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Skeletal Muscle Sodium Channel Is Affected by an Epileptogenic β1 Subunit Mutation

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The syndrome of generalized epilepsy with febrile seizures plus type 1 (GEFS+) has been associated to the gene SCN1B coding for the sodium channel β1 subunit (Wallace, R. H. et al. (1998) *Nature Genetics* 19, 366–370). In patients, a mutation of the cysteine 121 to trpyptophane (C121W) would cause a lack of modulatory activity of the β1 subunit on sodium channels expressed in the brain, rendering neurons hyperexcitable. We have confirmed that the normal β1-modulation of type-IIA adult brain α subunits (BIIA) expressed in frog oocytes is defective in C121W. We observed that the mixture of wild-type and mutant β1 subunits is less effective than wild-type alone, suggesting that the mutant β1 subunit does bind the α subunit. However, we also observed a similar lack of modulation by C121W of the in adult skeletal muscle α subunit (SkM1). This finding is in contrast with the simple idea that the mutational effect observed in the oocyte expression system is the principal physiopathological correlate of GEFS+, because no skeletal muscle symptoms have been reported in GEFS+ patients. We conclude that the manifestation of the pathological phenotype is conditioned by the presence of susceptibility genes and/or that the frog oocyte expression system is inadequate for the study of the mutant β1 subunit physiopathology. © 2001 Academic Press

Key Words: **sodium channel; β1 subunit; GEFS+; epilepsy; frog oocytes.**

The sodium channel β1 subunit is a membrane integral protein that copurifies with the pore-forming α subunit. From its molecular identification (1), the sodium channel β1 subunit has been proposed to modulate the sodium channel inactivation when it is heterologously expressed with the adult skeletal muscle (SkM1) or brain (BIA, BIIA, and BIIIA) α subunits in *Xenopus* oocytes (for review see (2–4)).

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A pathological condition in humans, generalized epilepsy with febrile seizures plus type 1 (GEFS+; OMIM # 604236), an autosomal dominant hereditary disorder, is directly associated with a β1 subunit (5). The defect consists to a C-to-G transversion of nucleotide 387 of the SCN1B gene, predicted to resulting in a Cys to Trp substitution in residue 120. The change of the conserved Cys 121 most likely disrupts a putative disulfide bridge that normally maintains an extracellular immunoglobulin-like fold of hr β1 polypeptide (6). Coexpression of the C121W with the BIIA channel α subunit in *Xenopus laevis* oocytes demonstrated that a lack of the normal ability of the β1 subunit to modulate channel-gating kinetics, consistent with a loss-of-function allele (5). From these results, it has been concluded that the observed functional changes associated with the C121W mutation may cause persistent inward sodium currents in neurons resulting in a more depolarized membrane potential and hyperexcitability (for review see (7–10)).

GEFS+ patients do not exhibit cardiac or skeletal muscle symptoms (5). Since the same gene is known to code for β1 subunit that is also assembling heart and skeletal muscle sodium channels (11), we expected the tissue-specificity of GEFS+ pathology to be correlated with a differential effect of C121W β1-mutation on the β1 interaction with the brain or the skeletal muscle α isoforms. Therefore, we have analyzed the effect of the C121W mutation on the β1-modification of the inactivation of adult skeletal muscle sodium channels expressed in frog oocytes. Surprisingly, we demonstrate that, in this preparation, the negative effect of the C121W mutation is similar in brain and skeletal muscle channels.

METHODS

Mutagenesis sodium channel expression. Site-directed mutagenesis of the cDNA of rat sodium channel β1 subunit (psPNa-beta, generous gift from K. Imoto) was accomplished using the Pfu DNA polymerase using the QuikChange kit (Stratagene). The mutation was verified by sequencing 400 bp's in the flanking region near the mutagenic point (M-Medical, Florence).

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Plasmids containing the full-length cDNA coding for the adult rat skeletal muscle (rSkM1) (12) and the rat brain type-II (rBIIA) (13) sodium channel α-subunits were linearised with *Nhe*I and *Sal*I, respectively. Plasmids containing the cDNA coding for the wild-type (WT) and mutant (C121W) β1 were linearised with *Eco*RI. Linearised plasmids were transcribed *in vitro* with T7 polymerase using the capped mMessage mMachine kit (Ambion).

