Functional Association of the β_1 Subunit with Human Cardiac (hH1) and Rat Skeletal Muscle (μ1) Sodium Channel α Subunits Expressed in *Xenopus* Oocytes

H. BRADLEY NUSS, NIPAVAN CHIAMVIMONVAT, M. TERESA PÉREZ-GARCÍA, GORDON F. TOMASELLI, and EDUARDO MARBÁN

From the Department of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205

ABSTRACT Native cardiac and skeletal muscle Na channels are complexes of α and β_1 subunits. While structural correlates for activation, inactivation, and permeation have been identified in the α subunit and the expression of α alone produces functional channels, β_1-deficient rat skeletal muscle (μ1) and brain Na channels expressed in *Xenopus* oocytes do not gate normally. In contrast, the requirement of a β_1 subunit for normal function of Na channels cloned from rat heart or human heart (hH1) has been disputed. Coinjection of rat brain β_1 subunit cRNA with hH1 (or μ1) α subunit cRNA into oocytes increased peak Na currents recorded 2 d after injection by 240% (225%) without altering the voltage dependence of activation. In μ1 channels, steady state inactivation was shifted to more negative potentials (by 6 mV, $p < 0.01$), but the shift of 2 mV was not significant for hH1 channels. Nevertheless, coexpression with β_1 subunit speeded the decay of macroscopic current of both isoforms. Ensemble average hH1 currents from cell-attached patches revealed that coexpression of β_1 increases the rate of inactivation (quantified by time to 75% decay of current; $p < 0.01$ at -30, -40, and -50 mV). Use-dependent decay of hH1 Na current during repeated pulsing to -20 mV (1 s, 0.5 Hz) after a long rest was reduced to 16 ± 2% of the first pulse current in oocytes coexpressing α and β_1 subunits compared to 35 ± 8% use-dependent decay for oocytes expressing the α subunit alone. Recovery from inactivation of μ1 and hH1 Na currents after 1-s pulses to -20 mV is multiexponential with three time constants; coexpression of β_1 subunit decreased all three recovery time constants. We conclude that the β_1 subunit importantly influences the function of Na channels produced by coexpression with either the hH1 or μ1 α subunits.

Address correspondence to Eduardo Marbán, M.D., Ph.D., Division of Cardiology, 844 Ross Building, Johns Hopkins University, 720 North Rutland Avenue, Baltimore, MD 21205.

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INTRODUCTION

Sodium channels are pore-forming transmembrane proteins that selectively pass Na^+ ions down their electrochemical gradient in response to membrane depolarization. An inward flow of Na^+ ions generates the depolarizing current which initiates the rapid action potentials of nerve, skeletal muscle, and heart. Sodium channels in skeletal and ventricular muscle are complexes of a large α subunit (260 kD) and a smaller noncovalently associated β_1 subunit (38 kD for skeletal muscle, 36 kD for cardiac) (Sutkowski and Catterall, 1990; Isom, De Jongh, and Catterall, 1994). Mammalian brain Na channels are composed of three subunits; the additional β_2 (33 kD) subunit is covalently linked by a disulfide bond to the α subunit (Sutkowski and Catterall, 1990). The α subunit contains at least 24 membrane-spanning segments, while the smaller β_1 subunit has a lengthy extracellular domain, a short intracellular domain and a single membrane-spanning segment (Catterall, 1994).

The α subunit encodes a transmembrane protein with the essential functions of an Na channel, including voltage-dependent gating and Na^+ selectivity (Goldin, Snutch, Lubbert, Dowsett, Marshall, Auld, Downey, Fritz, Lester, Dunn, Catterall, and Davidson, 1986). However, β_1-deficient rat skeletal muscle and brain Na channels do not function normally (Krafte, Snutch, Leonard, Davidson, and Lester, 1988; Zhou, Potts, Trimmer, Agnew, and Sigworth, 1991; Cannon, McClatchey, and Gusella, 1993). The currents inactivate slowly compared to those recorded in native channels and the voltage dependence of inactivation is shifted to more positive potentials. Coinjection of rat brain and skeletal muscle α subunit RNA with the rat or human brain β_1 subunit RNA in *Xenopus* oocytes restores normal rapid inactivation, shifts the voltage dependence of inactivation to more negative potentials, increases the size of the current and relieves use-dependent decay (Auld, Goldin, Krafte, Marshall, Dunn, Catterall, Lester, Davidson, and Dunn, 1988; Isom, DeJongh, Patton, Reber, Offord, Charbonneau, Walsh, Goldin and Catterall, 1992; Cannon et al., 1993).

Despite biochemical evidence for a functional association of α and β_1 subunits in heart muscle and the abundant expression of β_1 subunits in heart as well as in skeletal muscle and brain (Sutkowski and Catterall, 1990; Makita, Bennett, and George, 1994; Wallner, Weigl, Meera, and Lotan, 1993), the requirement of β_1 for normal function of cardiac Na channels expressed in oocytes has been disputed. Cardiac Na channels expressed in oocytes from a subunit RNA have been reported to possess normal physiologic properties (Krafte, Volberg, Dillon, and Ezrin, 1991) and macroscopic current kinetics characteristic of native cardiac Na channels (Gellens, George, Chen, Chahine, Horn, Barchi, and Kallen, 1992; Satin, Kyle, Chen, Rogart, and Fozzard, 1992). In contrast to findings in skeletal muscle Na channels, it has been reported that the human β_1 subunit ($H\beta_{1A}$) does not functionally modify the human heart Na channel when both the hH1 α and $H\beta_{1A}$ subunits are coinjected into *Xenopus* oocytes (Makita et al., 1994). Our observations indicate that coexpression of β_1 with the hH1 α subunit in oocytes has several effects. First, coexpression dramatically increases the current density; second, inactivation is accelerated; and finally, use-dependent decay of the Na current is markedly reduced and recovery from inactivation is accelerated. Our data support an impor-

tant functional association between the β_1 subunit and both the hH1 and μ1 α subunits.

MATERIALS AND METHODS

Preparation of cRNA

In vitro transcription of complementary RNA (cRNA) for injection into oocytes was initiated by restriction enzyme digestion of the full-length cDNA constructs subcloned into the pSP64T* vector (Krieg and Melton, 1984). Plasmid DNA was linearized by digestion with XbaI (hH1), SalI (μ1), or EcoRI (β_1) which recognized sites beyond the 3′ noncoding region of the insert DNA. After phenol/chloroform (1:1) and chloroform/isoamyl alcohol (24:1) extractions, the linearized DNA was precipitated (3 M NaOAc, pH 5.2), washed with ethanol (70%) and vacuum dried. The in vitro transcription reactions were carried out by the addition of 2 μl SP6 RNA polymerase (GIBCO BRL, Gaithersburg, MD) to a buffer solution comprised of transcriptional buffer (20 μl), dithiothreitol (10 μl, 0.1 M), RNase inhibitor (2.5 μl), ribonucleotide triphosphates (10 μl of 10× stock, Promega Corp., Madison, WI), diguanosine triphosphate (10 μl), diethyl pyrocarbonate-deionized H_2O (35.5 μl), and DNA (10 μl) at 37°C for 6 h. Additional RNA polymerase (2 μl) was added after the first 3 h. After a short incubation (15 min, 37°C) with RQ1 DNase (4 μl; Promega Corp.), addition of 5 μl EDTA (0.5 M, pH 8.0) stopped the reaction. After extraction, precipitation and ethanol wash, the integrity of the RNA was verified by electrophoresis on an agarose gel (1%). The final concentration of hH1 and μ1 α subunit mRNA was adjusted to 1 μg/μl, while β_1 subunit RNA was left undiluted at concentrations ranging from 5–6 μg/μl. The clones for hH1 (Gellens et al., 1992), μ1 (Trimmer, Cooperman, Tomiko, Zhou, Crean, Boyle, Kallen, Sheng, Barchi, Sigworth, Goodman, Agnew, and Mandel, 1989) and rat brain β_1 (Isom et al., 1992) were provided by R. G. Kallen (University of Pennsylvania, Philadelphia, PA), Gail Mandel (State University of New York at Stony Brook, Stony Brook, NY) and W. A. Catterall (University of Washington, Seattle, WA), respectively. A large portion of the 3′ untranslated region of the hH1 cDNA vector was eliminated using PCR mutagenesis because it contained ambiguous sequence.

