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The intracellular segment of the sodium channel β1 subunit is required for its efficient association with the channel α subunit

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Abstract

Sodium channels consist of a pore-forming α subunit and auxiliary β1 and β2 subunits. The subunit β1 alters the kinetics and voltage-dependence of sodium channels expressed in *Xenopus* oocytes or mammalian cells. Functional modulation in oocytes depends on specific regions in the N-terminal extracellular domain of β1, but does not require the intracellular C-terminal domain. Functional modulation is qualitatively different in mammalian cells, and thus could involve different molecular mechanisms. As a first step toward testing this hypothesis, we examined modulation of brain $Na_V1.2a$ sodium channel α subunits expressed in Chinese hamster lung cells by a mutant β1 construct with 34 amino acids deleted from the C-terminus. This deletion mutation did not modulate sodium channel function in this cell system. Co-immunoprecipitation data suggest that this loss of functional modulation was caused by inefficient association of the mutant β1 with α, despite high levels of expression of the mutant protein. In *Xenopus* oocytes, injection of approximately 10 000 times more mutant β1 RNA was required to achieve the level of functional modulation observed with injection of full-length β1. Together, these findings suggest that the C-terminal cytoplasmic domain of β1 is an important determinant of β1 binding to the sodium channel α subunit in both mammalian cells and *Xenopus* oocytes.

Keywords: β subunits, functional modulation, mammalian cells, mutagenesis, sodium channel, Xenopus oocyte.
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Voltage-gated sodium channels in mammalian excitable cells consist of a pore-forming α subunit (260 kDa) and two auxiliary subunits, a non-covalently associated β1 subunit (36 kDa) and a disulfide-linked β2 subunit (33 kDa) (Catterall 2000). Expression of α alone in heterologous cell systems is sufficient for formation of a functional sodium channel, confirming its central role in voltage-dependent channel gating and ion permeation (Noda *et al*. 1986). In contrast, the auxiliary β subunits modulate channel expression levels and functional properties (Isom *et al*. 1992, 1995a) and mediate interactions between sodium channels and other extracellular and intracellular proteins (Srinivasan *et al*. 1998; Xiao *et al*. 1999; Malhotra *et al*. 2000; for review see Isom, 2001). The β1 subunit strongly affects the time course and voltage-dependence of cloned sodium channels expressed in *Xenopus* oocytes. Microinjection into oocytes of RNA encoding brain or skeletal muscle α subunits results in expression of functional sodium channels that adopt an abnormally slow type of gating behavior, characterized by a slow inactivation rate and slow recovery from inactivation (Krafte *et al*. 1990; Moorman *et al*. 1990; Zhou *et al*. 1991). Coexpression of β1 shifts most channels from this slow gating mode to a fast type of gating behavior that more closely resembles the behavior of sodium channels in neurons and muscle cells (Isom *et al*. 1992; Patton *et al*. 1994; Chang *et al*. 1996). This shift from slow to fast gating results in ≥ fivefold faster macroscopic current time course, a negative shift in the voltage-dependence of activation and steady-state inactivation and a steepening of the inactivation curve.

The β1 subunit consists of a large extracellular N-terminal domain with an Ig loop, a single transmembrane segment and a small cytoplasmic C-terminal domain (Isom and Catterall 1996). Site-directed mutagenesis studies have demonstrated that β1 subunits with deletions or point

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Abbreviations used: CHL, Chinese hamster lung; STX, saxitoxin; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; $Na_V1.2a$, type-IIA sodium channel α subunit; $β1_{STOP}$, sodium channel β1 construct with deletion of 34 amino acids at C-terminus.