Oocytes were surgically extracted from anaesthetized *Xenopus laevis* frogs, and isolated enzymatically by treatment with collagenase-A (Sigma). Stage IV and V oocytes were injected with 50 nl cRNA solutions in DEPC-treated water. α-cRNA (rSkM1 or rBIIA) contained 125 ng/μl of cRNA. β1-cRNA (WT or C121W) contained 150 μg/μl of cRNA, or two to fourfold dilutions of the later for β1 dose-response measurements. For WT-C121W competition measurements, both WT and C121W β1-cRNA were coinjected to the highest concentration (150 ng/μl). Injected oocytes were incubated for 5 to 7 days in Barth's solution at 18°C.

Electrophysiological recording. Sodium currents were measured from the whole oocyte using a two-electrode voltage clamp designed and constructed in our laboratory. Borosilicate glass micropipettes (Hilgemberg) were filled with 3 M KCl and had a resistance of 0.2 to 0.6 MΩ. The oocyte bathing solution (normal frog Ringer) had the following composition (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8, HEPES 10, pH = 7.4. In some cases, when the expression of sodium currents was too high (>20 μA), compromising the accuracy of the voltage-clamp, despite a large percentage of series resistance compensation, the currents were reduced by substituting part of the extracellular Na^+ by K^+, to a final NaCl concentration of 30 mM. No differences in the inactivation properties were detected in low Na^+ solutions. The output of the voltage-clamp amplifier was filtered by a low-pass 4-pole Bessel filter with a cut-off frequency of 5 kHz and sampled at 10 kHz. The oocytes were normally kept at a holding membrane potential of −100 mV, Pulse stimulation and data acquisition used 16 bit D-A and A-D converters (ITC-16, Instrutech), and were controlled by a Macintosh microcomputer with the Pulse software (Heka Electronik). Linear responses were estimated from sub-threshold stimulations, partially compensated analogically and digitally subtracted with a standard P/4 protocol. All measurements were done at a controlled temperature of 15 ± 0.5°C.

Data analysis. Data were analyzed with a Macintosh microcomputer using custom software developed in the IgorPro program (Wavemetrics). Statistical comparisons were done with a Student's *t*-test, and statistical significance was defined by $P < 0.05$. The results were expressed as mean ± sem (number of measures). A simplex method was used for curve fitting. Data for each α and β1 subunit combination were obtained from 3 to 6 different batches of oocytes.

RESULTS

Following a step depolarization from a holding potential, $V_h = -100$ mV, the sodium current mediated by the expression of α-rSkM1 or α-rBIIA alone shows a fast activation and a biphasic inactivation, as illustrated in Figs. 1A and 2A. The falling phase of the current is well fitted by a double-exponential function as $I(t) = a_1\exp(-t/\tau_1) + a_2\exp(-t/\tau_2)$, with fast and slow time constants, τ_1 and τ_2, differing by about one order magnitude, and with a ratio of the two amplitudes a_1 and a_2 fairly independent of the depolarizing voltage (14, 15). It has been proposed that the two components are due to the separate contribution of a mixed population of channels that at the time of the stimulus are either in a fast or in a slower mode of inactivation.

