Macroscopic Properties of Spontaneous Mutations in Slow-Channel Syndrome: Correlation by Domain and Disease Severity

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KEY WORDS AChR; Ca^{2+} permeability; channelopathy; desensitization; neuromuscular; nicotinic

ABSTRACT The slow-channel syndrome (SCS) is a neuromuscular disorder characterized by fatigability, progressive weakness, and degeneration of the neuromuscular junction. The SCS is caused by missense mutations in the four subunits of the skeletal muscle acetylcholine receptor (AChR), which leads to altered channel gating, prolonged neuromuscular postsynaptic currents, and impaired neuromuscular transmission. Although a diverse set of mutations in different functional domains of the AChR appear to be associated with symptoms of widely ranging severity, there is as yet no mutant channel property or combination that explains the variations in disease severity. By observing the recovery time of AChR from desensitization, the authors determined that this process is significantly enhanced in SCS channels. In addition, as expected, the authors found that SCS macroscopic decay currents in transfected HEK293 cells are slower than wild type currents. While slight differences in relative Ca^{2+} permeability between some SCS mutations were identified, they did not correlate with apparent disease severity. These results suggest that of the different AChR kinetic features studied, only recovery from desensitization and slow postsynaptic currents correlate with the severity observed in the different mutations of this syndrome. **Synapse 60:441–449, 2006.**

INTRODUCTION

Nicotinic acetylcholine receptors (AChRs) are ligand-gated ion channels present in both the nervous system and muscle. These receptors play a major role in fast synaptic transmission in the CNS and at the neuromuscular junction (NMJ). In the adult NMJ, AChRs are composed of four homologous subunits: 2α, 1β, 1δ, and 1ε, each of which has four transmembrane domains. Several genetic disorders resulting from mutations in these ion channel proteins impair synaptic transmission and lead to neurological or neuromuscular disease (Bertrand et al., 1998; Engel et al., 1999). The slow-channel syndrome (SCS) is a neuromuscular disorder characterized by fatigability, progressive weakness, and degeneration of the NMJ. The SCS is caused by missense mutations in the four subunits of the skeletal muscle AChR (Fig. 1). SCS mutations alter AChR gating, prolong neuromuscular synaptic currents, and impair neuromuscular transmission (Croxen et al., 1997, 2002; Engel et al., 1996; Gomez et al., 1996a,b, 2002b; Milone et al., 1997; Ohno et al., 1995b; Sine et al., 1995). In all, 15 mutations have been identified. Most SCS mutations occur in the pore-forming second transmembrane domain (M2), only three mutations have been found in the first transmembrane domain (M1), and two in the ligand binding domain (Croxen et al., 1997; Engel et al., 1996; Wang et al., 1997).

Single-channel kinetics studies have determined that the mutations in SCS change the gating properties of the channel, leading to increased duration of channel bursts. These studies have demonstrated that this change is due to several possible effects on gating

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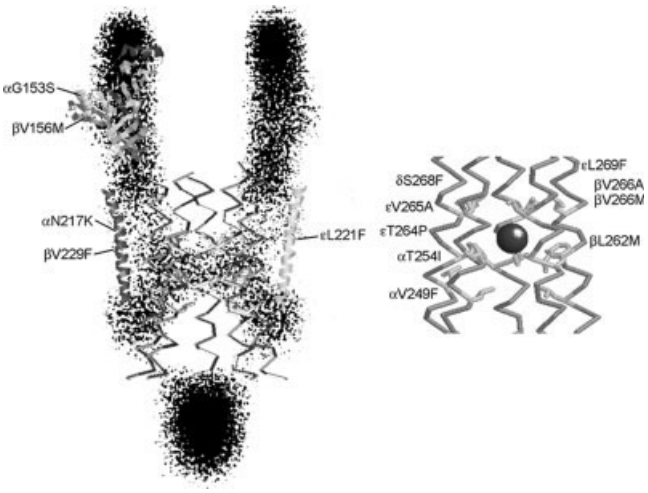

Fig. 1. Schematic diagram of slow-channel congenital myasthenic syndrome mutations explored in this study.

mechanisms, mainly resulting from a decrease in the probability of closure of the biliganded channel. This diverse set of mutations in different functional domains of the AChR is associated with symptoms of widely ranging severity. However, a broad panel of different SCS mutations has never been studied systematically to ascertain whether any single property or combination of properties can account for variations in severity. While the sophisticated methods and models used to characterize the mutations have provided useful data to gain insight into structural and functional aspects of the AChR molecule, such kinetic analyses are sometimes difficult to extrapolate to the macroscopic level to explain synaptic events. Because of the ensemble of events and frequency of activation of AChR in the NMJ, single-channel studies may not be a reliable method for determining the effect of these AChR channel mutations on synaptic transmission.