mutations in specific regions of the extracellular domain do not modulate the functional properties of sodium channels expressed in oocytes (Chen and Cannon 1995; Makita *et al*. 1996; McCormick *et al*. 1998, 1999). In contrast, mutant β1 subunits lacking the entire intracellular C-terminal domain retain the capacity to modulate channel function in oocytes. Together, these data suggest that the specific determinants in the extracellular domain of β1 are necessary and sufficient for regulation of channel function, whereas the intracellular C-terminal domain is not involved in this process. However, because these results were obtained in frog oocytes, some caution is in order in extrapolating them to mammalian excitable cells. There is no evidence that the β1-mediated shifts in modal gating seen in frog oocytes also occur in neurons or muscle cells, and thus it is not clear whether the molecular mechanisms of functional modulation are the same in oocytes as in mammalian cells. Indeed, when cloned α subunits are transfected into various cultured mammalian cell lines, the expressed sodium channels exhibit fast kinetic properties, even in the absence of β1 subunits (Ukomadu *et al*. 1992; West *et al*. 1992), perhaps reflecting different protein processing in mammalian cells versus oocytes (Buller and White 1990) or endogenous expression of β subunits in mammalian cell lines (Moran *et al*. 2000). Coexpression of cloned β1 subunits in mammalian cell systems does not appreciably alter the rate of channel inactivation, but causes negative shifts in the voltage-dependence of activation and inactivation (Isom *et al*. 1995b; Kazen-Gillespie 2000). Whether the molecular mechanisms that are responsible for this type of functional modulation are the same as those responsible for β1-dependent regulation of gating modes in oocytes has not previously been explored. To begin to address this question, we examined whether the intracellular domain of β1, which was previously shown not to be required for functional modulation in oocytes, is involved in modulation of brain type-IIA sodium channel α subunits ($Na_V1.2a$; see Goldin *et al*. 2001 for nomenclature of sodium channel α subunits) stably expressed in a Chinese hamster lung (CHL) cell line, previously shown not to express endogenous β1 subunits (Isom *et al*. 1995b). To our surprise, this deletion mutation resulted in loss of modulation of sodium channel function in CHL cells. Subsequent biochemical analysis indicated that this loss of functional modulation was caused by inefficient association of the truncated β1 subunit with the channel α subunit, despite high levels of expression of the mutant β1 protein. These results prompted us to re-examine functional modulation in oocytes. Results from experiments in which varying concentrations of wild-type or mutant β1 RNA were injected into oocytes along with a fixed concentration of sodium channel α subunit RNA suggest that the mutant β1 subunit was approximately 10 000 times less effective than the wild-type β1 at modulating sodium channel function. These results suggest that the C-terminal cytoplasmic domain of β1 is an important determinant of co-assembly of sodium channel α and β1 both in *Xenopus* oocytes and mammalian cells.

Materials and methods

Mutagenesis and expression of wild-type and mutant β1 subunits in SNaIIA cells

Deletion of the intracellular domain of the rat brain β1 subunit has been described previously (McCormick *et al*. 1998). Briefly, using standard PCR mutagenesis, a termination signal (TAA) was substituted for the isoleucine at position 166 in the wild-type sequence. This mutation resulted in deletion of 34 amino acids at the C-terminus of β1. We refer to this mutant construct as $\beta1_{STOP}$. For expression in mammalian cells, β1 and $\beta1_{STOP}$ cDNAs were subcloned into the eukaryotic expression vectors pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pBK-CMV (Stragagene, LaJolla, CA, USA), respectively. Both vectors encode G418 resistance. The wild-type and mutant β subunit constructs were transfected into cell line SNaIIA, a Chinese hamster lung-derived cell line stably expressing the rat brain $Na_V1.2a$ α subunit of the voltage-gated sodium channel (West *et al*. 1992, Isomet *et al*. 1995b). Transfection and cell culture were as previously described, using pSV2*Hyg as a co-transfected selectable marker (Isom *et al*. 1995b; Kazen-Gillespie 2000), to allow drug selection in the G418-resistant SNAIIA cell line. Following drug selection with hygromycin, surviving clones were analyzed by northern and western blots for β1 or $\beta1_{STOP}$ expression.

Electrophysiological recording in *Xenopus* oocytes and CHL cells

$Na_V1.2a$ (in vector pGEM2; Auld *et al*. 1988), β1 (in vector pcDNA3.1Zeo) and $\beta1_{STOP}$ (in vector pBKCMV) RNA transcripts were obtained using mMessage Machine *in vitro* transcription kits (Ambion, Inc., Austin, TX, USA). *Xenopus laevis* oocytes were harvested from anesthetized female frogs, defolliculated with collagenase and maintained as described (Li *et al*. 1999). Oocytes were injected with a 50-nL volume containing approximately 0.3 ng of $Na_V1.2a$ transcript, either alone, with 0.003–3 ng of β1 or 0.38–38 ng of $\beta1_{STOP}$ transcripts. RNA concentrations were estimated from the intensity of bands on ethidium bromide stained gels, compared with bands of known concentration. Injected oocytes were incubated at 18°C for 48 h, and then examined by two-electrode voltage clamp, as previously described (Li *et al*. 1999).