Expression in Xenopus laevis Oocytes

Adult female *Xenopus laevis* (Xenopus 1; Ann Arbor, MI or Nasco, Ft. Atkinson, WI) were anesthetized with 3-aminobenzoic acid ethyl ester (methanesulfonate salt, 0.17%, Sigma Chemical Co., St. Louis, MO). Sections of ovarian lobes were surgically removed through a small incision in the abdomen and placed in sterile ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6, with NaOH). The ovarian lobes were teased into small clumps of oocytes, placed into centrifuge tubes and rinsed in Ca^{2+}-free OR-2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, with NaOH). Two periods (30–45 min) of collagenase (1–2 mg/ml in Ca^{2+}-free OR-2, Type 1A; Sigma Chemical Co., St. Louis, MO) digestion with mild agitation on an orbital shaker freed individual oocytes from the ovarian lobes and removed the surrounding follicular cell layer. After digestion, stage V and VI oocytes were placed in ND-96 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (2 mM), and theophylline (0.5 mM).

Oocytes with a distinct equatorial band between the animal and vegetal poles were injected with 50–100 nl of mRNA using a 10-μl microinjector (Drummond Scientific Co., Broomall, PA) to express μ1 and hH1 channels. Oocytes were coinjected with 50–100 nl of mRNA solution containing a 1:1 volume ratio of α to β_1 subunit mRNA to express μ1-β_1 or hH1-β_1 channels. Since the β_1 subunit mRNA was more concentrated than the α subunit mRNA, β_1 was coinjected in five- to sixfold excess of the α subunit. Using this procedure we estimate that the amount of injected β_1 mRNA sufficed to enable the majority of the expressed α subunits to associate with a β_1 subunit (Cannon

et al., 1993). Injected oocytes were incubated for 1–5 d at room temperature until the desired expression level was achieved.

Two-Electrode Recording of Macroscopic I_{Na}

Macroscopic μ1 and hH1 Na currents were recorded using a two-microelectrode voltage clamp technique (Methfessel, Witzemann, Takahashi, Mishina, Numa, and Sakmann, 1986). The whole-cell recording solution was ND-96. Both voltage and current electrodes were pulled from 1.2-mm OD borosilicate glass (World Precision Instruments Inc., Sarasota, FL) on a two-stage vertical puller (Narishige Tokyo, Japan). When filled with 3 M KCl, the electrodes had resistances of 0.2–0.4 MΩ. Membrane potential was controlled by a two-electrode voltage clamp amplifier with a virtual ground circuit (Warner Instruments Corp., Hamden, CT). Current signals were low-pass filtered at 1 kHz by an 8-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and digitized on-line at 10–20 kHz with 12-bit resolution onto a personal computer. All electrophysiological recordings were obtained at room temperature (20–22°C).

Oocytes accepted for study had stable membrane potentials more negative than −25 mV and holding currents that remained <-0.01 μA at −100 mV for the duration of the experiment. During two-electrode voltage clamp recording, precautions were taken to avoid voltage escape or a loss of voltage control. Experiments with over-compensation artifacts evident as notches in the current elicited by steps to positive potentials or in the tail currents evoked upon repolarization were not analyzed. Likewise, experiments with artifacts such as an "abominable notch" (Cole, 1972) were also excluded from study. When determining the whole-cell current-voltage relationships, all experiments included in the analysis had currents that activated in a graded fashion during progressive 5-mV depolarizing steps. When determining the voltage dependence of steady state inactivation, the only experiments analyzed were those in which the times to peak current measured with all ($h_\infty = 1$) and half ($h_\infty = 0.5$) the channels available differed by <10%.

Single-Channel Current Recordings

Oocytes were first screened to determine the level of Na channel expression: those expressing macroscopic currents in excess of 4 μA were selected for patch clamp experiments. After screening, the vitelline membrane was microdissected away during incubation of the oocytes in a hyperosmolar solution (462 mOsm) comprised of 220 mM *N*-methyl-D-glucamine (NMG), 220 mM aspartic acid, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.2, with NMG.

Depolarizing high-potassium bath solution containing 140 mM KCl, 10 mM HEPES, pH 7.4, was used to zero the membrane potential. Pipettes were filled with recording solution consisting of 140 mM NaCl, 1 mM $BaCl_2$, 10 mM HEPES, 7.4 pH . Patch pipettes, fabricated from borosilicate glass, coated near the tip with Sylgard (Dow Corning, Midland, MI) and fire polished to adjust tip diameter, had resistances of 10–15 MΩ when filled with recording solution. An Axopatch 200 amplifier with CV-202 headstage (Axon Instruments, Foster City, CA) was used to record currents in the cell-attached configuration. Voltage pulse protocols, data acquisition and analysis were performed by custom-written software and executed on a personal computer. Current recordings were filtered at 2 or 5 kHz when sampling every 100 or 50 μs, respectively, and digitized on-line at 10–20 kHz with 12-bit resolution.

Capacity transients and passive leak currents were subtracted using a curve-fitted blank sweep as a leak template. Only fully resolved single-level openings were used to construct amplitude distributions. Amplitude histograms were fit by Gaussian functions (Origin, MicroCal Software Inc., Northampton, MA) to determine the unitary current amplitude at each test potential. Ensemble average currents were constructed from patches containing four or more and often in excess of 10 active channels as judged from the number of stacked openings. These data were collected by repeatedly cycling through a family of potentials from −60 to −20 mV using a 2-s repetition interval

and recording from each patch for at least 25 min. These records were idealized using a half-height criterion (Colquhoun and Sigworth, 1983) with three-point detection. The ensemble averages of the idealized currents at each potential were used for kinetic analysis.

Curve Fitting Functions

The macroscopic current-voltage relationships were fit with a combined Boltzmann and Goldman-Hodgkin-Katz equation (Hille, 1992) to determine half-activation potentials. Voltage dependence of inactivation relationships (h_∞ curves) were fit with a standard Boltzmann equation. The recovery from inactivation data were fit by exponential decay functions. A Gaussian function was used to fit the single-channel current amplitude histograms.

Statistical Analysis

All data are presented as means and standard errors. Significance of differences between two populations (α vs α-β_1) over a range of voltages was assessed using multivariate analysis of variance (MANOVA, Systat Inc., Evanston, IL). Data at individual voltages were compared by unpaired two-sided t tests. Differences were considered statistically significant when $p < 0.02$.

RESULTS

Coinjection of the β_1 Subunit Increases Functional Na Channel Expression

Sodium currents recorded in oocytes injected with RNA encoding the μ1 skeletal muscle Na channel α subunit were compared to I_{Na} recorded in oocytes coinjected with excess β_1 subunit. With the holding potential set at -100 mV, step depolarizations to voltages from -60 to 30 mV evoked families of currents shown in Fig. 1. The rate of macroscopic current decay is dramatically increased by coexpression with the β_1 subunit (Fig. 1 *A*). The similarity of the descending limbs of the peak current–voltage relationships (Fig. 1 *B*) suggests that the voltage dependence of activation in μ1 channels is not significantly influenced by the β_1 subunit. Indeed, the half-activation potentials equal -27 ± 1 mV for μ1 ($n = 10$ and 13) and -26 ± 1 mV for μ1-β_1 ($n = 4$ and 6). The ascending limbs of the current–voltage relationships are significantly different ($p < 0.001$), possibly due to rapid inactivation of μ1-β_1 currents at positive potentials. Coexpression with β_1 subunit increased the expression of peak I_{Na} measured one day after injection to -6.2 ± 0.9 μA ($n = 27$) from -1.9 ± 0.2 μA in μ1 Na channels alone ($n = 14$, $p < 0.001$, Fig. 1 *B, inset*). Na currents recorded two days after injection of the α subunit alone (-6.0 ± 0.7 μA, $n = 40$) equaled the level of expression that the coinjected (μ1-β_1) oocytes had achieved in just one day and did not significantly increase further by day 3.

