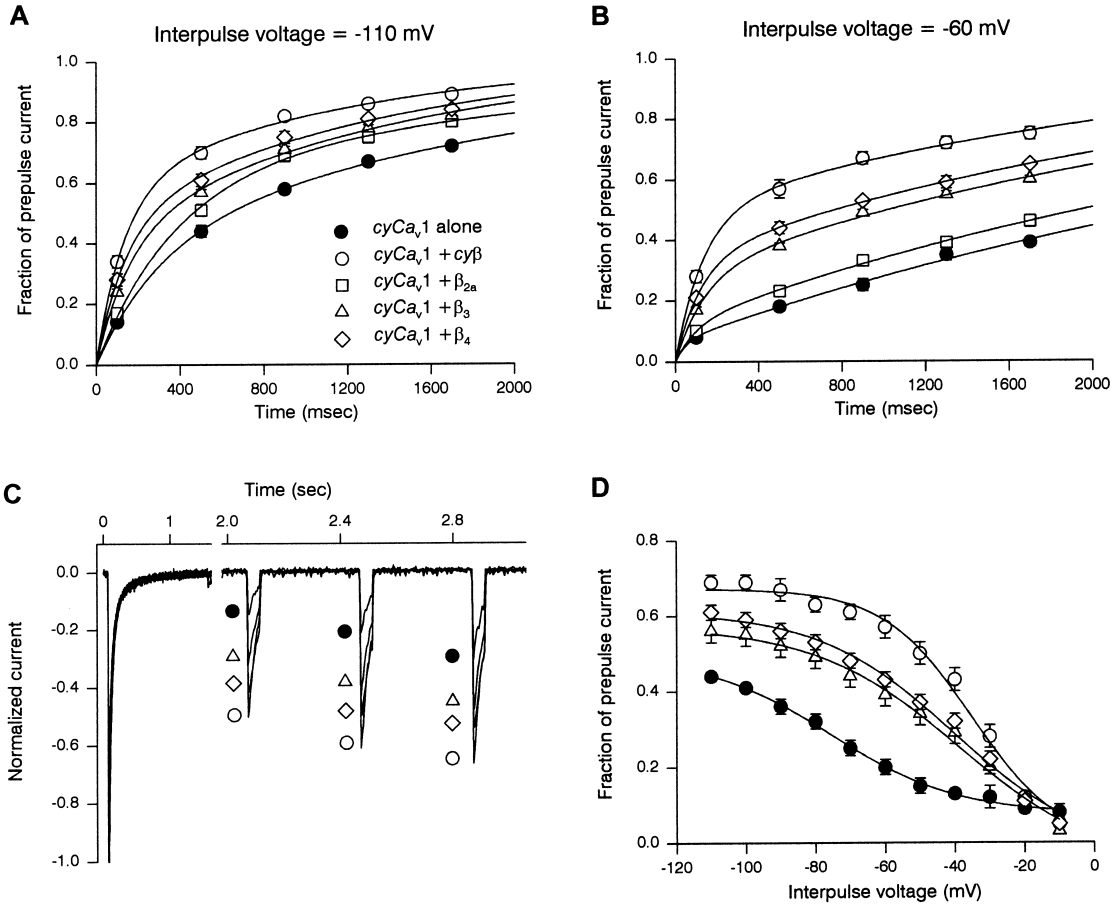

Fig. 2. Recovery of the *cy*Ca$_v$1 subunit. A: Recovery of *cy*Ca$_v$1 expressed alone or coexpressed with the *cy*β, β$_{2a}$, β$_3$, or β$_4$ subunits. The peak current elicited by the test pulse (+10 mV for 50 ms) after a step of variable duration to −110 mV is shown as the fraction of the peak current elicited by the prepulse (+10 mV for 1500 ms). B: Results of the protocol described in (A) when the interpulse voltage was −60 mV. C: Examples of the recovery of *cy*Ca$_v$1 expressed alone (closed circles) or in combination with *cy*β (open circles), β$_3$ (open triangles), or β$_4$ (open diamonds). Oocytes were stepped from a holding voltage of −110 mV to +10 mV for 1500 ms, then hyperpolarized to −60 mV for a variable length of time before a 50 ms test pulse to +10 mV. For each subunit combination, the currents produced by test pulses after interpulse intervals of 500, 900, and 1300 ms are combined into a single sweep to more clearly illustrate the pattern of recovery. Note the difference in time scale between the two halves of the figure. The currents are normalized with respect to the peak current generated by the prepulse. D: Voltage dependence of recovery from inactivation. Following a 1500 ms prepulse to +10 mV, the voltage was stepped to varying voltages for 500 ms before the 50 ms test pulse to +10 mV. Responses are expressed as the fraction of the peak current during the prepulse that was elicited by the test pulse. Symbols are as described in (A). $n = 7$–10 cells for all figures.