Sodium currents were recorded from CHL cells using the whole cell configuration of the patch clamp recording technique (Hamill *et al*. 1981). The bath solutions contained 130 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4 with NaOH. The pipette solution consisted of 105 mM CsF, 10 mM CsCl, 10 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.4 with CsOH. Other details of the procedures for whole cell recording from CHL-derived cell lines are described elsewhere (Kazen-Gillespie 2000). The voltage-dependence of channel activation was determined by applying depolarizating test pulses to a range of test potentials, from a holding potential of −90 mV. Peak current amplitude (I_{peak}) was measured at each test potential, and converted to conductance (g) according to $g = I_{peak}/(V_{rev} - V_{test})$, in which

V_{test} is the test potential and V_{rev} is the current reversal potential, determined by linear extrapolation of the straight line portion of the falling phase of the current–voltage relationship. The conductance values were normalized with respect to the maximal conductance, plotted as a function of V_{test}, and fit with the Boltzmann equation: $1/\{1 + \exp[(V_{test}\text{-}V_{1/2})/k]\}$, in which $V_{1/2}$ is the midpoint of the curve and k is a slope factor. Steady-state inactivation was examined by applying 100-ms long prepulses to a range of prepulse potentials, followed by a test pulse to 0 mV. The peak amplitude of currents evoked by the test pulses were normalized with respect to the largest currents, plotted as a function of prepulse potential and fit with the Boltzmann equation.

Single channel recordings were obtained using the cell-attached configuration (Hamill *et al*. 1981). The bath solution consisted of 140 mM K-Acetate, 5 mM NaCl, 4 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM $CdCl_2$, 25 mM glucose, 10 mM HEPES, pH 7.4 with KOH. The pipette solution was the same as the bath used for whole cell recordings. Data were acquired at a sampling rate of 50 kHz and filtered at 1.5 kHz. All test pulses were to −20 mV from a holding potential of − 100 mV. P_{null}, the probability that a single channel would fail to open during a depolarizing test pulse, was determined according to the n^{th} root of the number of null sweeps/the total number of sweeps in the experiment, where n is the number of channels in the patch, determined by the maximum number of superimposed openings. P_o, the channel open probability was estimated according to $P_o = I_{avg}/(ni)$, where I_{avg} is the mean current for each sweep, n is the number of channels in the patch, and i is the single-channel current amplitude.

[^{3}H]-Saxitoxin (STX) binding analysis

STX binding analysis was performed as previously described (Kazen-Gillespie 2000). Briefly, cloned cell lines were grown to confluency in T-225 tissue culture flasks, harvested by trituration and resuspended in ice-cold binding buffer. Intact cells were incubated at 4°C with 5 nM [^{3}H]STX (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in the presence or absence of 10 μM tetrodotoxin (TTX) (Calbiochem, LaJolla, CA, USA). The binding assay was terminated by vacuum filtration over GF/C filters (Fisher). Specific binding data were normalized to total cell protein using the BCA protein assay (Pierce, Rockford, IL, USA), with bovine serum albumin as the standard.

Co-immunoprecipatation of sodium channel α and β1 subunits

Membranes were prepared from SNaIIAβ1 and SNaIIAβ1_{STOP}7 cells as described previously (Malhotra *et al*. 2000). Membranes were solubilized in 1.25% Triton X-100 and the soluble fraction was incubated overnight at 4°C, either with 1.5 μg anti-SP11-II antibody (Alomone Labs, Jerusalem, Israel), which is specific for $Na_V1.2$ sodium channel α subunits, an anti-β1 antibody (1 : 500 dilution) directed to an extracellular epitope (Malhotra *et al*. 2000), or with non-immune serum. Protein A sepharose beads were added and the incubation continued for 2 h at 4°C. The protein A sepharose beads were pelleted and washed in 50 mM Tris–HCl, pH 7.5, 0.1% Triton X-100. Immunoprecipitates were eluted from the beads with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, separated on 10% acrylamide gels and transferred to nitrocellulose. The western blot was probed with an anti-β1 antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG at 1 : 100 000 dilution. Chemiluminescent detection of immunoreactive bands was accomplished with WestDura reagent (Pierce).