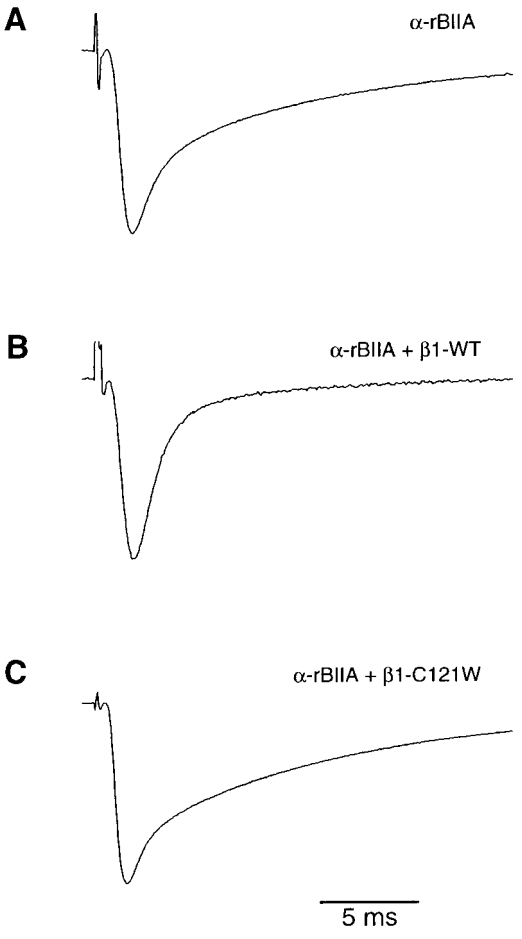

FIG. 1. Adult rat brain sodium channels (rBIIA) expressed in frog oocytes. The rBIIA α subunit alone (A) has a slow component in the inactivation phase, which is strongly reduced by the coexpression of WT β1 subunit (B). Differently, coexpression of C121W β1 subunit does not reduce the slow inactivation component (C). Traces were obtained from two-electrode voltage-clamp experiments, and currents were evoked by a −10 mV pulse from a holding potential of −100 mV.

Single channel recordings have shown that in the fast inactivating mode, which we shall call M1, the channels open once and briefly during the depolarizing stimulus, while in the slower mode, called M2, channels have late reopenings during the sweep (16–19).

To quantify the propensity of the channels to gate in either mode, we defined the parameter $P_{M2} = [a_2/(a_1 + a_2)] \times 100$, as a measure of the probability of finding a channel in mode M2 during any test stimulus. P_{M2} was measured from the double exponential fit of the decaying phase of the sodium current evoked by 30 to 50 ms depolarization to voltages between −20 and 0 mV. We previously reported that the estimates of P_{M2} are fairly independent of the test voltage (14, 15).

In the series of experiments reported here the expression of α-rBIIA cRNA yielded sodium currents with $P_{M2} = 16.7 \pm 2.8$ ($n = 30$) (Fig. 1A). As previously reported (1, 3, 6, 20, 21), the propensity of the channels