Using whole-cell patch-clamp technique, we studied the macroscopic properties of mutant and wild-type AChRs expressed in HEK293 cells. We characterized calcium permeability, desensitization rate, and channel open time. Evidence has suggested that variation in several of these parameters can profoundly alter AChR function. For example, mutations in the neuronal nicotinic AChR in nocturnal frontal lobe epilepsy lead to a decrease in Ca^{2+} permeability and overactive presynaptic terminals in inhibitory neurons (Kuryatov et al., 1997). Also in both the congenital slow- and fast-channel syndromes changes in the rates of desensitization are believed to be partially responsible for both impaired neuromuscular transmission and progressive muscle atrophy (Bhattacharyya et al., 1997; Milone et al., 1997; Sine et al., 1995). Finally, differences in the decay time of macroscopic postsynaptic currents observed in this syndrome may explain in part the different levels of disease severity in SCS, presumably by through different degrees of cation overload.

In this study, we combined whole-cell patch clamp and fast-step ligand perfusion techniques to characterize for the first time a panel of AChR mutants associated with the SCS. We found, as expected, that SCS AChR macroscopic decay currents are significantly slower than WT. Moreover, we found that a significant number of SCS mutations desensitize faster than WT receptors. Finally, using ion substitution experiments, we showed that Ca^{2+} permeability does not, with one exception, vary significantly for most SCS mutants. These results improve the understanding of the manner in which the different SCS AChR mutations can alter different kinetic parameters of these receptors in a macroscopic setting. They suggest that no single change in channel property provides direct correlation with disease severity. Rather, the impairment in neuromuscular transmission and the different phenotypes observed in this syndrome are most likely related to a combination of effects on channel behavior ranging from enhanced desensitization to prolonged macroscopic postsynaptic currents.

MATERIALS AND METHODS

Cell culture and transfection

The SCS mutations were generated in mouse nicotinic AChR muscle subunits cDNAs cloned into the vector pcDNA/3 using site-directed mutagenesis (Quick-change, Strategene, La Jolla, CA). The mutation was confirmed by dideoxy nucleotide sequence analysis of the entire cDNA. For reversal potential studies, human embryonic kidney 293 (HEK293, Gibco BRL, Carlsbad, CA) cells were maintained in culture at 37°C, 5% CO_2 in DMEM containing 10% FBS, 0.05 mg/ml Gentamicin (Gibco BRL, Carlsbad, CA). Cells were transfected at ~50% confluency using the Effectene transfection reagent (Quiagen, Valencia, CA). For a 25-ml flask, a total amount of 1 μg of DNA was used, the transfection mix was composed of a ratio of 2α:β:ε:δ subunits. Cells expressing mature receptors were selected 36–48 h after transfection by using magnetic beads (Dynal Biotech, Lake Success, NY) coated with the mAb35 antibody (Tzartos and Lindstrom, 1980). Cells were replated into 60-mm petri dishes and recordings were performed 18–24 h after replating.

Calcium permeability

Recordings from HEK293 cells were obtained in whole cell configuration with an Axoclamp 200B amplifier and digitized through a Digidata 1320A (Axon Instruments, Sunnyvale, CA) at 22–23°C. The resistance of the patch electrodes was between 2–4 MΩ. The electrode solution was composed of 145 mM CsCl, 10 mM Hepes, and 5 mM 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetate (pH 7.3). Recordings

were initiated in extracellular solution containing 150 mM CsCl, 1 mM $CaCl_2$, and 10 mM HEPES (pH 7.3). To determine the reversal potential of the AChRs voltage ramp protocols were applied while a fast step perfusion system (Warner Instruments, Hamden, CT) applied an extracellular solution containing 100 μM ACh. This protocol was succeeded by repeating the ramp protocol with a solution containing 100 μM ACh. The data were acquired using pClamp 8 software (Axon Instruments) in a Pentium III personal computer. The value of the reversal potential was determined for the different mutations at 1 and 10 mM $CaCl_2$. The calcium permeability was determined by using an extended GHK equation used to calculate the relative ion permeabilities with respect to Cs^+ (Castro and Albuquerque, 1995):

$$\frac{P_{Ca}}{P_{Cs}} = \frac{[Cs^+]_O(1 - e^{\Delta V_{rev}F/RT})}{4[Ca^{2+}{}_1]_O e^{\Delta V_{rev}F/RT}(1 + e^{V_{rev1}F/RT})^{-1} - 4[Ca^{2+}{}_{10}]_O(1 + e^{V_{rev10}F/RT})^{-1}}$$

Permeability calculations were made using the Mathematica 4.1 (Wolfram Research, Champaign, IL) software. Statistical analyses of these results were made using the StatView (SAS institute Inc, Cary, NC) software package.

Desensitization

HEK293 cells were patch clamped in whole cell configuration in an extracellular solution composed of 37 mM NaCl, 2 mM $CaCl_2$, 10 mM HEPES, 1 mM $MgCl_2$, and 10 mM glucose (pH 7.4) and pipette solution composed of 110 mM CsCl, 5 mM MgATP, 14 mM EGTA, 20 mM HEPES, and 20 mM triethylamine (pH 7.2). Afterwards, cells were stimulated five times for 500 ms every second with 100 μM ACh. The percentage decrease between the first depolarization and last from cells that showed similar amplitudes to the ones observed in cells expressing WT receptors (~10 nA) was calculated. In addition, only cells bound to 3–5 mAb35 coated beads were used.