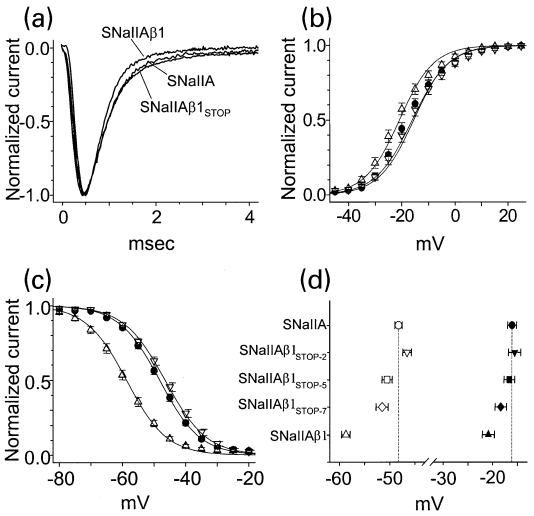

Fig. 1 Deletion of the C-terminus of β1 results in loss of functional modulation of sodium channels expressed in CHL cells. (a) Typical whole cell currents elicited in SNaIIA, SNaIIAβ1 and SNaIIAβ1_{STOP} cells by depolarization to 0 mV from a holding potential of − 90 mV. The current traces were normalized with respect to the current peaks, to make it easier to compare the current time courses in the different cells. (b and c) Activation (b) and steady-state inactivation (c) curves for SNaIIA (●), SNaIIAβ1 (△) and SNaIIAβ1_{STOP}2 (▽). The protocols for determining the voltage-dependence of activation and inactivation are described in the Materials and methods section. In this and subsequent figures, the data points and error bars show means ± SEM. The smooth lines are according to the Boltzmann equation (see Materials and methods) using mean values for $V_{1/2}$ and k determined from fits of the individual experiments. (d) Mean values for $V_{1/2}$ of activation (filled symbols) and steady-state inactivation (open symbols) for SNaIIA, SNaIIAβ1 and three different SNaIIAβ1_{STOP} cell lines.

Results

β1_{STOP} does not significantly alter the functional properties of sodium channels expressed in CHL cells

Our initial objective was to determine whether the cytoplasmic C-terminal domain of the sodium channel β1 subunit is involved in functional modulation of sodium channels expressed in mammalian cells. To examine this issue we transfected a CHL-derived cell line stably expressing the rat $Na_V1.2a$ α subunit (cell line SNaIIA; West *et al*. 1992; Isom *et al*. 1995b) with a mutant β1 construct, called β1_{STOP}, in which 34 amino acids were deleted from the C-terminus. We then isolated and examined three clonal cell lines, designated SNaIIAβ1_{STOP} 2, 5 and 7, respectively which stably expressed both $Na_V1.2a$ and

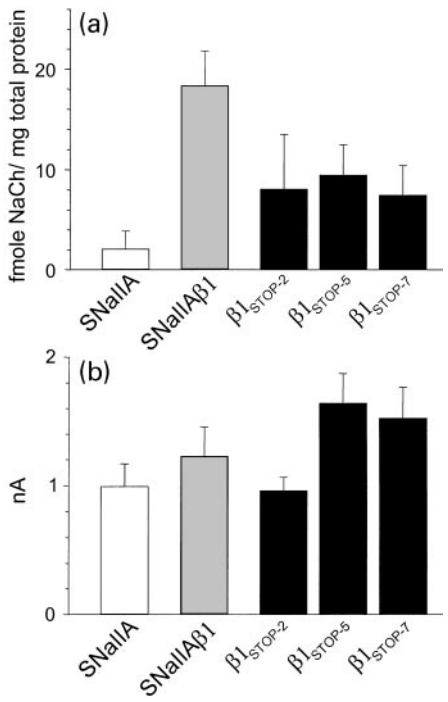

Fig. 2 Effects of coexpression of β1 or $\beta1_{STOP}$ on the levels of [^{3}H]-STX binding and whole cell sodium currents in CHL cells expressing sodium channel α subunits. (a) [^{3}H]-STX binding is increased by coexpression of either β1 or $\beta1_{STOP}$. The bars indicate means ± SEMs for SNaIIA ($n = 5$), SNaIIAβ1 ($n = 10$), SNaIIA$\beta1_{STOP}$2 ($n = 11$), SNaIIA$\beta1_{STOP}$5 ($n = 13$) and SNaIIA$\beta1_{STOP}$7 ($n = 11$) cells. (b) Coexpression of β1 or $\beta1_{STOP}$ has little or no effect on the amplitude of whole cell sodium currents in CHL cells. Currents were evoked by depolarization to + 10 from a holding potential of − 90 mV. Bars indicate means ± SEM for SNaIIA ($n = 76$), SNaIIAβ1 ($n = 64$), SNaIIA$\beta1_{STOP}$2 ($n = 55$), SNaIIA$\beta1_{STOP}$5 ($n = 31$) and SNaIIA$\beta1_{STOP}$7 ($n = 31$).