While the inactivation kinetics of the whole-cell Na currents recorded in oocytes injected with the hH1 α subunit RNA are intrinsically faster than those of μ1 Na currents, coexpression with the β_1 subunit further hastens the decay of hH1 Na currents during maintained depolarization (Fig. 2 *A*). (This kinetic effect is analyzed in detail below, based on patch recordings.) As was the case with μ1, coinjection of β_1 increased the hH1 peak current density (Fig. 2 *B, inset*). Families of Na currents were recorded in oocytes injected with either the hH1 α subunit alone or in tandem with the β_1 subunit at days 1, 2, 3, and 4 (hH1 only) after injection. Coinjection with the β_1 subunit increased the size of I_{Na} over the entire range of po-

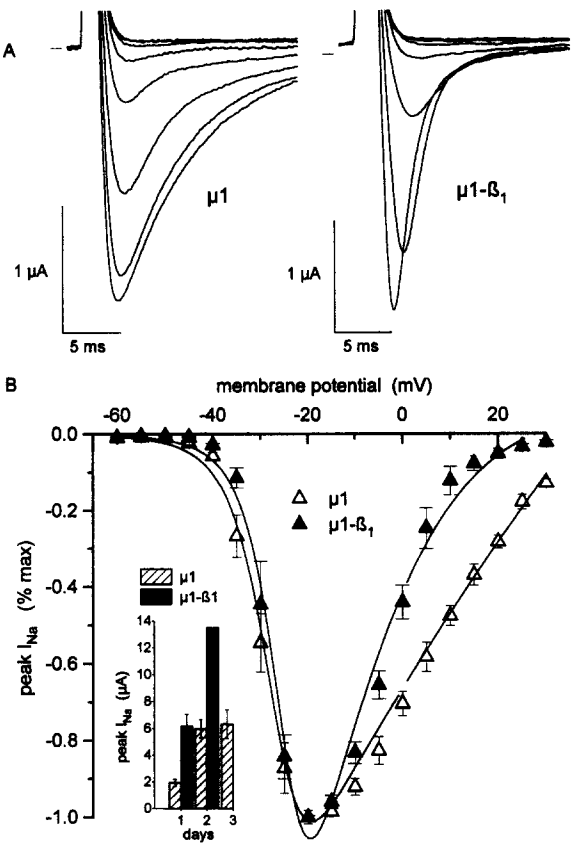

FIGURE 1. Macroscopic μ1 and μ1-β_1 currents (*A*), normalized current–voltage relationships (*B*) and comparison of expression levels of μ1 and μ1-β_1 Na channels (*B, inset*). Families of currents were elicited from −100 mV by progressive step depolarizations (40 ms) starting at −60 mV and ending at +30 mV. The currents shown were recorded at −60, −50, −45, −40, −35, −30, −25, and −20 mV. Measurements of peak current were made with respect to the baseline current at 40 ms and normalized to the largest peak current recorded. The mean data were fit to combined Boltzmann and Goldman-Hodgkin-Katz equations. The half-activation potentials equaled −27 ± 1 mV for μ1 (n = 10 and 13, *open triangles*) and −26 ± 1 mV for μ1-β_1 (n = 4 and 6, *solid triangles*). The bar graph (*B, inset*) illustrates that the level of μ1 current expressed increases more than twofold by coinjection of the β_1 subunit (see Results for details).

tentials (−55 mV to +20 mV, not shown). Peak I_{Na} measured in hH1-β_1 oocytes was always larger than that recorded in oocytes injected with the α subunit alone ($p <$ 0.001). When the means of the normalized current–voltage relationships were fit using combined Boltzmann and Goldman-Hodgkin-Katz equations (Fig. 2 *B*), there was no significant difference in the half-activation potentials for hH1 ($V_{1/2}$ = −6 ± 1 mV, n = 30) and hH1-β_1 ($V_{1/2}$ = −37 ± 1 mV, n = 8 and 14) channels.

Permeation Is Not Altered by β_1 Association

We used single-channel recordings to try to understand the mechanism of the changes in the macroscopic currents. Measurements from cell-attached patches indicate that unitary current amplitudes (i) are not altered by coexpression of the β_1 subunit with μ1 and hH1 α subunits. Amplitude histograms were constructed from clearly resolved openings to determine i for μ1 (Fig. 3) and hH1 (Fig. 4) channels at potentials between −70 and −10 mV. At any given potential, unitary current amplitudes of channels expressed from α only or from α-β_1 were indistinguishable. The slope conductance measured 32.3 ± 0.1 pS for μ1 α-only channels (n = 32) and 29.5 ± 0.2 pS for hH1 α-only channels (n = 25); neither was significantly al-

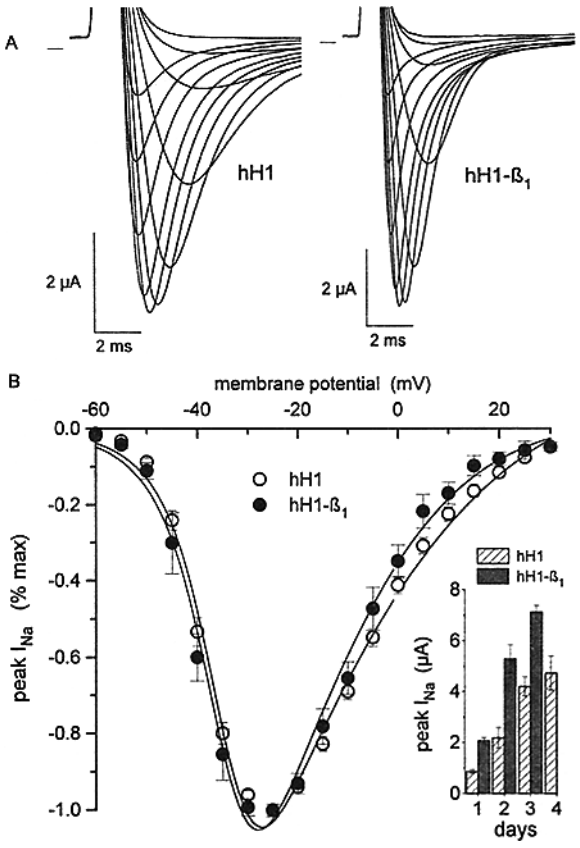

FIGURE 2. Macroscopic hH1 and hH1-β_1 currents (*A*), normalized current–voltage relationships (*B*) and comparison of expression of hH1 hH1-β_1 Na channels (*B, inset*). Families of currents were elicited from −100 mV by progressive step depolarizations (20 ms) starting at −60 mV and ending at +30 mV. Comparison of similarly sized currents (*A*) recorded at −60, −50, −45, −40, −35, −30, −25, −20, −10, 0, and +10 mV and shown at the same time base demonstrates that coexpressed channels have hastened inactivation kinetics. Normalized current–voltage relationships (*B*) for hH1 and hH1-β_1 macroscopic currents show no significant difference in the half-activation potentials for hH1 (−36 ± 1 mV, n = 30, *open circles*) and hH1-β_1 (−37 ± 1 mV, n = 8 and 14, *solid circles*) currents. The mean data were fit to combined Boltzmann and Goldman-Hodgkin-Katz equations to determine the half-activation potentials. A bar graph (*B, inset*) summarizes measurements of peak I_{Na} at successive days after injection of RNA into oocytes. The level of hH1 current expressed at days 1, 2, and 3 (day 1 = −0.9 ± 0.1 µA, n = 9; day 2 = −2.2 ± 0.4 µA, n = 24; day 3 = −4.2 ± 0.4 µA, n = 14) was significantly ($p < 0.001$) increased by coinjection with the β_1 subunit (day 1 = −2.1 ± 0.1 µA, n = 31; day 2 = −5.3 ± 0.6 µA, n = 17; day 3 = −7.1 ± 0.3 µA, n = 9) ($p < 0.001$).

tered by coexpression with the β_1 subunit (32.3 ± 0.2 pS for α1-β_1, n = 29 and 29.0 ± 0.2 pS for hH1-β_1 channels, n = 26). The lack of any changes in the open-channel properties indicates that the increase in whole-cell current reflects an increase in functional channels and/or changes in unitary gating, as considered further in the Discussion.