Decay currents

Cells were clamped using the extracellular and pipette solutions used in the desensitization experiments. Nondesensitizing concentrations of ACh (1 μM) were applied with the fast step perfusion system while applying the voltage-jump protocol showed in Figure 2. To calculate the decay time, a second-term exponential approximation was calculated by pClamp 8 software package (Axon Instruments).

RESULTS

Slower macroscopic nicotinic decay currents contribute to the SCS

In the intact NMJ, the mean time that two ACh molecules remain bound to the postsynaptic nicotinic receptors determines the decay time constant of the postsynaptic AChR, because diffusion and hydrolysis by acetylcholinesterase removes free ACh from the synaptic cleft rapidly (Anderson and Stevens, 1973; Katz and Miledi, 1973; Magleby and Stevens, 1972). To roughly estimate the current decay times of the SCS mutants, we expressed the receptors in HEK293 cells and activated them with a relatively low concentration of acetylcholine (1 μM). To obtain these decay currents, we used a protocol based on voltage-jump relaxation studies, in which, during continuous AChR activation, the cell receives a brief depolarizing stimulus followed by negative hyperpolarization (Figl et al., 1996). The second-term exponential approximation obtained from the decay phase of these currents τ is comparable with the burst duration recorded at the single-channel level (Auerbach et al., 1983; Figl et al., 1996). Although we found difficult to correlate the τ of these currents with previously reported values for the duration of single channel bursts, this technique provided us with a rough estimate for the duration of macroscopic currents in a nondesensitizing environment. Under these conditions, macroscopic decay currents in HEK293 cells should resemble those of miniature endplate currents.

First, we looked at the SCS ligand binding site mutations αG153S and αV156M. We found that both αG153S (708.3 ± 47.6 ms, $n = 10$) and αV156M (1079.6 ± 92.2 ms, $n = 10$) lead to a large increase of the decay time of the acetylcholine induced currents (Fig. 2) when compared with WT (352.5 ± 7.2 ms, $n = 10$). In addition, we studied the decay currents of mutations in both the M1 and M2 domains of the AChRs. Our data demonstrate that, except for the δS268F and αT254I mutations, SCS mutations significantly increase the decay time (βV229F 564.1 ± 20.0 ms, $n = 10$; αT254I 325.6 ± 23.0 ms, $n = 10$; βL262M 830.0 ± 30.0 ms, $n = 10$; εT264P 951.1 ± 95.6 ms, $n = 10$; βV266A 498.2 ± 20.0 ms, $n = 10$; δS268F 327.6 ± 15.0 ms, $n = 10$; and εL269F 840.1 ± 20.2 ms, $n = 10$). These findings confirm that the burst activity of mutated SCS AChR have a significant impact on the decay phase of macroscopic ACh induced currents.

M2 domain mutations desensitize when subject to fast acetylcholine stimulation

To characterize the sustained responses of mutant AChRs macroscopically, we devised an approach that employs brief pulses of ACh to HEK293 cells expressing mutant AChRs. We systematically stimulated HEK293 cells expressing each of 12 distinct SCS mutations with 500 ms pulses of 100 μM acetylcholine and determined the changes in the amplitude of peak currents between