$\beta1_{STOP}$. For comparison, we also examined the parent SNaIIA cell line and a cell line stably coexpressing $Na_V1.2a$ and full-length β1 (SNaIIAβ1) (Isom *et al*. 1995b; Kazen-Gillespie 2000). Figure 1(a) shows typical whole cell sodium currents elicited in SNaIIA, SNaIIAβ1 and SNaIIA$\beta1_{STOP}$ cells. Currents in SNaIIA cells, which express just the $Na_V1.2a$ subunit alone, exhibited rapid inactivation time course (Fig. 1a). This behavior is consistent with previous results (Ukomadu *et al*. 1992; West *et al*. 1992; Isom *et al*. 1995b), which show that, in contrast to channel behavior in oocytes, brain and muscle α subunits form fast sodium channels when expressed alone in various types of mammalian cells. Also consistent with previous results (Isom *et al*. 1995b; Kazen-Gillespie 2000), β1 caused at most a small speeding of current inactivation (Fig. 1a), which may have been at least in part secondary to a negative shift in the voltage-dependence of activation (see below). $\beta1_{STOP}$ did not alter current time course (Fig. 1a).

As shown previously (Isom *et al*. 1995b; Kazen-Gillespie 2000), coexpression of wild-type β1 in CHL cells caused a small negative shift in the voltage-dependence of activation (≈ − 4 mV) (Figs 1b and d) and a larger negative shift in steady-state inactivation (≈ − 11 mV) (Figs 1c and d). In contrast, the midpoints of the mean activation and inactivation curves for the three $\beta1_{STOP}$ lines were not significantly different than the curves for $Na_V1.2a$ alone (Figs 1b–d). The β1-dependent shift in steady-state inactivation gives the most robust measure of modulation of channel function in CHL cells (Figs 1c and d; Isom *et al*. 1995b). Therefore, the lack of significant shifts in inactivation curves for the three $\beta1_{STOP}$ cell lines indicates a large attenuation of functional modulation with deletion of the intracellular C-terminal domain.

β1 and $\beta1_{STOP}$ increase [^{3}H]-STX binding sites on the surface of CHL cells

In addition to altering sodium channel function, β subunits also increase channel expression levels (Isom *et al*. 1992, 1995a, 1995b; Kazen-Gillespie 2000). To assess the relative effects of β1 and $\beta1_{STOP}$ on the levels of sodium channel expression on the surface of CHL cells, we measured [^{3}H]-STX binding in intact cells. The results of these binding experiments are summarized in Fig. 2(a). [^{3}H]-STX binding for cell line SNaIIAβ1 was approximately ninefold higher than binding for cell line SNaIIA, indicating that coexpression of β1 caused a large increase in sodium channels on the cell surface. A modest increase in [^{3}H]-STX binding was observed in the three cell lines expressing $\beta1_{STOP}$. Based on these results, we expected to see a large increases in whole cell currents in SNaIIAβ1 cells compared with cells expressing $Na_V1.2a$ alone, and perhaps a smaller increases in currents in the three SNaIIA$\beta1_{STOP}$ cell lines. Surprisingly, however, current amplitude was not significantly larger for SNaIIAβ1, while only one of the three SNaIIA$\beta1_{STOP}$ lines showed significantly larger currents (Fig. 2b). What is responsible for this discrepancy between [^{3}H]-STX binding and electrophysiological data? One possibility is that β1, while increasing the number of channels on the cell surface, simultaneously decreases the probability of channel opening during a depolarizing test pulse. This could happen, for example, if β1 increased the likelihood that channels enter null gating modes, as has been described for dihydropyridine interactions with voltage-gated calcium channels (Hess *et al*. 1984). We tested this hypothesis by determining P_{null}, the probability that single sodium channels would fail to open during 40 ms long depolarizing test pulses to −20 mV in cell attached patch recordings from SNaIIA, SNaIIAβ1 and SNaIIA$\beta1_{STOP}$ cells. The results of these experiments are summarized in Figure 3. P_{null} was somewhat variable from patch to patch, ranging from approximately 0.4 to 0.8; however, there was no significant difference between patches from SNaIIA,