The Voltage Dependence of Inactivation Is Left-shifted in Oocytes Coinjected with the β_1 Subunit

Coexpression of rat brain IIA and skeletal muscle Na channels with the β_1 subunit has been shown to shift the voltage dependence of inactivation to more negative potentials, closer to the gating behavior found in native channels (Isom et al., 1992; Cannon et al., 1993). We confirmed these results in µ1-injected oocytes (Fig. 5)

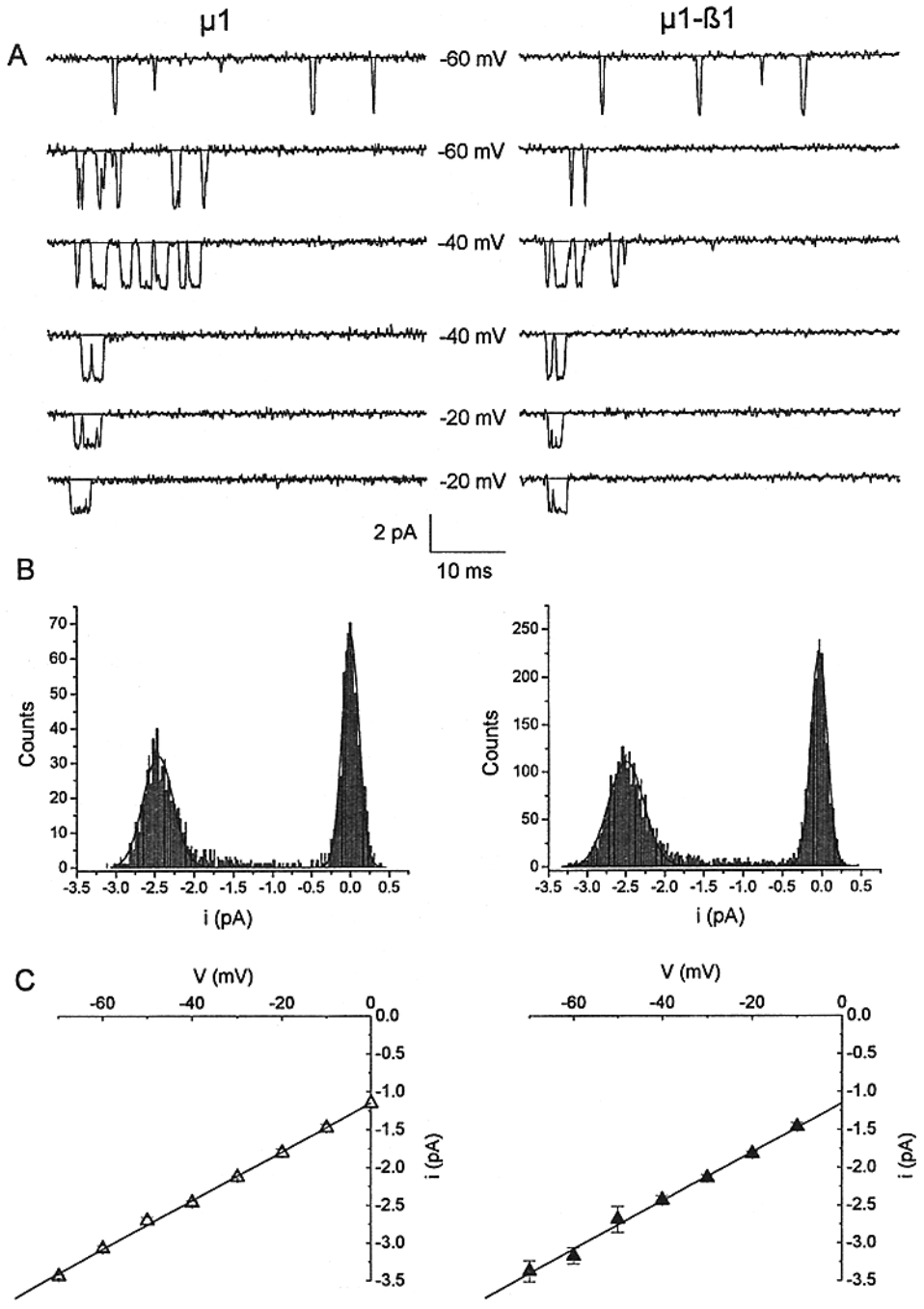

FIGURE 3. Single-channel currents (*A*), amplitude histograms (*B*) and unitary current–voltage relationships (*C*) for μ1 and μ1-β_1 Na channels. Single-channel current amplitude (*i*) determined by amplitude histograms (*B*) equaled −2.46 ± 0.03 pA for μ1 ($n = 8$) and −2.43 ± 0.06 pA for μ1-β_1 channels ($n = 6$) at −40 mV. The unitary current-voltage relationships (*C*) were not altered: slope conductance equaled 32.3 ± 0.1 pS in μ1 channels ($n = 32$) and 32.3 ± 0.3 pS in μ1-β_1 channels ($n = 29$).

and tested for a similar β_1 effect on the voltage dependence of inactivation in hH1-injected oocytes (Fig. 6). Peak I_{Na} availability was measured by a test depolarization to −20 mV after a 1-s conditioning prepulse. The prepulse potential was varied from −140 mV to progressively more depolarized potentials to inactivate greater numbers of channels. A repetition interval of 15 s allowed for full recovery of I_{Na} from the inactivation which occurs during a 1-s prepulse. Coinjection of β_1 resulted in a 6-mV hyperpolarizing shift in the half-inactivation voltages to −60 ± 1 mV ($n = 6$) from −54 ± 1 mV ($n = 8$) in μ1-injected oocytes ($p < 0.001$). The raw current tracings shown in Fig. 5 *A* illustrate a greater fraction of Na channels inactivated by a prepulse to −60 mV in μ1-β_1 than in μ1 oocytes.

For hH1, representative records illustrate that slightly more hH1-β_1 current is inactivated by a prepulse to −75 mV than in an hH1-alone oocyte (Fig. 6 *A*). Pooled data reveal that the voltage dependence of inactivation in oocytes expressing hH1 Na channels was shifted to more negative potentials by coinjection with the β_1 subunit (Fig. 6), albeit to a lesser extent than was the case for μ1. The half-inactivation potential was changed by only 2 mV, to −78 ± 1 mV in hH1-β_1 oocytes ($n = 9$) from −76 ± 1 mV in hH1 oocytes ($n = 9$, p = NS).

Coexpression of the β_1 Subunit Speeds Activation and Inactivation

The decay of macroscopic μ1 current during maintained depolarization was increased in oocytes coexpressing α and β_1 subunits (Figs. 1 and 5). Coexpression of the β_1 subunit also speeds up the decay of macroscopic hH1 Na currents, albeit to a lesser extent than for μ1 Na currents (Figs. 2 and 6). Recordings from cell-attached patches containing μ1, μ1-β_1, hH1, and hH1-β_1 Na channels verified that the observed differences in the inactivation kinetics of the macroscopic currents recorded using two-electrode voltage clamp were genuine. The ensemble average currents shown in Fig. 7 were normalized and superimposed to facilitate the comparison of inactivation kinetics. The increased rate of inactivation of ensemble average currents (Fig. 7 *A*, shown at −40 mV) recorded from oocytes coexpressing α and β_1 subunits is dramatic for μ1 Na channels over a wide range of voltages (−60 to 0 mV, data not shown). Similarly, in Fig. 7 *B* the ensemble averages (shown at −30, −40, and −50 mV) of coexpressed hH1-β_1 channels clearly inactivate more rapidly than ensembles constructed from α-only channels (hH1).

The rates of inactivation of hH1-β_1 and hH1 channels were compared by measuring the times to 75% decay of ensemble average currents (Fig. 8 *A*, T_{75}) at potentials between −60 and −30 mV. We chose to quantify T_{75} because it is a simple model-independent measure that does not require the assumption of single- or multi-exponential current decay. Voltage-dependent inactivation of hH1 and hH1-β_1 channels is evident in the superimposed ensemble averaged current records (Fig. 7 *B*) and in the T_{75} plot (Fig. 8 *A*). While hH1 currents decay faster than μ1 currents, coexpression with the β_1 subunit nevertheless significantly speeds the rate of inactivation of hH1-β_1 ensemble average currents at −50, −40, and −30 mV ($p = 0.01$, 0.01, and 0.004, respectively, by unpaired two-sided t test). For example, at −40 mV hH1-β_1 ensemble averaged currents decayed to 25% of their peak amplitude in 1.5 ± 0.3 ms ($n = 5$) as compared to 2.8 ± 0.3 ms ($n = 6$) for hH1. These data confirm that the β_1-induced hastening of inactivation of hH1 Na channels underlies the ac-