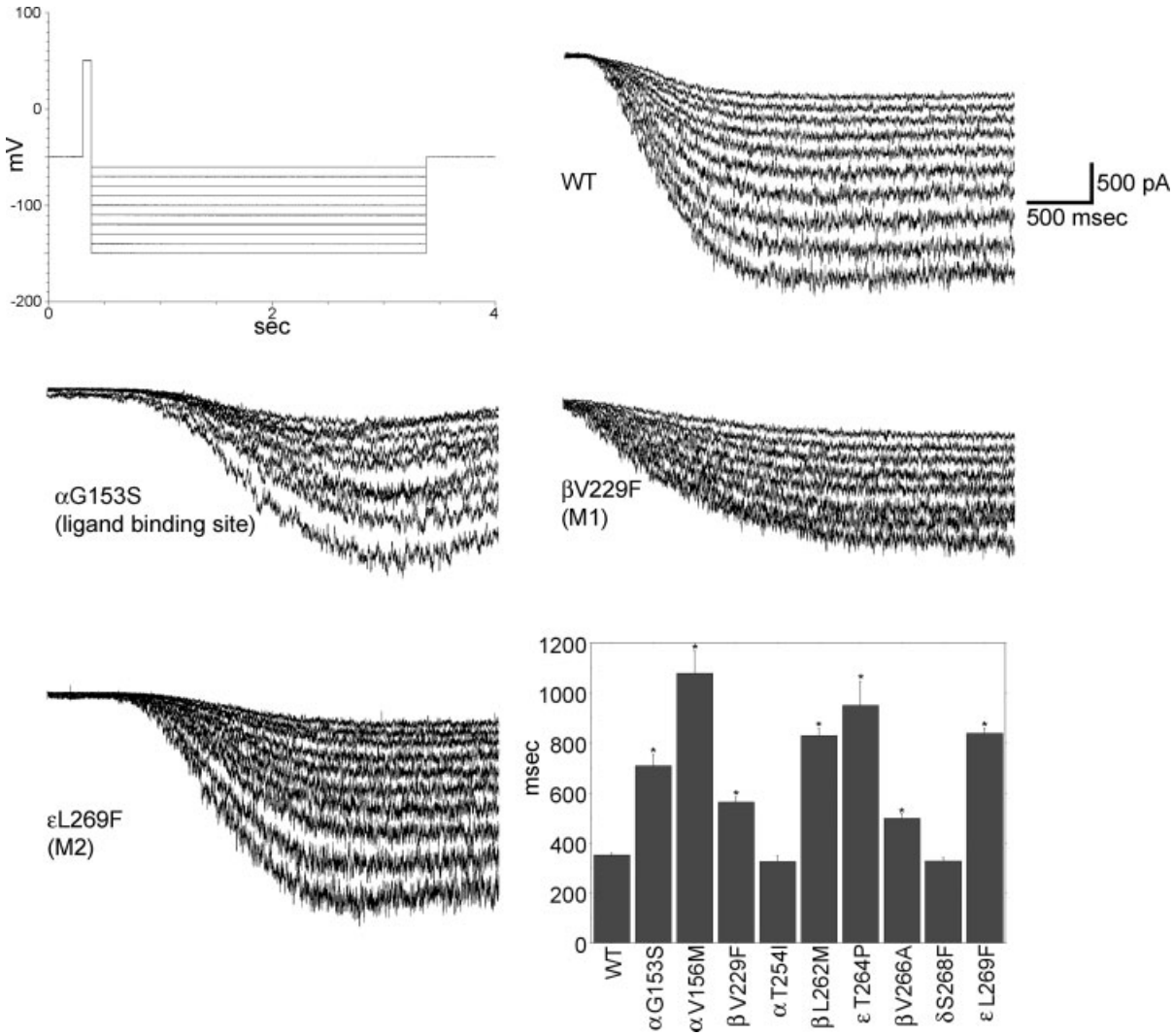

Fig. 2. Macroscopic decay currents of slow-channel syndrome (SCS) are significantly slowed by most SCS mutations. Decay current of human embryonic kidney cells (HEK293) expressing acetylcholine (1 μM) activated AChR were studied by applying a brief hyperpolarizing stimulus (50 mV) followed by a series of voltage steps. All of the ligand binding site and first transmembrane domain (M1) mutations exhibit long decay times (αG153S 708.3 ± 47.6 ms, αV156M 1079.6 ± 92.2 ms, βV229F 564.1 ± 20.0 ms, n = 10) when compared with WT receptors (352.5 ± 7.2 ms, n = 10). All but two of the pore-forming second transmembrane domain (M2) mutations have slowed decay currents (βL262M 830.0 ± 30.0 ms, εT264P 951.1 ± 95.6 ms, βV266A 498.2.7 ± 20.0 ms, εL269F 840.1 ± 20.2 ms, n = 10), although αT254I and δS268F displayed currents that were not significantly different from the ones observed in normal receptors (αT254I 325.6 ± 23.0 ms, and δS268F 327.6 ± 15.0 ms, n = 10).

the first and last responses (Elenes and Auerbach, 2002). Several of the M2 domain mutations exhibited macroscopic desensitization when compared with WT (35% ± 3%, n = 4). The results obtained from cells expressing αV249F (94% ± 1%, n = 4) agree qualitatively with studies cited previously (Milone et al., 1997). In addition, the M2 mutations (αT254I 85% ± 2%, n = 3; βL262M 86% ± 3%, n = 3; βV266A 87% ± 1%, n = 4; δS268F 50% ± 3%, n = 3; εV265A 79% ± 3%, n = 3; and εL269F 65% ± 3%, n = 3) desensitized nearly as rapidly (Fig. 3). Other mutants, such as εT264P (32% ± 3%, n = 3) and βV229F (33% ± 7%, n = 4), behaved exactly as WT AChRs in this paradigm. Mutations at the ligand binding site (αG153S 85% ± 6%, n = 3 and αV156M 78% ± 2%, n = 3) had the tendency to desensitize nearly as fast as M2 mutations. In addition, after the removal of the ligand, the time of recovery to resting potential was longer than that observed in WT and other SCS channels (data not shown). This suggests that the enhanced desensitization may be due to increased affinity (Croxen et al., 1997) between the ligand and the ligand binding site in these AChR mutations.

Ca^{2+} permeability remains mostly unchanged in SCS mutations

Histochemical studies of muscle biopsy material obtained from patients and a transgenic mouse model of