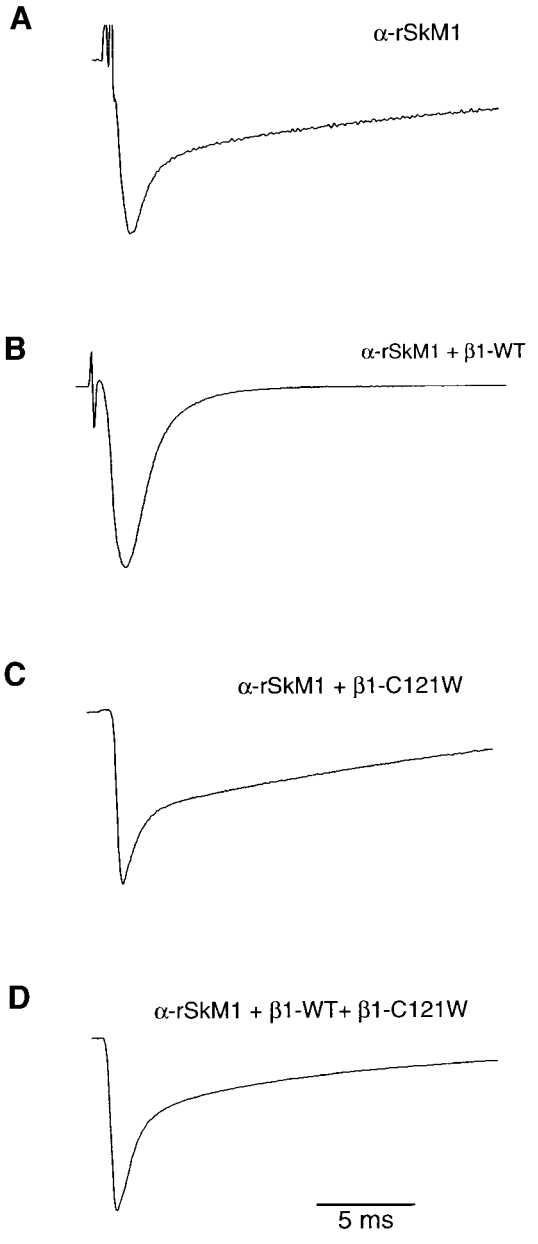

FIG. 2. Adult rat skeletal muscle sodium channels (rSkM1) expressed in frog oocytes. The rSkM1 α subunit alone (A) has a slow component in the inactivation phase, which is strongly reduced by the coexpression of WT β1 subunit (B). Differently, coexpression of the C121W β1 subunit does not reduce the slow inactivation component (C). The coexpression of the rSkM1 α subunits with a mixture of WT and C121W β1 subunits produce an intermediate phenotype, where the slow component is only partially reduced. All traces were obtained from two-electrode voltage-clamp experiments, and currents were evoked by a −10 mV pulse from a holding potential of −100 mV.

for the mode M2 is strongly reduced ($P < 0.0002$) in oocytes coinjected with WT β1-cRNA (Fig. 1B), resulting in a $P_{M2} = 4.8 \pm 2.5$ ($n = 27$). The dependence of P_{M2} on the amount of coinjected WT β1-cRNA is shown in Fig. 3A (filled symbols). It is seen that P_{M2} decreases for increasing amounts of coinjected β1-cRNA, approaching a plateau at the maximum concentration of β1-cRNA that have used, corresponding to a molar ratio of β1:α cRNA larger than 10:1.

Similarly, injection of α-rSkM1 cRNA also expresses sodium currents with a relatively high propensity for mode M2 ($P_{M2} = 11.8 \pm 1.3$; $n = 53$), that is significantly reduced ($P < 0.0001$) by the coexpression of 150 ng/μl of WT β1-cRNA (20, 22, 23) (Fig. 2B). As shown in Fig. 3B (filled symbols), the effect of the WT β1 subunit on the modal inactivation of α-rSkM1 also depends on the cRNA concentration (22), and also in this case the injection of 50 nl of β1-cRNA 150 ng/μl appears to produce a near maximal depression of mode M2 ($P_{M2} = 4.8 \pm 0.9$; $n = 27$).

As reported by others (5), we also observed that the C121W mutation of β1 apparently fails to modulate the modal inactivation of the α-rBIIA channels (Fig. 1C), yielding a $P_{M2} = 22.5 \pm 2.5$ ($n = 38$), that is not statistically different ($P > 0.12$) from control levels without the β1 subunit (Fig. 3A, open symbols). This

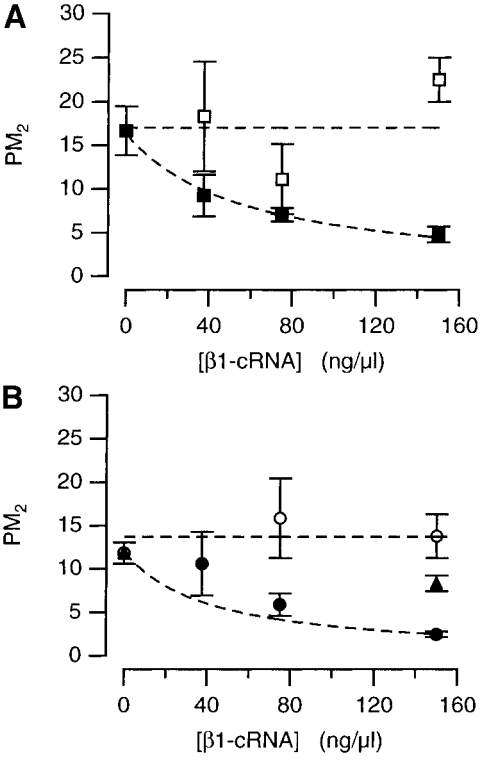

FIG. 3. The propensity of the sodium current to inactivate in the slow mode, P_{M2}, is plotted as a function of the cRNA concentration coding for the β1 subunit injected in the oocyte. It is shown by the effect of coexpression of WT (filled symbols) and C121W β1 subunit (empty symbols) on the currents expressed by rBIIA (A) and rSkM1 (B) α subunits. The broken lines are empirical interpolations of data with PM2 = A + [B/(1 + [cRNA]/k)], for clarity of the presentation. The triangle in panel B represents data obtained from the coexpression of the rSkM1 α subunit with a mixture of WT and C121W β1 subunit, both injected as 150 ng/μl cRNA. Data represent means and bars are sem.

