IS3 peptide-formed ion channels in rat skeletal muscle cell membranes

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Abstract A 22-mer peptide, identical to the primary sequence of domain I segment 3 (IS3) of rat brain sodium channel I, was synthesized. With the patch clamp cell-attached technique, single channel currents could be recorded from the patches of cultured rat myotube membranes when the patches were held at hyperpolarized potentials and the electrode solution contained NaCl and 1 μM IS3, indicating that IS3 incorporated into the membranes and formed ion channels. The single channel conductances of IS3 channels were distributed heterogeneously, but mainly in the range of 10–25 pS. There was a tendency that the mean open time and open probability of IS3 channels increased and the mean close time decreased with the increasing of hyperpolarized membrane potentials. IS3 channels are highly selective for Na^+ and Li^+ but not for Cl^- and K^+, similar to the authentic Na^+ channels.

Key words: Synthetic peptide; IS3 channel; Na^+ selective channel; Single channel recording; Cultured rat myotube membrane

1. Introduction

Sodium channels play a key role in generating and propagating action potentials in most excitable cells. In different animals and different tissues, Na^+ channels are composed of different numbers of subunits in which an α-subunit is the main component. An α-subunit is composed of four repeat domains (I, II, III, IV). Every repeat domain includes six transmembrane α-helixes (S1–S6) [1]. Between S5 and S6 there is a semi-transmembrane linker peptide, called SS1-SS2 peptide or P region [2].

It is generally agreed that the S4 segments of all voltage-gated ion channels are the main components of the voltage sensors [1,2]. But it has been uncertain which parts of the transmembrane segments are involved in forming the pore lining region of the Na^+ channel. Data from mutagenesis studies suggested that the P region and to some extent S6 comprise the selectivity filter and a part of the pore lining of the Na^+ channel [3–8]. Recently the first X-ray crystal structure of K^+ channel from *Streptomyces lividans* (KcsA) supported this idea [9]. On the other hand, some synthetic peptides corresponding to certain transmembrane segments of the Na^+ channel could be incorporated into lipid bilayers and could form ion channels. Analysis of the characteristics of these synthetic channels also provided important information of structure-function relationships in Na^+ channels [4,10,11].

Synthetic peptide IS3, corresponding to repeat I segment 3 from rat brain Na^+ channel type I, could form cation selective ion channels in lipid bilayers [11]. Here it is reported that IS3 peptide incorporates into the patches of cultured rat myotube membranes and forms a Na^+ selective channel.

2. Materials and methods

2.1. Cell culture

The preparations were cultured myotubes from myocytes of the thigh muscle of 1–3 day Sprague-Dawley rats. The methods of cell culture were described by Yaffe [12] and modified by X.P. He et al. [13]. The cultured myotubes were used 5–10 days after plating.

2.2. Peptide synthesis and purification

The methods of IS3 peptide synthesis were described in detail previously [14]. Briefly, first, several small peptide segments were synthesized, and then beginning from the C-terminus, the IS3 peptide was synthesized by means of segment condensation. The crude product was purified by HPLC, first on Protein Park 125 and 60. Then a preparative reversed-phase HPLC (SynChropac C18, 250×10 mm) was used for further purification, using 40% acetonitrile in 0.1 M NH_4HCO_3 aqueous solution as the mobile phase. Its purity was confirmed by amino acid analysis and fast atom bombardment mass spectrometry. The sequence of the synthetic IS3 peptide is DPWNWLDFTVITFAYVTEFVDL.

2.3. Patch clamp methods

With the cell-attached configuration, single channel currents were recorded from patch membranes on cultured rat myotubes by a patch clamp amplifier (List EPC-7, Germany), filtered at 3 kHz, digitized at 10 kHz and stored in a PC 386 computer using a Labmaster TL-1 interface (Axon Instruments, USA) and pCLAMP version 5.5.1 software. Standard single channel analysis was performed also using pCLAMP software. All experiments were carried out at room temperature (21–24°C). Records with >500 openings in a continuous 15 s recording were valid for analysis.

The extracellular bathing solution was (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 2, HEPES 10, pH 7.2. The standard pipette solution was (in mM): NaCl 168, EDTA 5, HEPES 5, IS3 (1 μM, IS3 was dissolved in DMSO and diluted with NaCl solution, DMSO:NaCl solution = 0.5:100, v/v), pH 7.2.