β$_{2a}$ subunit, which had the weakest effect of the β subunits tested.

Because the interpulse voltage used in this experiment was strongly hyperpolarizing (−110 mV), we examined the possibility that β subunits may not significantly alter the rate of channel recovery when the interpulse voltage is closer to physiological potentials. At an interpulse voltage of −60 mV, the effects of β subunits on recovery were more marked, however, and differences among the various β subunits were more pronounced (Fig. 2B). Examples of recovery of *cy*Ca$_v$1 after an interpulse potential of −60 mV are shown in Fig. 2C. Recovery of *cy*Ca$_v$1 was slower after repolarization to −60 mV; although the fast time constant was shorter than that at −110 mV, its contribution to recovery was much smaller (Table 1). The *cy*Ca$_v$1 subunit expressed alone reached approximately 40% of its peak current after 1600 ms of repolarization, a delay modified only slightly by the β$_{2a}$ subunit, but comparable recovery was seen in the presence of β$_3$ or β$_4$ after 500 ms, and in the presence of *cy*β after only 200 ms. The effect of the *cy*β, β$_3$, and β$_4$ subunits was manifested primarily as a greater proportion of recovery accounted for by the fast component.

The difference between the experiments at the two interpulse voltages demonstrated that the effects of β subunits upon recovery from inactivation are voltage-dependent. We investigated the voltage dependence further by measuring recovery after an interpulse of constant length (500 ms) and varying voltage. The results (Fig. 2D) indicate that the effects of β subunits upon recovery of the *cy*Ca$_v$1 subunit from inactivation are most pronounced at voltages between −40 and −80 mV. The *cy*β, β$_3$, and β$_4$ subunits each shifted the midpoint of the voltage relationship by 25–30 mV as well as raising the maximal degree of recovery (Table 1).

To determine whether the recovery of mammalian α$_1$ subunits can be similarly modulated, we examined the effects of β subunit coexpression on the human Ca$_v$2.3 subunit. hCa$_v$2.3 also exhibits a biexponential pattern of recovery, but the fraction accounted for by the fast component was greater at in-