result is consistent with the simple interpretation of the physiopathological effects of the GEFS+ mutation as due to lack of inhibition of the M2 mode of the brain isoforms of the sodium channel α subunit. Quite surprisingly, however, we discovered that the C121W mutation loses also the ability to inhibit the M2 mode of the muscle isoform α-rSkM1 (Fig. 2C). The expression of C121W with the later phenotype shows a high value of $P_{M2} = 13.8 \pm 2.5$ ($n = 27$), that is also not statistically different ($P > 0.45$) from controls (see Fig. 3B, open symbols).

The lack of modulation of C121W-β1 for both isoforms does not seem to result from a reduced interaction, since we see no tendency of the reduction of P_{M2} to decrease with in increasing amounts of C121W-cRNA injection (Figs. 3A and 3B, empty symbols). One possibility for explaining the lack of modulatory effect of the C121W β1 subunit is that this polypeptide is not able to assemble with the α subunit in general, or in the frog oocyte expression system in particular. To test this possibility, we coinjected oocytes with 150 ng/μl of WT β1-cRNA and 150 ng/μl of C121W β1-cRNA together with the α-rSkM1 cRNA. We found that the expressed mixture of WT and mutant β1 subunits produced an intermediate phenotype of sodium currents (Fig. 2D), yielding a $P_{M2} = 8.2 \pm 0.9$ ($n = 20$). This indicates that the C121W β1 polypeptide is indeed expressed and antagonizes the correct assembly of the WT β1 subunit with α subunit (6).

DISCUSSION

The association of GEFS+ type 1 syndrome with a sodium channel subunit (5) is very noteworthy because it represents the first case of a putative sodium channel genetic defect affecting the central nervous system (CNS). Several reviews have the GEFS+ mutation as a prototype of genetic CNS disorders produced by a primary sodium channel dysfunction (7–10). Several mutations of the sodium channel α subunits in skeletal muscle and heart, liked to muscular and cardiac diseases, had been identified in the last 12 years (24, 25), and all of them show an impaired inactivation as a primary functional defect. Even if the issue is still controversial, it is generally accepted that the functional role of the β1 subunit in the sodium channel complex is the modulation of inactivation (1, 2, 4). Therefore, following the original report of a lack of modulatory effect of the C121W mutant of β1 in the frog oocyte preparation, it was natural to consider theoretically such defective modulation as the primary cause of the neuronal hyperexcitability that is likely accompanying the epileptic attacks of GEFS+ patients (5).

The clinical report of the GEFS+ type 1 syndrome indicates that patients do not show any skeletal muscle or cardiac symptoms (5), despite the fact that the same β1 subunit is expressed also in skeletal and cardiac muscle (11) and likely functionally assembled with the specific α subunit isoforms expressed in these tissues (2–4). The simplest explanation for the apparently nondefective function of heart and skeletal muscle sodium channels could be a specificity of the mutated β1 to associate with the brain isoforms of the sodium channel α subunits. However, our present data show that the C121W mutation has a negative modulatory effect also on the α-SkM1 expressed in oocytes, and that probably the defective polypeptide competes with the normal β1 for the association with the α subunit, and this is in contradiction with the proposed isoform-specificity of the C121W β1 subunit.

There is a contradictory body of information on the functional role of the sodium channel β1 subunit in mammalian cells. It has been demonstrated that the transfection of α subunits alone in mammalian cells does not express channels with large P_{M2} values like as it does in oocytes (26–35), and that the coexpression of β1 subunit does not alter substantially the channels modal properties (26, 27, 34, 36). This rises the question of whether the observations reported from frog oocyte experiments are indeed useful to understand the sodium channel physiology or rather an epiphenomenon occurring in these particular cells. In the large family affected by GEFS+ type 1 the mutation produces distinct types of seizure in different members of the same family (5). These differences probably result from inheritance of the mutant gene in the context of other susceptibility genes (8), that may express only in the CNS, and confer functional role for the β1 subunit. One proposed possibility is that the β1 serves as communication link between intracellular and extracellular environments, as suggested by its interaction with extracellular matrix proteins (37) and cytoskeleton intermediate filaments (38).

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