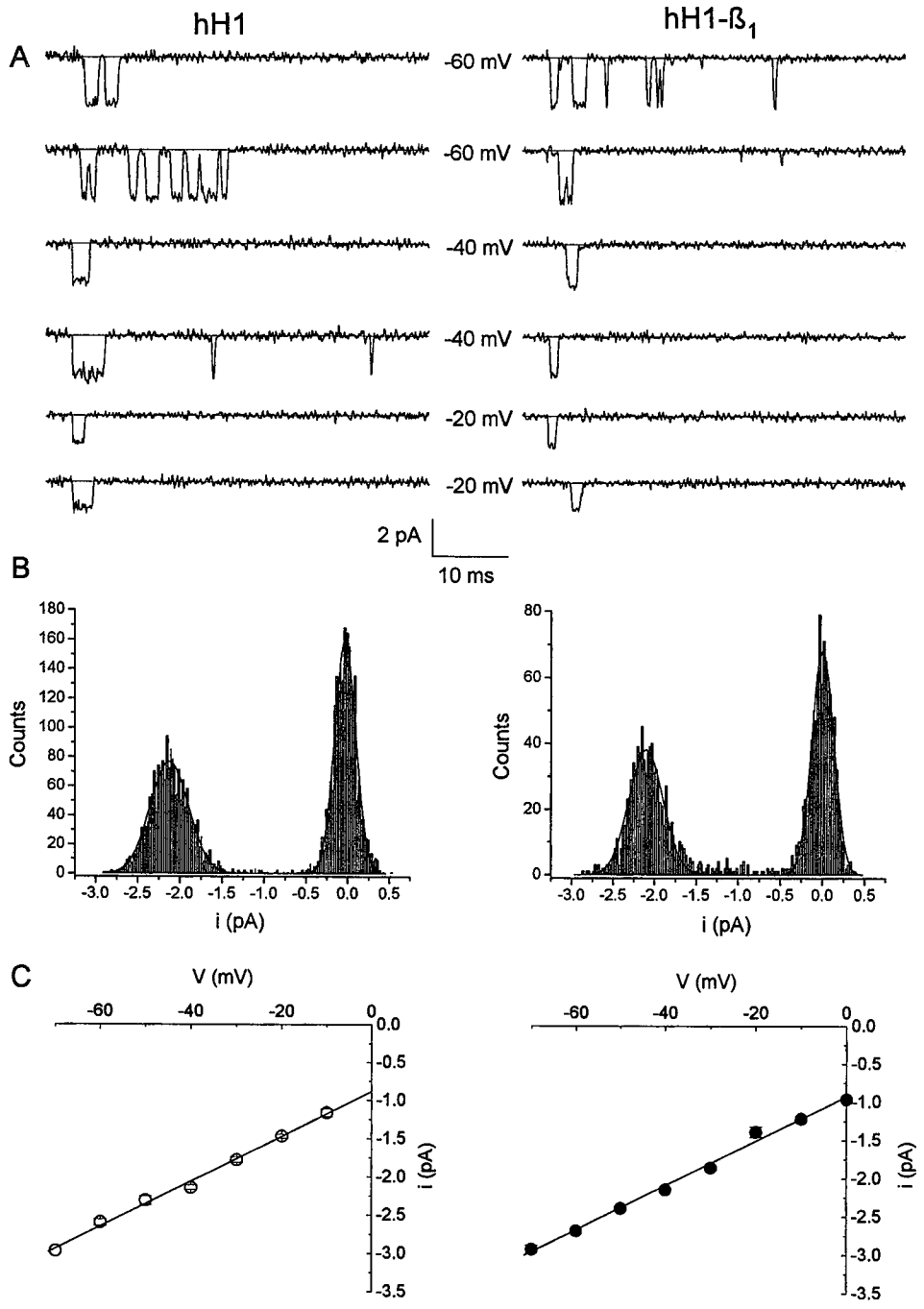

FIGURE 4. Single-channel currents (*A*), amplitude histograms (*B*) and unitary current-voltage relationships (*C*) for hH1 and hH1-β_1 Na channels. Single-channel current amplitude (*i*) determined by amplitude histograms (*B*) equaled -2.14 ± 0.03 pA for hH1 ($n = 6$) and -2.14 ± 0.02 pA for hH1-β_1 channels ($n = 6$) at -40 mV. The unitary current-voltage relationships (*C*) were not altered: slope conductance equaled 29.5 ± 0.2 pS in hH1 channels ($n = 25$) and 29.0 ± 0.2 pS in hH1-β_1 channels ($n = 26$).

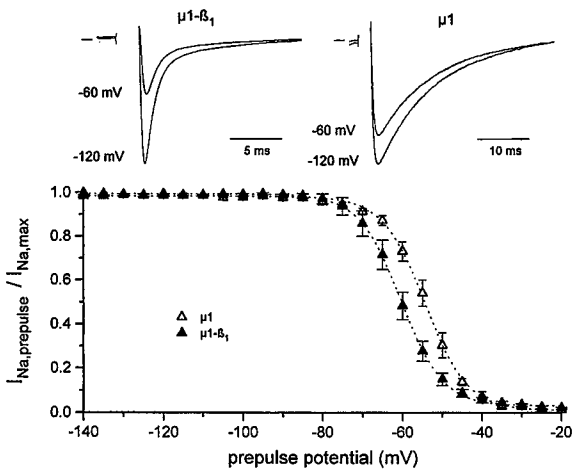

FIGURE 5. Voltage dependence of inactivation of μ1 and μ1-β_1 Na channels. Measurements of peak currents elicited at −20 mV immediately after a 1-s prepulse (−140 to −20 mV) define the relationship (*B*). Raw currents (shown with capacity transients blanked) in the upper panels (*A*) were recorded after prepulses to −120 and −60 mV. When the mean data were fit by a Boltzmann function, the half-inactivation potentials equaled −60 ± 1 mV for μ1-β_1 (n = 6, *solid triangles*) and −54 ± 1 mV for μ1 (n = 8, *open triangles*).

celerated decay of the macroscopic Na current observed in whole-cell recordings with two-electrode voltage clamp (Figs. 2 and 6).

The results in Fig. 7 *B* further suggest that the activation of hH1 may be accelerated by coexpression with β_1. Although the pooled data for the times to peak of the ensemble average currents (Fig. 8 *B*) did confirm a tendency for hH1-β_1 channels to activate faster than hH1 channels, the differences did not reach statistical significance (p = 0.06 by unpaired two-sided t test at −40 mV).

Relief of Use-dependent Decay of Peak Current by β_1 Subunit

Differences in the reduction of the peak current with repetitive pulsing provide strong evidence for a functional association between α and β_1 subunits in oocytes.

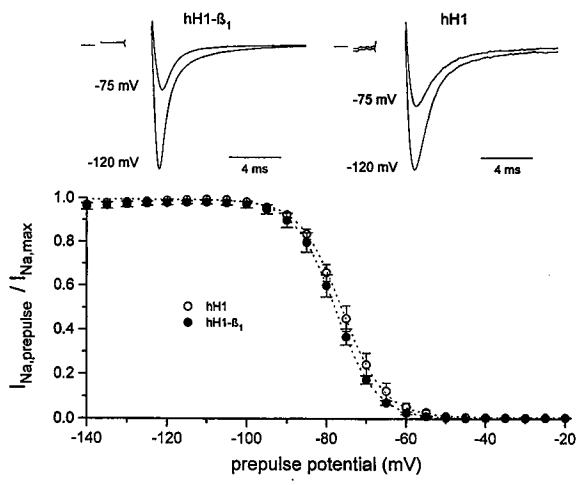

FIGURE 6. Voltage dependence of inactivation of hH1 and hH1-β_1 Na channels. Measurements of peak currents at −20 mV immediately after a 1-s prepulse (−140 to −20 mV) define the relationships. Raw currents (shown with the capacity transients blanked) in the upper panels were recorded after prepulses to −120 and −75 mV. When the mean data (*B*) were fit by a Boltzmann function, the half-inactivation potentials equaled −78 ± 1 mV for hH1-β_1 (n = 9, *solid circles*) and −76 ± 1 mV for hH1 (n = 9, *open circles*).