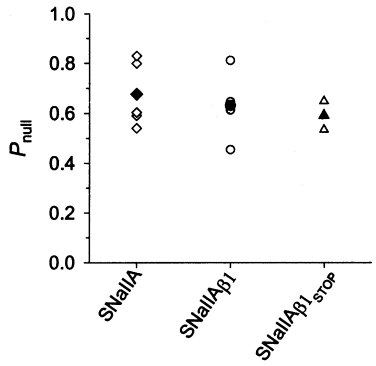

Fig. 3 P_{null} values for single sodium channels recorded in cell-attached patches from SNaIIA (diamonds), SNaIIAβ1 (circles) and SNaIIAβ1$_{STOP}$ (triangles) cells. The open symbols show P_{null} values for individual experiments, whereas the filled symbols indicate mean values for each cell type. Currents were elicited by 40 ms long depolarizations to − 20 mV from a holding voltage of − 100 mV.

SNaIIAβ1 and SNaIIAβ1$_{STOP}$ cells in the probability of observing nulls. β1 could also decrease current per channel by decreasing P_o, the open channel probability (e.g. by decreasing single channel open time or the number of times the channel opens in a burst) or by decreasing the single channel conductance. However, we did not observe significant differences between SNaIIA and SNaIIAβ1 lines either in open probability (mean P_o values were: SNaIIA: 0.0079 ± 0.0022, $n = 5$; SNaIIAβ1: 0.0060 ± 0.0008, $n = 4$) or in the amplitude of single channel currents (mean amplitudes at − 20 mV: SNaIIA: 1.07 ± 0.15 pA; SNaIIAβ1: 1.28 ± 0.18 pA). In summary, single channel analysis failed to detect a β1-induced change in channel properties that could account for the apparently contradictory effects of β1 on [^{3}H]-STX binding and sodium current amplitude.

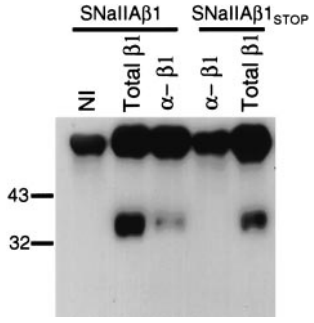

Fig. 4 Immunoprecipitation of sodium channel subunits expressed in SNaIIAβ1 and SNAIIAβ1$_{STOP}$ cells. (a) Proteins were precipitated from solubilized membranes prepared from SNaIIAβ1 (lanes 1–3) or SNaIIAβ1$_{STOP}$ (lanes 4 and 5) cells using either rat IgG (lane 1), anti-β1 antibody (lanes 2 and 5) or anti-sp11-II antibody (lanes 3 and 4), separated by SDS-PAGE, blotted to nitrocellulose and probed with anti-β1. Molecular weight markers are given in kDa.

β1$_{STOP}$ does not associate efficiently with αIIA in CHL cells

To examine the possibility that lack of detectable functional modulation of sodium channels by β1$_{STOP}$ in CHL cells results from poor association of the mutant β1 with the α subunit, sodium channel subunits were immunoprecipitated from solubilized membranes prepared from SNaIIAβ1 and SNaIIAβ1$_{STOP}$ cells using either anti-α or anti-β1 antibodies, separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-β1 antibody. Immunoprecipitation with anti-β1 from either SNaIIAβ1 and SNaIIAβ1$_{STOP}$ cells resulted in strong anti-β1 (Fig. 4, lane 2) or anti-β1$_{STOP}$ (Fig. 4, lane 5) immunoreactive bands, reflecting robust expression of wild-type and mutant β1 subunits in transfected CHL cells. For SNaIIAβ1, immunoprecipitation with anti-α antibody coimmunoprecipitated a fraction of the β1 subunit pool (Fig. 4, lane 3), indicating association between these two sodium channel subunits. In contrast, immunoprecipitation of αIIA from SNaIIAβ1$_{STOP}$ cells resulted in little or no detectable coimmunoprecipitation of β1$_{STOP}$ (Fig. 4, lane 4), suggesting much weaker association of the mutant β1 with $Na_V1.2a$. In some co-immunoprecipitation experiments with SNaIIAβ1$_{STOP}$ we detected a faint band corresponding to β1$_{STOP}$ (not shown); however, the intensity of this band was always weak compared with that observed for full-length β1. These data indicate that deletion of the C-terminus of β1 greatly reduces the efficacy of its association with $Na_V1.2a$ in CHL cells.