3. Results

3.1. Measurement of resting potentials

The resting potential of cultured rat myotubes was determined in extracellular solution with a conventional intracellular microelectrode (filled with 4 M KAc) to be -71.5 ± 2.0 mV ($n = 11$). This value is in agreement with the results of others [15,16].

3.2. IS3 peptide was incorporated into myotube membranes and formed ion channels

With standard pipette solution in the electrode, after tight

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Abbreviations: IS3 peptide, a synthetic peptide corresponding to the transmembrane segment 3 of repeat I from rat brain Na^+ channel type I

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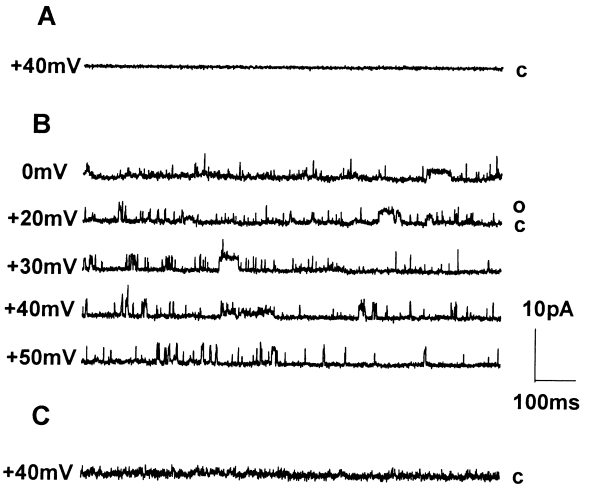

Fig. 1. Single channel currents recorded from cell-attached patches of cultured rat myotube membranes at various pipette potentials indicated on the left; upward deflection is the inward current. A: Majority of the control experiments: 168 mM NaCl in electrode solution without IS3 peptide; only record at +40 mV pipette potential is shown. B: Recordings at various pipette potentials when the electrode solution contains 168 mM NaCl and 1 μM IS3. C: Majority of the experiments with 168 mM NaCl and 1 μM scrambles IS3 in electrode solution; only record at +40 mV pipette potential is shown.

seal formation, the patches of myotube membranes were held at the resting potential and hyperpolarized potentials. In control experiments (pipette solution without IS3), there was no detectable current fluctuation (Fig. 1A) in 12 out of 14 records. While the pipette solution contained 1 μM IS3, inward single channel currents were observed (Fig. 1B) within 2 min in 22 out of 28 records. The probability of the occurrence of single channel currents was 0.14 (control) versus 0.79. From the statistical analysis, IS3 peptide was incorporated into cell membranes and formed ion channels.

To test the specificity of the IS3 sequence to generate the channel currents, another 22-mer peptide was synthesized by solid-phase methods, which had the same amino acid composition as the IS3 peptide but a computer-generated scrambled sequence (PVDFETVYFATIVTFLDWNWLD) and was predicted to retain helical structure. When this scrambled IS3 replaced the IS3 peptide in the pipette solution, there was no current fluctuation in 13 out of 15 records at the resting and hyperpolarized membrane potentials, as shown in Fig. 1C. The occurrence probability of the single channel currents was statistically different from that of the records with the IS3 peptide. Therefore, the specific sequence of the IS3 peptide is required to form an ion channel.

3.3. Characteristics of single channel currents

The amplitudes of the single channel currents through IS3 channels increased with increasing hyperpolarized membrane potentials, as shown in Fig. 1B. The single channel currents