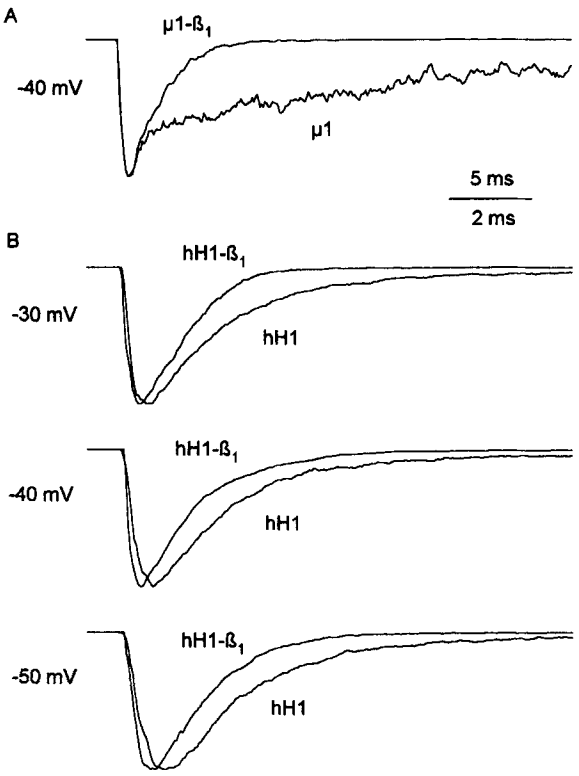

FIGURE 7. Ensemble average currents of μ1 and μ1-β_1 (*A*) and hH1 and hH1-β_1 (*B*) Na channels. Ensemble average currents were constructed from macropatch recordings from cell-attached patches during repeated families of depolarizations (−60 to −30 mV, 0.5 Hz) while holding at −140 mV between voltage steps. Ensemble average currents for α-only and α-β_1 channels are superimposed and normalized to peak current. Peak μ1 and μ1-β_1 ensemble averages (*A*) shown at −40 mV measured −1.2 and −2.0 pA, respectively. Peak hH1 and hH1-β_1 ensemble averages (*B*) shown at −30, −40, and −50 mV for the same two patches, measured −8 to −11 pA for the patch containing hH1 channels and −11 to −17 pA for the patch containing hH1-β_1 channels.

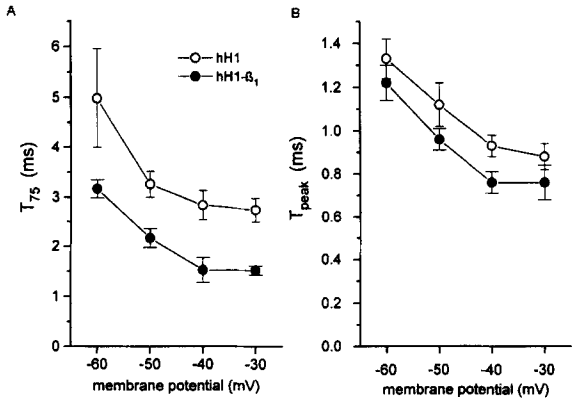

FIGURE 8. Analysis of inactivation (*A*) and activation (*B*) kinetics of ensemble average hH1 vs hH1-β_1 currents. Measurements of the time to 75% decay of current (*A*, T_{75}) and time to peak current (*B*, T_{peak}) were made from ensemble averages obtained at potentials between −60 and −30 mV of hH1-β_1 channels (*solid circles*) compared to hH1 (*open circles*) channels. The decrease in T_{75} was significant at −50, −40, and −30 mV (p = 0.01, 0.01, and 0.004, respectively, n = 6); the β_1-induced reduction in T_{75} measured at −60 mV did not reach significance because of the large variability in the decay phase of the hH1 ensemble averages, which were the smaller and noisier at −60 mV compared to hH1-β_1. The reduction in T_{peak} did not reach statistical significance at any potential studied (p = 0.06 at −40 mV, n = 6).

Currents were elicited by trains of short (Fig. 9 *A*, 10 ms) and long (Fig. 9 *B*, 1 s) voltage steps to −20 mV after a long rest (>45 s) at a repetition interval of 2 s. Peak I_{Na} was measured for each of the first 20 pulses in the train. This protocol provides an indirect measure of the accumulation of channels in the inactivated state. The extent of use dependence is a function of a particular channel type and its ability to recover from fast and slow inactivation. Let us first consider the μ1 channel. In the absence of the β_1 subunit, a substantial reduction in the peak current is already evident by the second voltage pulse in the train (Fig. 9). Second pulse μ1 currents equaled 91 ± 2% and 55 ± 1% of the first short and long pulses, respectively. With continued pulsing, the decay became even more pronounced, so that at pulse 20 the percentage of μ1 current remaining measured 84 ± 3% and 46 ± 5% of the first pulse current for short and long pulse durations, respectively. The comparable steady state values for oocytes coexpressing the β_1 subunit equaled 98 ± 1% and 83 ± 2% of the first pulse current. Thus, the presence of the β_1 subunit significantly reduced the use-dependent decay of the peak μ1 current by both short and long duration pulse trains ($p < 0.001$, $n = 6$).

The same experimental protocol was used to compare the use-dependent properties of hH1 and hH1-β_1 channels (Fig. 9). In contrast to the results with μ1, trains

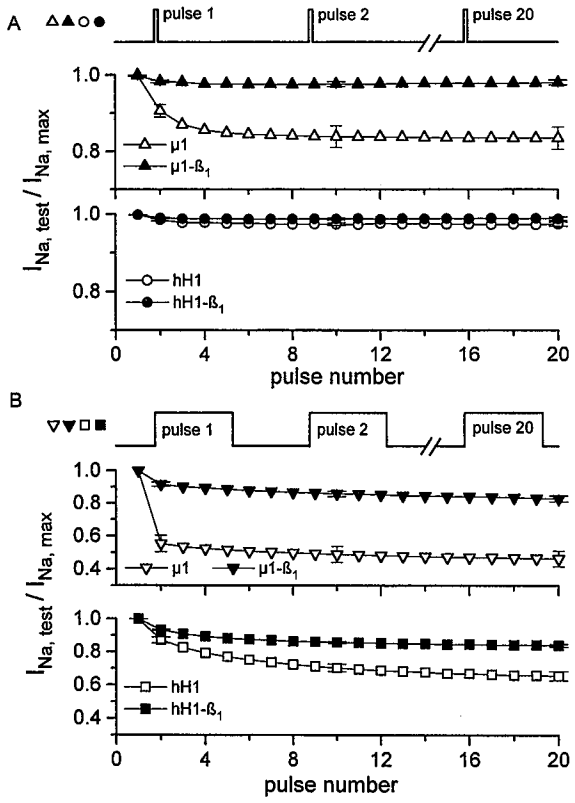

FIGURE 9. Use-dependent decay of peak μ1 and hH1 Na currents is reduced by coinjection with β_1. Step depolarizations at 0.5 Hz of 10 ms (*A*) or 1 s (*B*) were initiated after a long rest as illustrated by the voltage pulses shown above the plots. Measurements of peak currents were normalized to the first pulse current and plotted as a function of pulse number. Decay of μ1 current (*open triangles*) is pronounced even with 10-ms pulses (*A*). Coexpression of the β_1 subunit dramatically reduces use-dependent decay of μ1-β_1 current (*solid triangles*). Although the decay of hH1 current which occurs during a train of short pulses is small (3%), decay of hH1 current with 1 s pulses (*B, open squares*) is pronounced. Coexpression of the β_1 subunit dramatically reduces use-dependent decay current during the 1-s pulse train (*B, solid squares*, see Results for details).

of short pulses (Fig. 9, *upper panels*) resulted in a very small decrement of hH1 current (97 ± 1%, $n = 10$). The inclusion of the β_1 subunit produced a small but significant amount of relief of the use-dependent decay of peak hH1-β_1 current (99 ± 1%, $n = 10$, $p < 0.001$). The β_1-induced reduction in use dependence was more apparent when trains of long-duration pulses (Fig. 9 *B*) were used. Pulse 2 hH1 current was reduced to 87 ± 2% ($n = 10$) of the first pulse current. Use-dependent decay of hH1 current developed progressively during the remainder of the pulse train such that, by pulse 20, peak hH1 current was reduced to 65 ± 3% ($n = 10$) of the first post-rest peak current. The β_1 subunit dramatically reduced the use-dependent properties of hH1 Na channels as it did in μ1 Na channels. The relief of use-dependent decay by the β_1 subunit was first evident at pulse 2 where the hH1-β_1 current equaled 93 ± 1% ($n = 11$) of the first pulse current. With continued stimulation the progressive decay of hH1-β_1 current was less pronounced, such that the pulse 20 hH1-β_1 current was 84 ± 1% ($n = 11$) of the first pulse. The significant decrease ($p < 0.001$) in the amount of use-dependent decay of hH1-β_1 currents by both short- and long-duration pulse trains further supports a functional association of the β_1 subunit with hH1 Na channels.