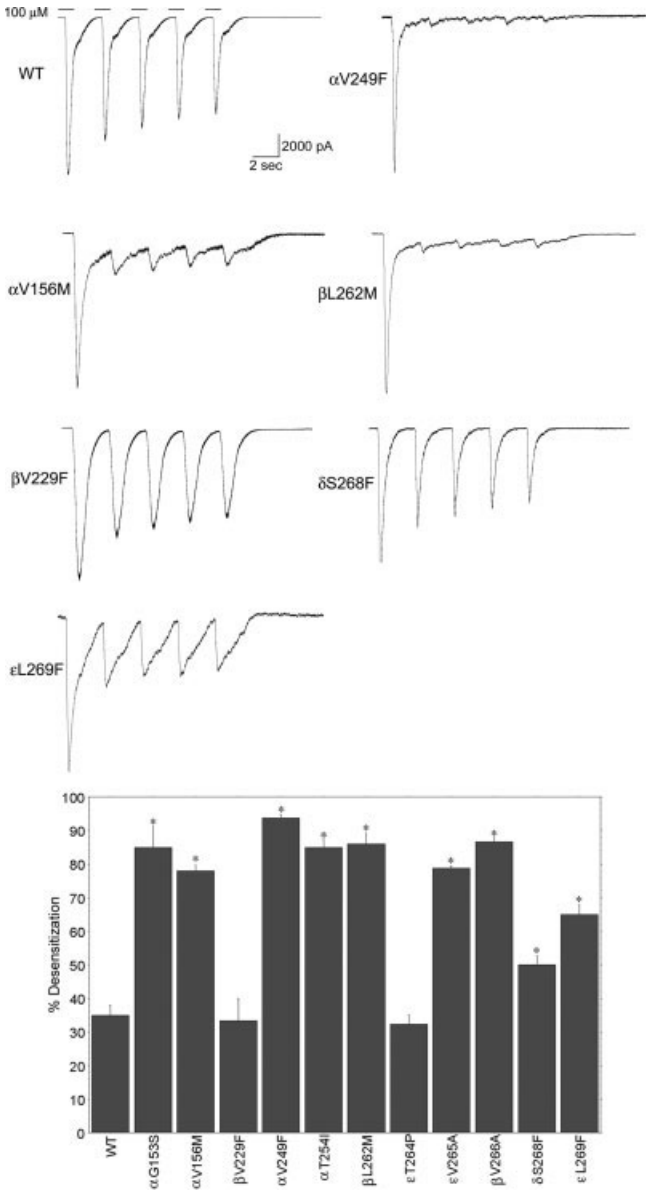

Fig. 3. SCS mutations have a reduced rate of recovery from desensitization. HEK293 cells expressing the different AChR were stimulated five times with acetylcholine (500 μM) for 500 ms every second. The rate of recovery based on the amplitude between the first stimulus and the last is expressed as % desensitization. The vast majority of SCS mutations studied (αG153S 85% ± 6%, αV156M 78% ± 2%, αT254I 85% ± 2%, βL262M 86% ± 3%, βV266A 87% ± 1%, δS268F 50% ± 3% ($n = 3$), εV265A 79% ± 3% ($n = 3$), and εL269F 65% ± 3% ($n = 3$), $P < 0.01$, $n \geq 3$) recovered from desensitization much more slowly than WT receptors (35% ± 3%, $n = 4$). Only the mutants εT264P (32% ± 3%, $n = 3$) and βV229F (33% ± 7%, $n = 4$) had rates of recovery that were similar to WT AChR.

SCS demonstrate accumulations of calcium around the endplate region. Using ionic substitution methods in voltage clamped HEK293 cells, we tested whether SCS mutations differed in their effect on calcium permeability. We expressed 12 different SCS AChR mutations in HEK293 cells and determined the reversal potentials of the activated receptors in both solutions containing 1 and 10 mM $CaCl_2$. By applying the measured reversal potentials in the extended version of the GHK equation described in the methods section, we calculated the relative Ca^{2+} permeability of receptors expressing each mutation (Fig. 4). The permeability value obtained of 0.44 ± 0.11, $n = 5$, PCa^{2+}/PCs^{+} in WT receptors matches the previously reported estimates (Castro and Albuquerque, 1995; Cens et al., 1997; Ragozzino et al., 1998). Surprisingly, no spontaneous M2 mutation had any significant effect on Ca^{2+} permeability compared with WT AChRs (αT254I 0.63 ± 0.20, $n = 3$ PCa^{2+}/PCs^{+}; βL262M 1.00 ± 0.50, $n = 3$ PCa^{2+}/PCs^{+}; εV265F 0.32 ± 0.02, $n = 3$ PCa^{2+}/PCs^{+}; βV266M 0.72 ± 0.14, $n = 3$ PCa^{2+}/PCs^{+}; δS268F 0.60 ± 0.20, $n = 4$ PCa^{2+}/PCs^{+}; and εL269F 0.78 ± 0.12, $n = 3$ PCa^{2+}/PCs^{+}). We were not able to study the mutant AChR, αV249F, because of extremely fast desensitization. However, these findings suggest that the spontaneous missense mutations in the pore forming domain that affect gating kinetics and desensitization in SCS do not alter Ca^{2+} selectivity in these receptors. Similarly, estimates of Ca^{2+} permeability for the ligand binding site mutations αG153S (0.87 ± 0.18, $n = 4$ PCa^{2+}/PCs^{+}) and αV156M (0.99 ± 0.28, $n = 3$ PCa^{2+}/PCs^{+}) also revealed no significant differences in the permeability of these receptors from WT, supporting the notion that these residues are not involved in the movement of this cation.