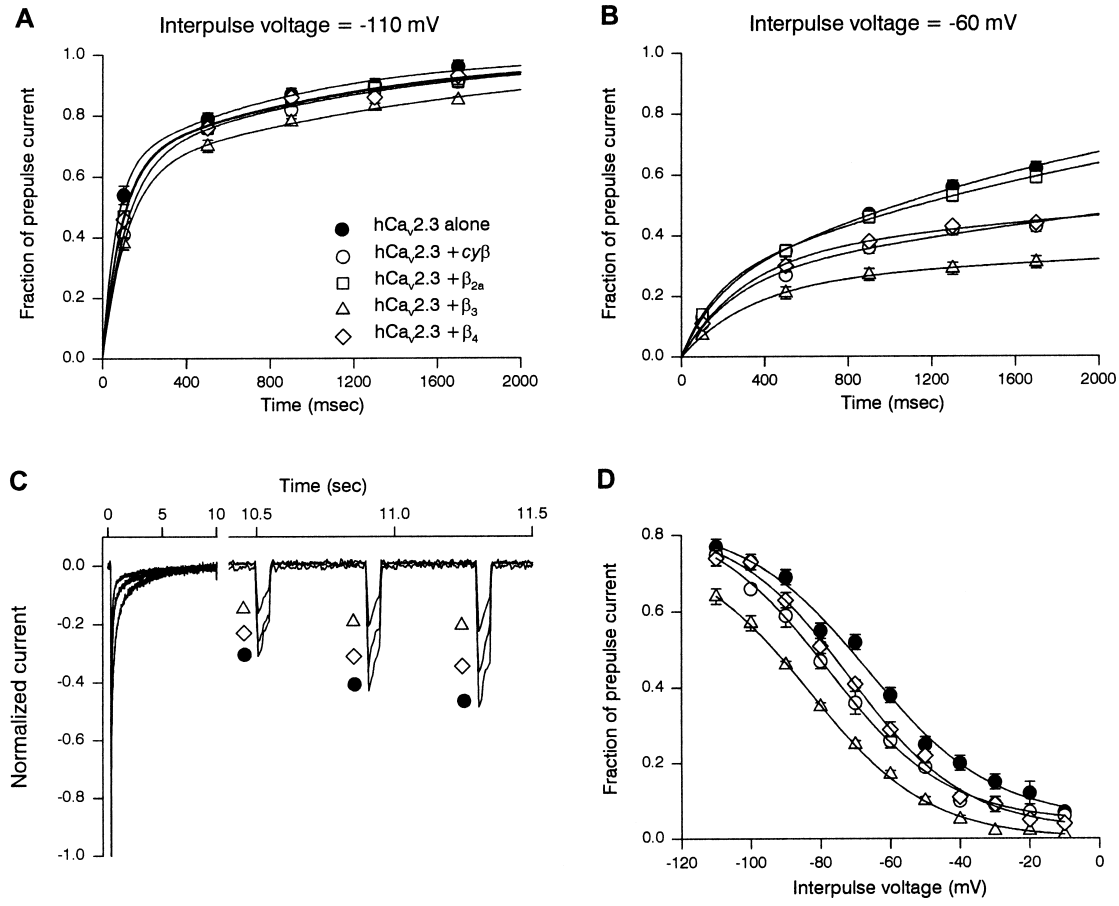

Fig. 3. Recovery of currents carried by the human $Ca_v2.3$ subunit. Protocols were as described in Fig. 2, with the exception that a 9.5 s prepulse was used to inactivate the current (16.2 s for the $Ca_v2.3+\beta_{2a}$ combination). A: Recovery of $Ca_v2.3$ expressed alone or coexpressed with the *cy*β, β_{2a}, β_3, or β_4 subunits after an interpulse of −110 mV. B: Recovery of the subunit combinations shown in (A) after an interpulse of −60 mV. C: Examples of recovery of the $hCa_v2.3$ subunit expressed alone (closed circles) or coexpressed with β_3 (open triangles) or β_4 (open diamonds). The currents shown were elicited by test pulses after a 9.5 s prepulse to +10 mV followed by a 500, 900, or 1300 ms interpulse step to −60 mV. Note the difference in time scale between the two halves of the figure. D: Voltage dependence of recovery. Symbols are as described in (A); the $hCa_v2.3+\beta_{2a}$ combination was not tested in this experiment. $n=4$–10 cells.

terpulse voltages of either −110 or −60 mV. In contrast to *cy*Ca_v1, the recovery of $hCa_v2.3$ was slowed by coexpression of any β subunit. The effects of the β subunits were minimal at an interpulse voltage of −110 mV (Fig. 3A), but quite evident at −60 mV (Fig. 3B,C), at which the difference among the treatments became more marked after repolarizations of longer duration. At −60 mV, β_3 was most effective at slowing recovery, followed by β_4 and β_{2a}. The *cy*β subunit also slowed recovery in a manner similar to that of β_4. The effects of the β subunits were manifested as an increase in the time constants for each component, although the proportion of recovery accounted for by the fast component was somewhat greater in the presence of a β subunit. The β subunit modulation of recovery was voltage-dependent (Fig. 3D), although to a smaller degree than that seen for *cy*Ca_v1, and was reflected as a shift in the midpoint of the recovery curve.

4. Discussion

Previous studies have established several modulatory actions of calcium channel β subunits upon α_1 subunits (for a review see [3]). In addition to its augmentation of α_1 subunit expression in the plasma membrane [17–19] and effects upon regulation by G proteins or phosphorylation [20], the β subunit shifts the current–voltage relationship and steady-state inactivation of the α_1 subunit, alters the rates of channel activation and inactivation, and affects prepulse facilitation and closed-state inactivation [1,2,21–23]. Here we demonstrate that not only can β subunits also modulate the recovery of the α_1 subunit from inactivation, but that the direction and degree of modulation depend upon the subtypes of both the α_1 and β subunit. Other actions of β subunits vary according to which β subtype is associated with the α_1 subunit. The β_{1b}, β_3, and β_4 subunits accelerate the rate of inactivation of $hCa_v2.3$ and shift steady-state inactivation to more hyperpolarized potentials, for example, while the β_{2a} subunit exerts the opposite effects [4,6,22]. The β_{2a} subunit is also unique among mammalian β subtypes in its inability to enhance prepulse facilitation of $Ca_v1.2$ [24] or preferential closed-state inactivation of $Ca_v2.1$, $Ca_v2.2$, and $Ca_v2.3$ [23], and in our experiments had the weakest effect upon the recovery of either α_1 subunit from inactivation. Differential modulation by β isoforms is significant in light of the findings that native N-type ($Ca_v2.2$; [8]), P/Q type ($Ca_v2.1$; [7]), and neuronal L-type channels ($Ca_v1.2$, $Ca_v1.3$; [9]) each contain a clearly identified α_1 subtype, but may include any of three or four of the β