Coexpression with the β_1 Subunit Speeds Recovery from Inactivation

Two-pulse experiments designed to quantify the time course of repriming provided further evidence that the β_1 subunit alters inactivation gating in μ1 and hH1 Na channels expressed in oocytes. A standard two-pulse protocol was used to examine the recovery from inactivation at −100 mV. Prepulses to −20 mV (1 s) were sufficient to recruit the process of slow inactivation (Rudy, 1978). As the repriming of channels occurred rapidly at −100 mV, interpulse intervals were varied from 1 ms to 2 s. In these experiments, the oocytes were held at −100 mV for a long enough time (15 s for μ1-β_1, 45 s for μ1, 15 s for hH1-β_1 and 30 s for hH1 Na channels) to allow full recovery of peak current between trials. The amount of recovery was assessed by the ratio of the second pulse current to the first pulse current (Fig. 10). Coexpression with the β_1 subunit significantly abbreviated the time required for repriming of current (μ1-β_1 vs μ1, $p < 0.001$; hH1-β_1 vs hH1, $p < 0.001$). The recovery from inactivation data were only fit adequately by third-order exponential decay functions in which all three time constants were decreased to describe the repriming of α-β_1 channels compared to α-only channels (see legend to Fig. 10 for fitting parameters).

DISCUSSION

Modification of Na channel function by the β_1 subunit has been established for brain and skeletal muscle Na channels expressed in oocytes (Krafte, Goldin, Auld, Dunn, Davidson, and Lester, 1990; Isom et al., 1992; Cannon et al., 1993). We have performed parallel sets of experiments on μ1 and hH1 Na channels that confirm the functional association of the β_1 subunit with the rat skeletal muscle α subunit and provide the first evidence that β_1 subunit RNA coinjected with cardiac α subunit RNA modifies the function of the oocyte-expressed Na channels.

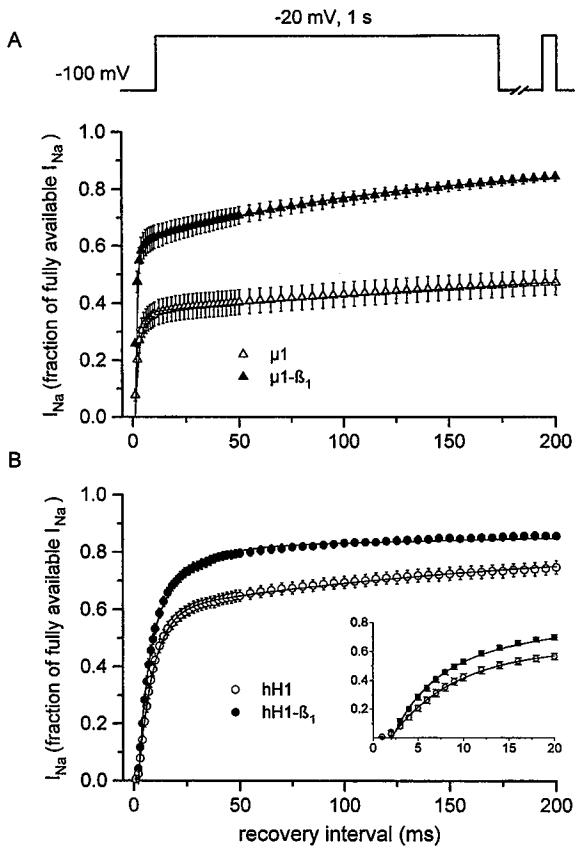

FIGURE 10. Recovery from inactivation of μ1 vs μ1-β_1 (*A*) and hH1 vs hH1-β_1 (*B*) Na currents. A standard two-pulse protocol was used to compare recovery from inactivation of α-only and α-β_1 channels at the holding potential of -100 mV as shown in the voltage pulse (*top*). Channels were inactivated (-20 mV, 1 s) and recovery measured at -20 mV (40 ms) after a variable recovery interval (see Results for details). The repriming data were best fit by a third order exponential decay function. The time constants were obtained by repetitive fitting sessions and then fixed while the amplitudes of each exponential were allowed to float for the final fitting. The fitting parameters for recovery of μ1 channels are: t1 = 2.1 ms, A1 = -0.36, t2 = 350 ms, A2 = -0.17, t3 = 3,000 ms, A3 = -0.47. Repriming of μ1-β_1 currents (*A*) was markedly hastened: t1 = 1 ms, A1 = -0.60, t2 = 100 ms, A2 = -0.23, t3 = 900 ms, A3 = -0.17. In contrast to μ1, hH1 α-only channels reprime rapidly (t1 = 7 ms, 1 = -0.59, t2 = 160 ms, A2 = -0.19, t3 = 1,800 ms, A3 = -0.21) although after an obvious deactivation period (1–2 ms, see *B, inset* at faster time base). Similar to its effects on repriming of μ1 channels, coinjection with β_1 further speeds recovery from inactivation of hH1 channels (*B*). The fitting parameters for hH1-β_1 repriming are: t1 = 4 ms, A1 = -0.45, t2 = 16 ms, A2 = -0.38, t3 = 900 ms, A3 = -0.17.

Modulation of μ1 Channels by β_1 Subunit

Early reports of functional expression of cloned mammalian Na channels by injection of α subunit RNA (from rat brain and skeletal muscle) into oocytes noted the anomalously slow inactivation of the macroscopic current (Trimmer et al., 1989; Noda, Ikeda, Suzuki, Takeshima, Takahashi, Kuno, and Numa, 1986). The functional properties of Na channels have been shown to be more like those of native channels when expressed by injection of unfractionated RNA (Krafte et al., 1988), by coinjection of α subunits with β_1 subunit δRNA (Cannon et al., 1993; Isom et al., 1992), or by transient transfection into mammalian cells such as HEK 293 cells (Ukomadu, Zhou, Sigworth, and Agnew, 1992) or CHO cells (Scheuer, Auld, Boyd, Offort, Dunn, and Catterall, 1990). The rate of current decay, the voltage depen-

dence of inactivation, and the recovery from inactivation in skeletal muscle and brain Na channels are directly modulated by the β_1 subunit (Cannon et al., 1993; Krafte et al., 1988). We coexpressed a β_1 subunit with μ1 Na channels in oocytes to confirm that these channel properties were modified by the functional association of the β_1 subunit. In our laboratory, coexpression of the rat brain β_1 subunit with the μ1 α subunit increased peak I_{Na} more than 225%, shifted the voltage dependence of inactivation by 6 mV in the hyperpolarizing direction, increased the rate of macroscopic current decay, reduced the use-dependent decrease of peak I_{Na} during repetitive short- (10 ms) and long- (1 s) duration voltage steps, and dramatically accelerated recovery from inactivation.

Both the functional properties of the macroscopic current and the underlying gating behavior of single channels are modified by coinjection (Krafte et al., 1988; Krafte et al., 1990; Zhou et al., 1991). Differences in distinct gating modes underlie the rapid inactivation of μ1 channels in HEK 293 cells compared with α subunits expressed in oocytes (Ukomadu et al., 1992). Mode 1 gating, characterized by brief openings and few reopenings, predominates in HEK 293 cells (Ukomadu et al., 1992) and in coinjected oocytes (Zhou et al., 1991), while mode 2 gating, characterized by multiple reopenings or bursting behavior, occurs more frequently in oocytes expressing the α subunit alone. Since both gating modes are encoded by the α subunit (Moorman, Kirsch, VanDongen, Joho, and Brown, 1990), the association of the β_1 subunit may alter the equilibrium between modes 1 and 2 towards mode 1 behavior (Zhou et al., 1991).

Expression of Cardiac Na Channels

Expression of mammalian cardiac Na channels in oocytes has not rigorously examined the requirement of the β_1 subunit to reproduce endogenous channel function (gating), yet the expressed channels are reported to possess the physiologic and pharmacologic properties of the native channels (Gellens et al., 1992; Chahine, Chen, Barchi, Kallen, and Horn, 1992; Satin et al., 1992; Krafte et al., 1991). Makita et al. (1994) found no evidence for a functional association between the hH1 α subunit and the β_1 subunit: the human β_1 subunit ($H\beta_{1A}$) cloned from heart and skeletal muscle did not alter the kinetics or the voltage dependence of inactivation of hH1 Na channels expressed in oocytes. We have examined the functional properties of hH1 Na channels expressed in oocytes with and without the β_1 subunit to establish whether β_1 modulates hH1 Na channel function. In line with our results in μ1 Na channels, coexpression of rat brain β_1 subunit with hH1 Na channels increased peak I_{Na} by 240%, accelerated the decay of macroscopic currents, and reduced use-dependent decay of I_{Na} during trains of voltage steps. Data collected from cell-attached patches confirmed that changes in the macroscopic inactivation kinetics, observed under two-electrode voltage clamp, were a genuine result of β_1 subunit association: times to 75% decay of hH1-β_1 ensemble average currents were shorter than for hH1. We have shown that coexpression with the β_1 subunit modifies inactivation kinetics, repriming and other functional properties of hH1 Na currents recorded in *Xenopus* oocytes. These results support a physiologically relevant functional association of β_1 subunit with the human cardiac Na channel α subunit.