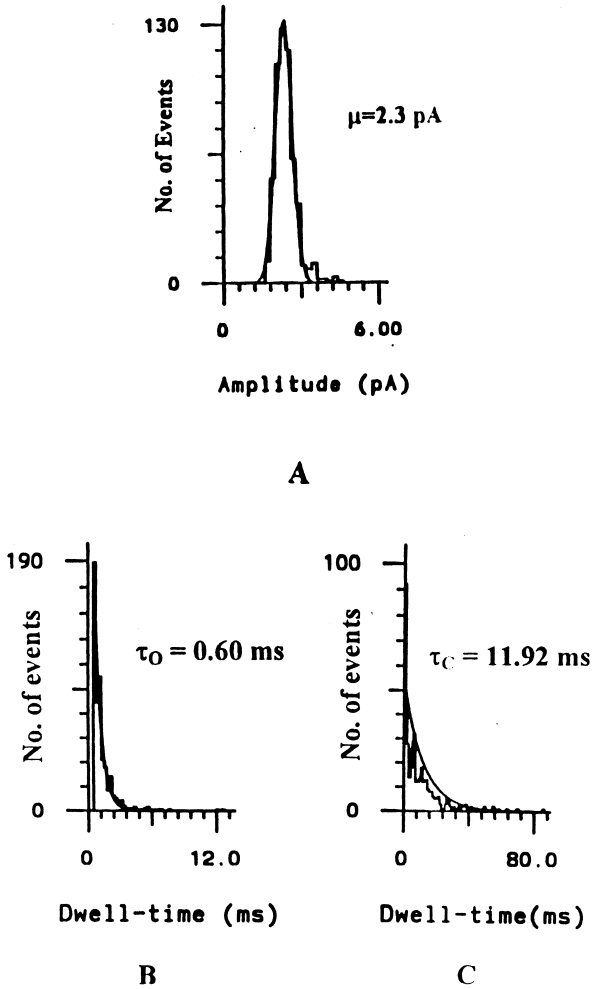

Fig. 2. Current amplitude histogram (A), open time histogram (B) and close time histogram (C) based on the single channel recording at +30 mV pipette potential of Fig. 1B.

were measured from the Gaussian fit curves of current amplitude histograms (Fig. 2A). The current-voltage curves of each recording were plotted, based on the least-squares fit to the points (see Fig. 3, 168 mM NaCl). The single channel conductances (γ) were calculated from the slopes of the curves; the distribution of γ was heterogeneous, but the majority were in the range of 10–25 pS (see Table 1).

The mean open times and close times of IS3 channels with a conductance of 10–25 pS were measured respectively based on the single exponential fitting curves of open time histograms (Fig. 2B) and close time histograms (Fig. 2C), and open probability was calculated using pCLAMP, shown in Table 2. Except between +40 mV and +50 mV, the corresponding kinetic characteristics between two adjacent voltages had no statistical difference. But it seemed that the mean open times and the open probability increased, and the mean close times de-

Table 1
The distribution of the single channel conductances of the IS3 channel and its relative frequency of occurrence

γ (pS)	Relative frequency of occurrence (%)	Number of experiments	Average conductance (pS)
10.0–25.0	68	15	16.9 ± 4.1
25.0–40.0	14	3	29.7 ± 4.1
> 40.0	18	4	49.2 ± 9.1

Student's *t*-test showed that there was statistical difference ($P < 0.05$) between each pair.

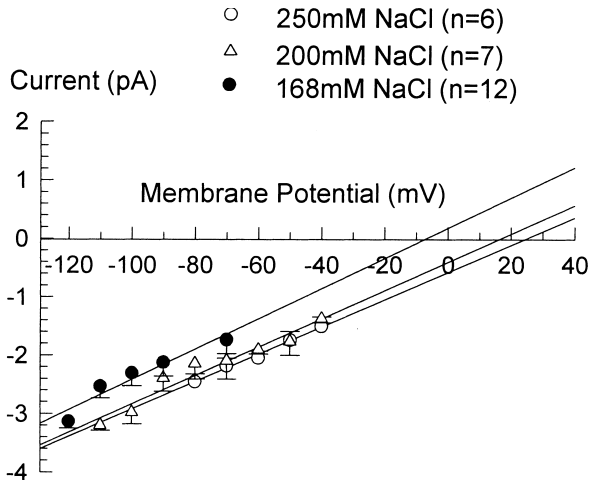

Fig. 3. *I-V* curve of IS3 channels with different concentrations of NaCl in electrode solution. Membrane potential = resting potential (−71 mV)−pipette potential.

creased with the increasing of the hyperpolarized membrane potentials.

3.4. Ion selectivity of the IS3 channel

In these experiments, the recorded currents are inward. Since there are only Na^+ and Cl^- in the electrode solution, these inward currents might be due to Na^+ influx or Cl^- efflux. Which ion carried the current through the channel? The concentration of NaCl in electrode solution was increased from 168 mM to 200 mM and 250 mM respectively. As shown in Fig. 3, the single channel current amplitudes recorded at the same membrane potentials increased with increasing NaCl concentration, and the extrapolating reversal potentials shifted to more positive membrane potentials. On the basis of the Nernst equation, the results suggest that IS3 channels are selective for Na^+ over Cl^-. In other experiments ($n = 15$) where only 168 mM NaCl was replaced by 200 mM choline chloride in the electrode solution, no detectable current fluctuations could be observed at various pipette potentials (0 to +40 mV), as shown in Fig. 4. It seems that the IS3 channel is impermeable to Cl^-.