isoforms. The association of different β isoforms with a given α_1 subunit does not appear to be random, but varies according to tissue distribution [9] or developmental stage [25].

A surprising finding of these studies was that the direction of modulation differs between the two α_1 subunits. Coexpression of β subunits accelerated recovery of the *cy*Ca_v1 subunit, but slowed recovery of the h$Ca_v2.3$ isoform. *cy*Ca_v1, a homologue of mammalian L-type α_1 subunits, exhibits significant variation in the cytoplasmic loop between domains I and II that contains the primary site for β subunit binding, but is clearly modulated by all three mammalian β subunits studied. Thus, the recovery of mammalian L-type α_1 subunits may be accelerated by certain β subunit isoforms in a similar manner. The degree of modulation exerted by each β subtype also differed between the two α_1 subunits. *cy*Ca_v1 recovered most quickly in the presence of the *cy*β subunit, followed by β_3 and β_4, which produced comparable enhancement, and the effect of β_{2a} was seen only at strongly polarized interpulse potentials. In contrast, the β_3 subtype had a more marked effect upon the h$Ca_v2.3$ subunit than β_4, which was similar to *cy*β, and β_{2a} had essentially no effect on recovery. The order of potency of the three mammalian β isoforms on the recovery of h$Ca_v2.3$ is similar to that exerted on preferential closed-state inactivation of the $Ca_v2.2$ subunit [23]. Because closed-state inactivation reflects the ease of transition from an intermediate closed state to an inactivated state, whereas recovery from inactivation represents the transition from an inactivated state to a closed state from which the channel can again be activated, β subunits may affect the modulation of the two responses via a similar mechanism.

The interaction between α_1 and β subunits is governed primarily by highly conserved domains in each subunit [26,27], but the present results suggest that modulation of recovery by the β subunit is mediated by a separate interaction site. The three mammalian β subunits studied are nearly identical in peptide sequence within the primary interaction domain, yet their effects upon recovery of the channel vary. The differential effects of β subunits upon inactivation have been linked to two distinct and variable regions external to the primary β interaction domain [4,6], and subtype-specific interaction sites have been found for certain β subunits [28].

Since our experiments were designed to separate recovery from inactivation from other phenomena, such as closed-state inactivation or prepulse facilitation, that can affect the measurement of recovery, we employed extended depolarizing and repolarizing pulses that may not mimic physiological conditions. Nevertheless, the effects of β subunits were evident after interpulses of moderate duration and potential, and are likely to be physiologically relevant. Patil et al. [23], in their description of preferential closed-state inactivation of neuronal calcium channels, argue that calcium channel inactivation may contribute to the synaptic depression seen during a series of action potentials. Differential rates of channel recovery would also affect the availability of channels, leading to altered entry of calcium and effects upon calcium-dependent processes such as docking of synaptic vesicles. The functional importance of recovery from inactivation has been demonstrated by mutations in the human $Ca_v2.1$ subunit that are associated with familial hemiplegic migraine. Each of four identified mutations has its most pronounced effects upon the recovery of the channel [15,29]. Alternatively spliced forms of the $Ca_v1.2$ subunit also differ in their rate of recovery [30] and in their sensitivity to the effects of isradipine upon recovery [31]. In addition, the calcium channel γ subunit has recently been shown to slow recovery of the $Ca_v1.2$ subunit [32]. Our findings establish that recovery of mammalian calcium channels can also be modulated by β subunits. The differential effects exerted by β subunit isoforms may reflect an additional mechanism by which calcium current heterogeneity is generated in vivo.

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