Functional modulation of sodium channels in oocytes is much weaker with β1$_{STOP}$ than with full-length β1

Co-immunoprecipitation data suggest that the reason β1$_{STOP}$ does not detectably alter the properties of sodium channels expressed in CHL cells is that it associates poorly with the α subunit. In *Xenopus* oocytes, on the other hand, several previous studies have shown that β1$_{STOP}$ or similar deletion constructs modulate channel function in a manner similar to full-length β1 (Chen and Cannon 1995; Makita *et al*. 1996; McCormick *et al*. 1998, 1999). Do these findings indicate that association of the mutant β1 subunit is more efficient in oocytes than in CHL cells? To address this question, we assessed modulation of sodium channel function in oocytes injected with a fixed concentration of $Na_V1.2a$ RNA and different concentrations of β1 or β1$_{STOP}$ RNA. The results of these experiments are summarized in Figure 5, which plots the rate of whole cell current inactivation as a function of the ratio of moles β1 or β1$_{STOP}$ RNA/mole $Na_V1.2a$ per oocyte. For oocytes expressing $Na_V1.2a$ alone, the time from current peak to 50% inactivation was approximately 3 ms (Figs 5a–c). The rate of current decay was progressively faster with progressively higher concentrations of injected β1, with the maximal effect observed ≥ 0.35 moles β1/mole α (Figs 5a and c). In contrast, 12.5 mol β1$_{STOP}$/mole α was without effect on the rate of

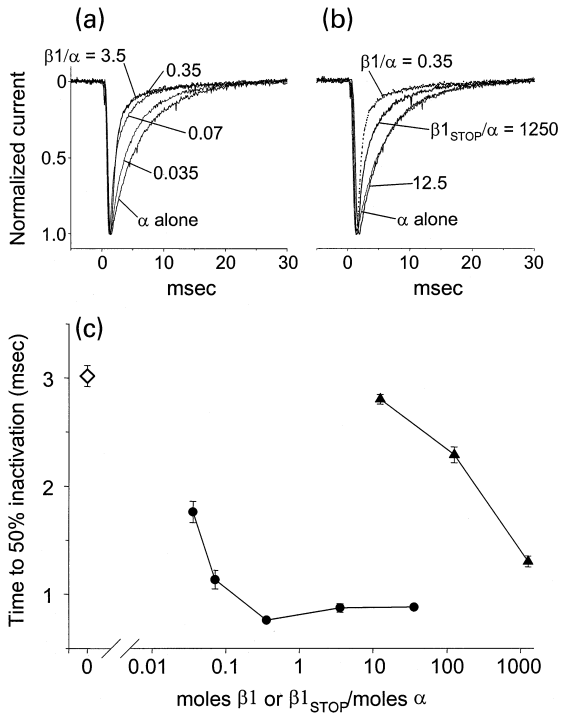

Fig. 5 $\beta1_{STOP}$ is much less potent than full-length β1 at modulating the functional properties of sodium channels expressed in oocytes. (a and b) Example traces in oocytes injected with varying molar ratios of β1 and $Na_V1.2a$ (a) or $\beta1_{STOP}$ and $Na_V1.2a$ (b). The dotted trace in (b) shows for comparison the current from panel (a) with a 0.35 molar ratio of β1 to $Na_V1.2a$. (c) Dose–effect relationships for decay rates of whole cell sodium currents recorded in oocytes injected with $Na_V1.2a$ alone (◇) or with varying amounts β1 (●) or $\beta1_{STOP}$ (▲). The symbols show mean ± SEM values for the time from the current peak to the point where current had decayed to 50% of its peak value, plotted as a function of the ratio of the moles β1 or $\beta1_{STOP}$/mole $Na_V1.2a$.

current decay, and even 1250 mol $\beta1_{STOP}$/mole α did not elicit the full effect observed with full-length β1 (Figs 5b and c). Overall, the dose–response curve for $\beta1_{STOP}$ was shifted to RNA concentrations approximately four orders of magnitude higher than for full-length β1. These data suggest that in oocytes, as in mammalian cells, deletion of the intracellular C-terminus of β1 profoundly affects the efficiency of β1 association with $Na_V1.2a$.