Finally, we studied permeability in SCS mutations in the first transmembrane domain that forms the outer part of the pore, remote from the channel gate. As with the other SCS mutations, neither αN217K (0.37 ± 0.11, $n = 6$ PCa^{2+}/PCs^{+}) nor εL221F (0.37 ± 0.12, $n = 6$ PCa^{2+}/PCs^{+}) had significant effects on Ca^{2+} permeability. However, the adjacent mutation, βV229F, significantly increased Ca^{2+} permeability. The value of 1.6 ± 0.16, $n = 6$ PCa^{2+}/PCs^{+} showed a four-fold increase in the amount of calcium that is moved through the pore. This somewhat surprising result suggests that this residue might be near a region in the M1 domain that contributes to determining the ion selectivity of the pore. The results obtained demonstrated that, with the exception of βV229F, mutations in SCS do not alter the Ca^{2+} permeability of AChR.

DISCUSSION

When muscle from human biopsies and murine SCS models are stained using glyoxal-bis-2-hydroxy-anil, a histochemical dye that detects free calcium ion in the millimolar range, intense labeling appears around the NMJ region (Gomez et al., 2002a; Ohno et al., 1995a; Vohra et al., 2004). The accumulation of free Ca^{2+} ions at postsynaptic sites suggests that this cation may play a major role in the degenerative process observed in SCS muscle endplates. It has been suggested that the slow kinetics of endplate currents influence the overall quantity of cations moving to the sarcoplasm, leading