Comparison of Primary Structure

The primary structure of the hH1 α subunit sequence is most similar (>90% primary structure identity) to rat heart (RH1) and to the equivalent isoform found in denervated rat skeletal muscle (rSkM2) (Gellens et al., 1992). However, the extent of structural homology between μ1 (rSkM1) and hH1 is much lower (59% amino acid sequence identity) (Gellens et al., 1992). The greatest homology resides within the membrane-spanning regions of hH1 and μ1, while the interdomain linkers I-II and II-III are divergent (<25% structural identity) (Gellens et al., 1992). Thus, it is not surprising that the electrophysiologic properties of μ1 and hH1 Na channels differ. Nevertheless, the functional effects of β_1 subunit coexpression are fundamentally conserved in the two isoforms, hinting that the region which associates with β_1 will prove to be at least partially homologous.

Although this study has provided functional evidence for interactions between the rat brain β_1 subunit and the human heart and rat skeletal muscle α subunits, the structural homology amongst the β_1 subunit isoforms is so high that similar results are anticipated with coinjection of the corresponding tissue- and species-specific β_1 subunits. A very high degree of structural homology among brain, heart and skeletal muscle β_1 subunits has been reported (Sutkowski and Catterall, 1990; Makita et al., 1994). Sutkowski and Catterall (1990) used antigen affinity chromatography to purify anti-β_1 subunit antibodies to rat brain β_1 and found that these antibodies also recognized β_1 in rat heart and skeletal muscle. Makita et al. (1994) reported that human heart and skeletal muscle β_1 cDNA nucleotide sequences are identical and possess 96% sequence identity to rat brain β_1 which has the identical cDNA sequence as rat heart β_1. In fact, the β_1 subunit has been mapped to a single gene (Tong, Potts, Rochelle, Seldin, and Agnew, 1993; Makita et al., 1994). There is also functional evidence that all β_1 subunits modulate Na channel properties in similar ways. Zhou et al. (1991) found that coinjection of β_1 RNA from either skeletal muscle or brain with μ1 RNA increased a fast component of macroscopic μ1 current by inducing alterations in modal gating behavior. Bennett, Makita, and George (1993) obtained similar results using rat heart β_1 to restore the normal gating behavior of the human skeletal muscle Na channel. These findings suggest that brain, heart, and skeletal muscle β_1 subunits associate with and modulate Na channel α subunit function in similar ways without demonstrating tissue specificity.

hH1-β_1 Channels More Closely Reproduce Endogenous Channel Function

A comparison of results obtained in this study to the functional properties reported for native heart channels indicate that coexpressed hH1 Na channels composed of α and β_1 subunits behave like cardiac channels in vivo. In isolated cardiac myocytes or Purkinje fiber cells, the half-inactivation potentials occur at −80 mV, nearly the same as hH1-β_1 in oocytes (Satin et al., 1992; Lee, Matsuda, Reynertson, Martins, and Shibata, 1993), or at more hyperpolarized potentials (Jia, Furukawa, Singer, Sakakibara, Eager, Backer, Arentzen, and Wasserstrom, 1993; Makielski, Sheets, Hanck, January, and Fozzard, 1987). The decay of whole-cell Na currents recorded in cardiac cells is extremely fast: when fit as a biexponential process, native I_{Na} decays with a fast time constant of inactivation of ~2 ms at −40 mV, which decreases

to <1 ms at more positive potentials (Satin et al., 1992; Brown, Lee, and Powell, 1981; Makielski et al., 1987). Coexpressed hH1-β_1 channels produce macroscopic and ensemble average currents that more closely reproduce such rapid inactivation kinetics than do channels expressed from the hH1 α subunit alone. Native heart Na channels demonstrate no use-dependent decay of peak current even at stimulation rates of 1–4 Hz (Bean, Cohen, and Tsien, 1983; Lee et al., 1993; Jia et al., 1993). Although repetitive pulsing (0.5 Hz) in oocytes produced only a small decrement of hH1 current, hH1-β_1 currents were even more resistant to such use-dependent effects. At hyperpolarized potentials, recovery from inactivation in native heart Na channels can be described by two exponential terms, with the larger portion of recovery occurring with a fast time constant of 3–6 ms (Jia et al., 1993; Brown et al., 1981). Recovery from inactivation by hH1-β_1 channels is similar to that in native channels: the amplitude of the fast component is largest, with a time constant equal to 4 ms (Fig. 10 *B*) compared to 7 ms for hH1 channels. Thus, many important features of endogenous cardiac Na channel gating, including rapid macroscopic current decay, the virtual absence of use-dependent decay of the peak current, and rapid recovery from inactivation, are more closely reproduced by hH1 α-β_1 than hH1 α-only Na channels.

Coexpression of β_1 Affects Gating but Not Permeation

Permeation in μ1 and hH1 channels is not altered by coexpression with the β_1 subunit: there were no significant differences in the unitary current amplitudes or the slope conductances of the coexpressed channels vs those encoded by α only. The whole-cell experiments reveal that the primary effect of the β_1 subunit association with hH1 and μ1 channels involves inactivation gating: β_1 speeds macroscopic current inactivation and increases the rate of recovery from inactivation. The apparent explanation for the enhancement of macroscopic inactivation is that β_1 modifies entry into and return from the inactivated state. Within the limitations of the data collected from cell-attached patches in this study (no one-channel patches), hH1-β_1 channels had shorter mean open times (0.89 ± 0.07 ms at −40 mV, $n = 6$) compared to hH1 channels (1.10 ± 0.10 ms, $n = 6$), and fewer reopenings. Both of these changes in single-channel gating would tend to hasten macroscopic current decay.

Given the lack of changes in permeation, there are two potential mechanisms by which coexpression with the β_1 subunit increases hH1 macroscopic current density: an increase in the number of functional channels, and/or an increase in the unitary open probability. We were able to estimate the maximal open probability of hH1 and hH1-β_1 channels from the single-channel data obtained in this study. The single-channel probability of opening is $P_o = I/Ni$ where I is the size of the peak ensemble average current, N is the number of active channels contained in the patch, and i is the unitary current amplitude. Measurements of P_o at two different potentials (−40 mV, −20 mV) indicate that coexpression with the β_1 subunit does not significantly increase hH1 channel open probability. At −40 mV, P_o equaled 0.22 ± 0.05 in hH1-β_1 channels ($n = 4$) and 0.21 ± 0.04 in hH1 channels ($n = 5$). While P_o tended to increase at −20 mV in hH1-β_1 channels (0.36 ± 0.08, $n = 4$) compared to hH1 channels (0.26 ± 0.06, $n = 3$), the effect did not reach statistical signifi-

cance by one-way ANOVA. Even if genuine, these modest increases in open probability do not come close to accounting for the 2.4-fold increase in macroscopic current in hH1-β_1 oocytes relative to those expressing the α subunit alone. Thus, the predominant effect is an increase in the number of functional channels.

It is instructive to compare the mechanism of the increased expression of hH1 Na current by β_1 subunit interactions to analogous results in Ca channels. Neely, Wei, Olcese, Birnbaumer, and Stefani (1993) reported that coexpression of the β subunit with the α_{1C} subunit does not increase the expression of Ca channels: measurements of gating currents and estimates of channel densities were not significantly different between coinjected oocytes and oocytes injected with the α_{1C} subunit alone. A different picture emerges from studies in mammalian HEK293 cells, where coexpression of α_{1C} and β increases the densities of Ca current and dihydropyridine receptors (Pérez-García, Kamp, and Marbán, 1995) while augmenting the gating currents (Kamp, Pérez-García, and Marbán, 1995). The latter results closely parallel those reported here, suggesting that α subunits may generally facilitate the packaging and assembly of functional ion channels.

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