Discussion

β1 requires its cytoplasmic domain for efficient association with α

Several previous studies have shown that specific determinants in the extracellular domain of β1 are both necessary and sufficient for shifting sodium channels expressed in oocytes from slow gating modes to fast gating modes (Chen and Cannon 1995; Makita *et al*. 1996; McCormick *et al*. 1998, 1999). In contrast, functional modulation was still observed after deletion of the intracellular C-terminal domain of β1. Thus it was concluded that this segment is not involved in functional modulation. The main finding of this study is that this cytoplasmic domain of β1, while perhaps not directly involved in regulation of channel function, nevertheless is necessary for efficient association of β1 with the sodium channel α subunit. This conclusion is supported by data from CHL cells showing little or no α-$\beta1_{STOP}$ coimmunoprecipitation, and from whole cell recordings in CHL cells, which show loss of functional modulation. Additional supporting evidence comes from titration experiments in *Xenopus* oocytes, which demonstrate that approximately 10 000 times more $\beta1_{STOP}$ RNA is required to achieve speeding of current time course comparable to that observed with full-length β1.

We can think of at least three possible explanations for the effects of C-terminal deletion on β1 association with α. One possibility is that determinants in the cytoplasmic domain of β1 are directly involved in protein–protein interactions that hold the β1 and α subunits together. Loss of these determinants would cause β1 to bind to α with lower affinity, an effect that presumably can be overcome in oocytes by injecting very high concentrations of RNA and thus expressing high levels of $\beta1_{STOP}$ protein. A second possibility is that specific signals in the C-terminus are involved in targeting β1 to the appropriate cellular compartment for assembly with α. For example, recent data suggest that the cytoplasmic domains of sodium channel β subunits are involved in interactions with components of the cytoskeleton (Malhotra *et al*. 2000). These or other interactions could be important for appropriate subcellular targeting of β1. Finally, the mutant protein may be detected as abnormal by the quality control mechanisms of the cell and routed to a degradation pathway rather than being targeted to the correct cellular compartment for association with α.

Discrepancy between STX binding and current levels

A surprising finding of this study was that large increases in STX binding sites on the cell surface caused by β1, and to a lesser extent $\beta1_{STOP}$, did not result in comparable increases whole cell sodium currents. Although previous studies have reported moderate increases in whole cell currents with coexpression of β1 in either mammalian cells (Isom *et al*. 1995; Kazen-Gillespie 2000) or oocytes (Isom *et al*. 1992, 1995a), these modest increases are not comparable to the large increases in binding sites described here. We have examined the possibility that this discrepancy is explained by a β1-mediated decrease in the probability of channel opening during depolarizing test pulses. These experiments reveal no difference in the probability of channel opening between cell lines with or without β1, and thus argue against

this explanation. The data do not rule out the possibility that β1 induces transitions to a silent gating mode with a long duration that would be difficult to detect in typical patch experiments lasting 10–20 min or that β1 facilitates expression of a subset of permanently silent sodium channels. Interestingly, a similar phenomenon has been described in PKA-deficient PC12 cell lines (Ginty *et al*. 1992). Exposure of PKA-deficient PC12s to NGF or bFGF resulted in large increases in sodium channel α subunit mRNA and STX binding sites, but no change in sodium current density. It was suggested that PKA acts at a post-translational level to influence whether or not expressed channels will function. Similarly, it is possible that β1 co-assembly influences the likelihood that α subunits, expressed on the cell surface will act as functional sodium channels.

Conclusion

At present very little is known about the cellular mechanisms responsible for association of sodium channel α and β1 subunits. For example, it is not known whether this is an early event in protein processing, as is the case for potassium channel β subunits (Shi *et al*. 1996), or a late event, as is the case for sodium channel β2 subunits (Schmidt and Catterall 1986). The results presented here suggest that the C-terminal domain of β1 may play a role in α-β1 assembly and thus provide data that begin to address the molecular mechanisms responsible for this process.

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