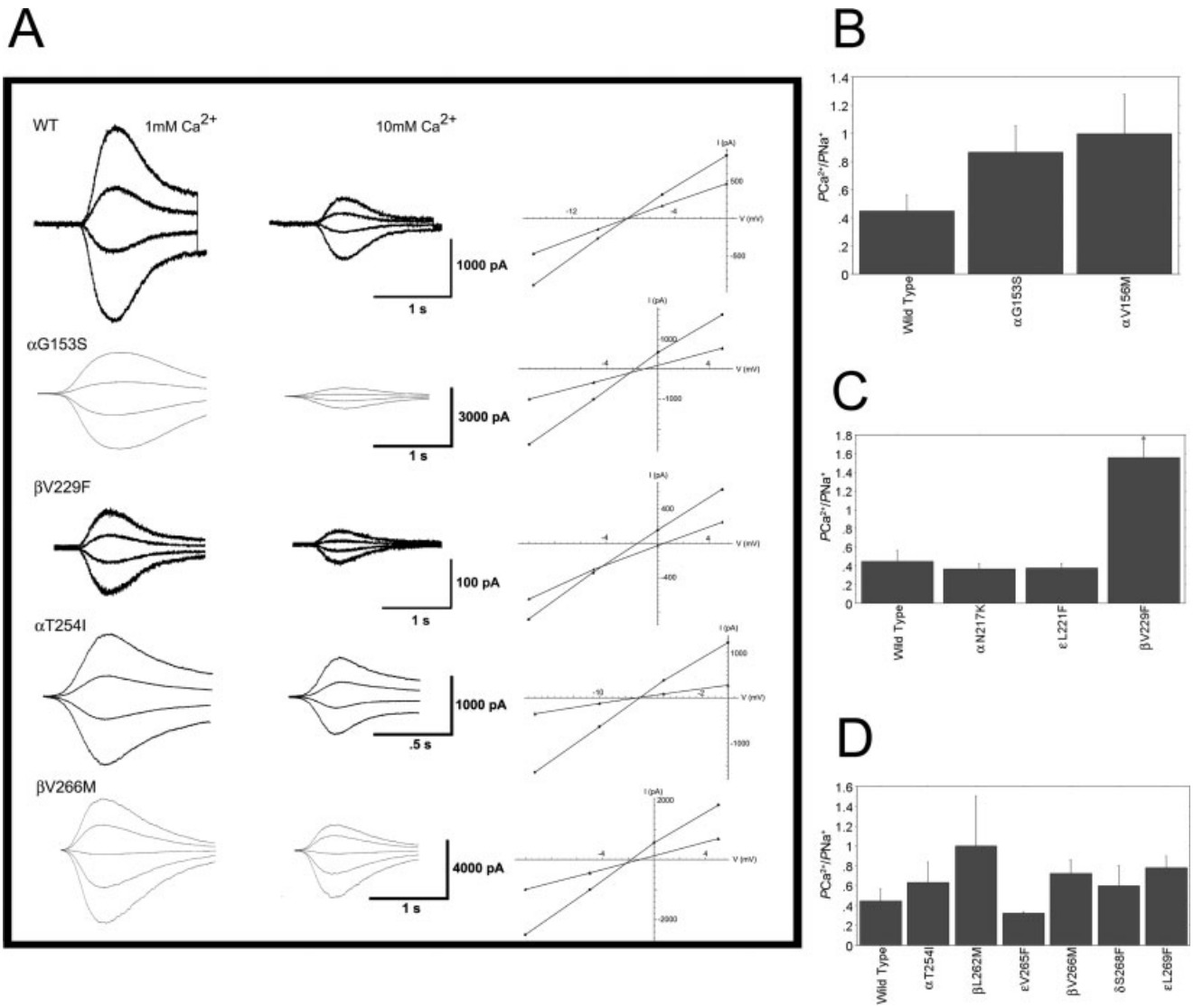

Fig. 4. Changes in calcium permeability cannot account for pathogenicity in SCS. Ca^{2+}/Cs^{+} permeability (PCa^{2+}/PCs^{+}) for both WT and SCS AChR was determined by recording the reversal potentials at two different concentrations of extracellular Ca^{2+} (**A**), and solving the value from a modified Goldman–Hodgkin–Katz equation. PCa^{2+}/PCs^{+} of ligand binding site mutations (αG153S 0.87 ± 0.18 PCa^{2+}/PCs^{+}, and αV156M 0.99 ± 0.28 PCa^{2+}/PCs^{+}, $n \geq 3$) was slightly but not significantly higher (**B**) than the permeability value observed in WT receptors (0.44 ± 0.11, $n = 5$ PCa^{2+}/PCs^{+}). Although most M1 mutations had permeability values similar to the ones observed in WT AChR (αN217K 0.37 ± 0.05, $n = 6$ PCa^{2+}/PCs^{+}, and εL221F 0.37 ± 0.05, $n = 6$ PCa^{2+}/PCs^{+}), the β subunit mutation βV229F (1.6 ± 0.16, $n = 6$ PCa^{2+}/PCs^{+}) had a permeability value three times larger than normal receptors (**C**). Finally, there were no significant differences between most M2 SCS mutations (αT254I 0.63 ± 0.20 PCa^{2+}/PCs^{+}, βL262M 1.00 ± 0.50 PCa^{2+}/PCs^{+}, εV265F 0.32 ± 0.02 PCa^{2+}/PCs^{+}, βV266M 0.72 ± 014 PCa^{2+}/PCs^{+}, δS268F 0.60 ± 0.20 PCa^{2+}/PCs^{+}, and εL269F 0.78 ± 0.12 PCa^{2+}/PCs^{+}, $n \geq 3$) and WT receptors despite their association with severe disease severity (**D**).

to formation of these Ca^{2+} deposits (Gomez et al., 1997, 2002a). The role of the mutant receptors and cholinergic activity in this process in SCS is further supported by the dependence of this process on synaptic activity. Exercise increases the number and intensity of glyoxal-bis-2-hydroxy-anil stained endplates (Gomez et al., 2002a). Axotomy of the motor nerve leads to the complete disappearance of the accumulated of calcium in the subsynaptic area of the muscle fibers (Groshong et al., submitted).

Changes in the permeation properties of different ligand gated channels including AChR have also been linked to different models of Ca^{2+} excitotoxicity (Glaznner et al., 2000; Kuryatov et al., 1997; Sattler and Tymianski, 2001). Previous studies associated calcium overload with changes in the Ca^{2+} permeability of the mutated receptor, although the correlation was only found with synthetic M2 mutations. The number of naturally occurring mutations studied was very limited and increments in Ca^{2+} permeation were not

observed in any of them (Gomez et al., 2002a). Distinct subunit combinations, most notably the developmental switch from δ to ε, can lead to changes in Ca^{2+} permeability of the AChR. This change is believed to be important for regulation of gene expression (Castro and Albuquerque, 1995; Dani, 1993; Witzemann et al., 1987). Most neuronal nicotinic receptor subtypes are more permeable to Ca^{2+} than muscle receptors. Changes in Ca^{2+} permeability due to mutations in the vicinity of the M2 domain of neuronal AChRs are believed to be responsible for the molecular phenotype of the frontal lobe nocturnal epilepsy (AFLNE) (Bertrand et al., 1998; Figl et al., 1998; Kuryatov et al., 1997). After an extensive study of a large panel of SCS mutations, we found little differences in the Ca^{2+} permeability of these mutated channels, except for the βV229F mutation. In this M1 mutation, we observed an almost three-fold increase in Ca^{2+} permeability (Table I). This change in AChR permeability is not as profound than the up to 20-fold increase observed in one of the AFLNE mutations (Ragozzino et al., 1998). However, given that individuals in this SCS kindred suffered from a very mild form of disease, it is likely that differences in channel divalent cation permeation alone do not play a prominent role in the pathogenesis of SCS.

From a structural standpoint, the changes in the Ca^{2+} permeability caused by the M1 mutation, βV229F, support the notion that the ion selectivity and inward rectification properties of AChRs are determined by a small number of residues in the rings flanking the pore, and not amino acids located nearby the hydrophobic gate (Galzi et al., 1992; Gunthorpe and Lummis, 2001; Haghighi and Cooper, 2000). The lack of effect of the similar mutation, εV265F, on permeability suggests that polar residues distal to the pore-forming region can contribute to the movement of this cation across from the extracellular space into the channel pore. This finding further expands our understanding of the M1 domain and the different roles it plays during channel activation.