Could K^+ pass through the IS3 channel? The electrode solution was used with the following composition (in mM): KCl 150, EGTA 5, HEPES 5, DMSO 0.5% (v/v), 1 μM IS3 and 2 mM $BaCl_2$. 2 mM BaCl2 was added to block the anomalous rectifier K^+ channels in the membrane of cultured rat myotubes [15]. When the patches of myotube membranes were held at the resting potential and various hyperpolarized potentials (up to +50 mV), single channel current fluctuations were observed in only three out of 21 recordings, while in control experiments under the same conditions but without IS3 in the electrode solution, current fluctuations were present in one out of 10 recordings. From the statistical analysis, IS3

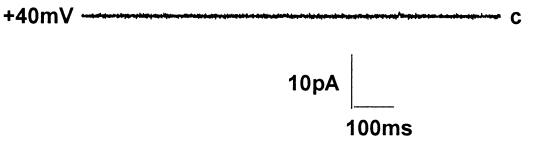

Fig. 4. No current fluctuations could be observed when 200 mM NaCl was replaced by 200 mM choline chloride with 1 μM IS3 in the electrode solution, recorded at 0 to +40 mV pipette potentials. Only recording at +40 mV pipette potential is shown.

channel is not selective for K^+ or is far less permeable to K^+ than to Na^+.

As for Li^+, it is quite different when 168 mM NaCl in the standard pipette solution was replaced by 126 mM LiCl and pipette potentials were held from −10 mV to +20 mV. In control experiments (pipette solution without IS3), there was no detectable current fluctuation in 14 out of 15 recordings. When the pipette electrode contained 0.4 μM IS3, the inward single channel currents were observed within 2 min in 12 out of 21 recordings, as shown in Fig. 5. The occurrence probability of single channel currents was 0.07 (control) versus 0.57. From the statistical analysis, IS3 channel is permeable to Li^+. The single channel conductance is mainly in the range of 40–45 pS. The average conductance is 45.2 ± 9.6 pS.

4. Discussion

4.1. IS3 peptide formed ion channels in rat cultured myotube membranes

In the cell membranes of cultured rat myotubes, there are several endogenous ionic channels: delayed rectifier K^+ channels [13], acetylcholine receptors [13], anomalous rectifier K^+ channels [15], Na^+ channels [16], $Ca2^+$-activated K^+ channels [17], two kinds of Cl^- channels [18,19]. Their single channel currents could be recorded under certain conditions. But under our experimental conditions – membrane patches held at hyperpolarized potentials, only Na^+ and Cl^- in the standard electrode solution and EDTA used to chelate divalent cations – all endogenous channels mentioned above could not be active. In addition IS3 channels could be formed in lipid bilayers [11], therefore the participation of any endogenous receptor could be excluded. It was concluded that IS3 peptide could incorporate into myotube membranes and form ion channels.

4.2. Comparison of IS3 channels formed in rat cultured myotube membranes with those in planar lipid bilayers

It is interesting to compare the characteristics of IS3 channels formed in lipid bilayers [11] with those in cultured rat myotube membranes. In both cases the distribution of single channel conductances is heterogeneous, but the most frequent events are in the range of 15–25 pS (in lipid bilayers) and 10–25 pS with an average of 16.9 pS (in myotube membranes). These values are very similar to the γ value of authentic my-

Table 2
The kinetic characteristics of IS3 channels at different pipette potentials

Pipette potential	0 mV	+20 mV	+30 mV	+40 mV	+50 mV
Mean open time (τ_o, ms), $n = 15$	0.54 ± 0.05	0.72 ± 0.10	0.64 ± 0.06	0.90 ± 0.16	1.34 ± 0.29
Mean close time (τ_c, ms), $n = 15$	27.05 ± 8.56	23.84 ± 4.72	19.47 ± 6.69	19.89 ± 4.59	12.38 ± 3.37
Mean open probability (P_o, %) $n = 15$	2.4 ± 0.1	2.6 ± 0.1	4.1 ± 0.6	4.2 ± 0.2	8.2 ± 0.3

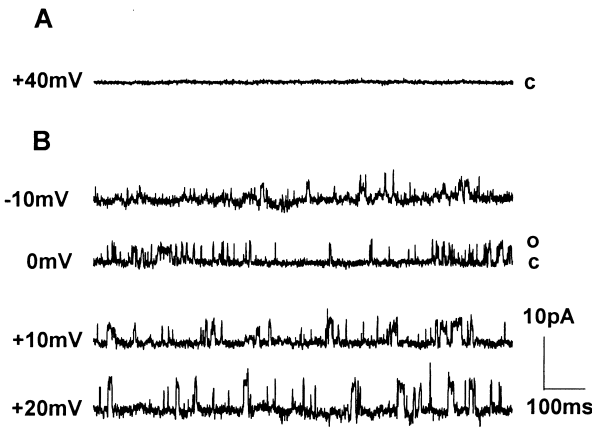

Fig. 5. Single channel currents recorded from cell-attached patches of cultured rat myotube membranes at various pipette potentials indicated on the left; upward deflection is the inward current. A: Majority of the control experiments: in the absence of IS3, 126 mM LiCl in electrode solution; only record at +40 mV pipette potential is shown. B: Recordings at various pipette potentials when the electrode solution contains 126 mM LiCl and 1 μM IS3.

otube Na^+ channels, 18 pS [16]. The heterogeneity of the conductances may be due to the formation of different quaternary structures during the self-assembly process, or to a different aggregation number [20]. From energetic consideration it is suggested that a bundle of four amphipathic α-helices is a plausible structure for the IS3 channel [11], and perhaps this structure gives the most frequent conductances in both bilayers and myotube membranes.

However, there is some difference between them. The mean open time of IS3 channels in lipid bilayers is longer than that in myotube membranes and the mean close time is shorter in lipid bilayers than that in myotube membranes. The channel open probability is greater in lipid bilayers. Moreover, IS3 channels in bilayer are cation selective but lack discrimination between Na^+ and K^+ [11]. But in myotube membranes, IS3 channels are much more permeable to Na^+ than to K^+ and even more to Li^+ than to Na^+, similar to the natural Na^+ channels. So IS3 channels formed in myotube membranes are Na^+ selective. The different properties of IS3 channels in lipid bilayers and in myotube membranes may be due to the different lipid composition of the membranes. Channel properties may depend on membrane lipid composition [21]. For example, melittin formed voltage sensitive anion selective channels in lipid bilayers [22], while in cultured *Xenopus* embryonic myocyte membranes, it formed ion channels with Na^+ selectivity and no voltage dependence [23]. The IS3 channels surrounded by natural lipid composition in cultured rat myotube membranes should be more like the authentic Na^+ channels in some respects.

4.3. Possible role of S3 peptide in sodium channels

Doyle et al. [9] using an X-ray diffraction method solved the crystal structure of one K^+ channel protein, KcsA K^+ channel, which spans the cell membrane only twice. Their work confirms that the P region forms the selectivity filter, and the inner helix corresponding to the S6 segment of the shaker K^+ channel forms the lining of the inner part of the pore. For the sodium channel, though no crystal structure has been published, mutagenesis confirmed that the P region forms the outer mouth of the pore and the selectivity filter [4–8]. But which segment is involved in the lining of the inner part of the pore is still uncertain. Montal and his collaborators first suggested that S3 could be the pore forming segment of the Na^+ channel [11]. Later they systematically studied the channel forming activity of synthetic peptides corresponding to segments S1–S6 of the voltage gated, dihydropyridine sensitive Ca^{2+} channels. They found S2 and S3 could form ion channels in lipid bilayers, but only channel S3 was modulated by enantiomers of Bayk 8644, showing the pore properties of the natural Ca^{2+} channel [24]. Brullemans et al. [10] suggested that S45, the linker between the S4 and S5 segments of domain IV from the electric eel Na^+ channel, is located in the inner part of the permeation pathway. The present work shows that the possibility of S3 peptides forming the inner part of the pore lining of the Na^+ channel could not be excluded. In order to elucidate the functional significance of the various segments of voltage gated channels, much more work needs to be done.

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