AChR desensitization, a phenomenon first studied in frog NMJ, is responsible for the reduction in the size of endplate currents after repetitive stimulus of the muscle AChR in the absence of acetylcholinesterase (Bhattacharyya et al., 1997; Elenes and Auerbach, 2002; Jones and Westbrook, 1996; Magleby and Pallotta, 1981). Although little is known about the molecular mechanisms of desensitization, a two-gate mechanism comparable with the one observed in other ion channels has been suggested (Auerbach and Akk, 1998). Different factors can influence how fast AChRs desensitize, including the duration of ligand exposure and conformational changes in the receptor protein structure that alter the stability of the different desensitized states (Auerbach and Akk, 1998; Elenes and Auerbach, 2002). Increased desensitization of the

TABLE I. Clinical correlation comparing electrophysiological properties of the mutations and different pathological features

	αG153S	βV156M	αN217K	βV229F	εL221F	αV249F	αT254I	βL262M	βV266A	βV266M	δS268F	εT264P	εV265A	εL269F
Disease severity	Mild	Mild	Mild	Mild	Mild	Severe	Mild	Severe	Severe	Severe	Severe	Severe	Severe	Severe
Age of onset	infant–57	14	8–57	18–30	17–40	infant	16	infant	infant	childhood	infant	infant	infant	infant
Ca^{2+} permeability	+	+	+	++	+	n/a	+	+	+	+	+	+	+	+
Desensitization (%)	++	++	n/a	+	n/a	++	++	++	++	n/a	++	+	++	++
τ (ms)	++	++	n/a	++	n/a	n/a	+	++	++	n/a	+	++	n/a	++
Clinical correlation	low	low	good	low	good	good	med	good	good	low	low	med	med	good

+Values not significantly different when compared to WT.
++Values significantly different when compared to WT.

mutated receptors during synaptic activity could have profound effects on the ability of the muscle fiber to reach its depolarizing threshold. Paradoxically, this may also actually diminish the effect of a prolonged channel opening on cation overload. Enhanced desensitization has only been documented for αV249F (Milone et al., 1997). The study of the desensitization rates of AChRs at the macroscopic level provided us with useful information about how these receptors react to tetanic ligand stimulation with acetylcholine, especially over short time scales. Fast desensitization of receptors could be partially responsible for the weakness phenotype observed in many of the SCS patients, as it is in at least one murine model of SCS (Bhattacharyya et al., 1997; Milone et al., 1997).

Our studies showed that a large number of mutations in the M2 domain exhibit significant changes in the rate of desensitization when studied with this protocol. This observation suggests that mutations around the gate region of the receptor are capable of shifting the dynamic equilibrium of the channel between the open state and desensitized state. There is a modest correlation between these mutations and patients who suffer from severe muscle weakness and fatigue (Table I). The desensitizing feature could play a major role during the early stages of the disease or patients with early onset of disease (Table I). Reduced ability of mutated receptors to respond to synaptic activity, due to enhanced desensitization, could have a profound effect on the ability of the muscle to reach its depolarizing threshold. The inability of AChRs to recover from their desensitized state, especially during periods of repeated synaptic activity, could lead to serial and progressive decrease of AChR currents, and reduction of the safety factor of neuromuscular transmission. Alternatively, desensitization could, in theory, protect muscle cells from the prolonged opening of the postsynaptic AChRs. Until individual SCS mutants are tested that differ in only single properties this question cannot be fully resolved.

Finally, it has been suggested that the slowed kinetics of endplate currents might influence the overall quantity of cations moving to the sarcoplasm. The electrophysiological hallmark of SCS is slow decay of endplate currents. Although it is known that the altered gating of the mutated channels influences the endplate currents, the effect of a panel of mutations on the macroscopic decay time of the mutated AChR currents has not been compared until now. The results obtained in this experiment suggest that the prolonged decay currents exhibited by SCS mutations may be partially responsible for the cation overload observed in this syndrome. In addition, slowed currents lead to prolonged depolarization of the endplate region which may disturb voltage dependent binding and gating of AChRs (Auerbach et al., 1996). Curiously, relaxation current experiments for mutations αT254I and δS268F have values that are similar to the ones observed in WT AChR expressing HEK293 cells. These findings contradict results obtained from single channel patch-clamp and muscle electrophysiology studies of these SCS mutations (Croxen et al., 1997; Gomez et al., 2002b). Anomalies in voltage-jump relaxation experiments and their interpretation have been previously reported (Figl et al., 1996). The relation between the rate opening constant and the steady state response, and how these values change in relation to each other when studied under this experimental paradigm, remains unclear. We have shown that, unlike other SCS mutations, the rate of opening of δS268F is greatly decreased (Gomez et al., 2002b). This change, and perhaps the behavior of these mutations under different concentrations of ligand, could explain the relatively fast τ observed in these experiments.

This is the first time different parameters of such large array of SCS have been compared side by side. The combined study of these parameters demonstrated that there is a strong trend between both the decay time of nicotinic AChR currents, the rate of desensitization and the severity of the disease, but, as in other syndromes, no single change in SCS AChR function that can serve as a universal predictor for disease severity. Nevertheless, these studies provide evidence that, unlike previously thought (Gomez et al., 2002a) Ca^{2+} permeability does not play a role in this disorder. Second, it gives us a better understanding of the nature of different kinetic parameters of these mutated channels.

This study confirms that several AChR channel properties that affect macroscopic currents are altered in SCS. Such changes may greatly alter synaptic transmission, but because each SCS mutation exhibits changes in several properties, the relative contribution of each change to disease is difficult to estimate. Nevertheless, these results demonstrate the utility of studying mutations in macroscopic configuration and provide a platform for future comparisons of additional SCS mutations.

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