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Accelerated desensitization of nicotinic receptor channels and its dependence on extracellular calcium in isolated skeletal muscles of streptozotocin-diabetic mice

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1 To elucidate the influence of the diabetic state on desensitization of nicotinic acetylcholine (ACh) receptor channels, we investigated the time course of the decrease in amplitude of ACh potentials elicited by iontophoretic application to isolated diaphragm muscle of streptozotocin-diabetic mice. We also investigated time- and extracellular Ca^{2+}-dependent changes in the channel opening frequency of ACh-activated channel currents and the involvement of protein kinases by use of the cell-attached patch clamp technique in single skeletal muscle cells.

2 When ACh potentials were evoked at 10 Hz, the decline in trains of ACh potentials was accelerated in the diabetic state.

3 The time-dependent decrease in the channel opening frequency of diabetic muscle cells was greatly accelerated compared with normal cells in 2.5 mM Ca^{2+} medium.

4 This accelerated decrease in channel opening frequency was restored by pretreatment with a protein kinase C inhibitor, staurosporine (10 nM) but neither a protein kinase A inhibitor, H-89 (3 μM) nor a calmodulin kinase II inhibitor, KN-62 (5 μM) were able to restore the fall in opening frequency.

5 These results demonstrate that in the diabetic state the desensitization of nicotinic ACh receptor channels may be greatly accelerated by activating protein kinase C, which is caused by an increase in the amount of available intracellular Ca^{2+}.

Keywords: Nicotinic acetylcholine-receptor channel; desensitization; streptozotocin-diabetes; calcium; protein kinase C; single channel recording

Introduction

Diabetes mellitus causes complications including a disorder in skeletal muscles and peripheral nerves (Pain & Garlick, 1974). These changes involve both structural and metabolic defects, and include enzymatic disorders of the plasma membrane (Das *et al.*, 1976; Hegarty & Rosholt, 1981). A reduction in the conduction velocity of motor nerves is most frequently observed (Olson *et al.*, 1981; Gillon & Hawthorne, 1983). Few studies have been conducted on the function of the neuromuscular synapse in the diabetic state, although morphological abnormalities of the neuromuscular synapse in diabetes have been demonstrated (Coers & Hildebrand, 1965).

The function of the neuromuscular synapse is modulated by various protein kinase-induced phosphorylations of the nicotinic acetylcholine receptor (AChR) (Huganir *et al.*, 1986; Ochoa *et al.*, 1989). The activation of cyclic AMP-dependent protein kinase (PK-A) or protein kinase-C (PK-C) results in desensitization of nicotinic AChR in skeletal muscle cells (Eusebi *et al.*, 1985; Huganir *et al.*, 1986). The aim of the present study was to investigate whether the desensitization of the nicotinic AChR channel in streptozotocin (STZ)-induced diabetic mice occurs differently from that in normal mice. Therefore, we investigated the time course of the decrease in the amplitude of ACh potentials, and time- and extracellular Ca^{2+}-dependent changes in the channel opening frequency of ACh-activated channel currents in skeletal muscle cells of diabetic mice by use of the cell-attached patch clamp technique. The involvement of PK-C, PK-A, and calmodulin kinase II was also examined.

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Methods

Animals

Male mice (ddY strain, 4 weeks) were injected with a bolus of STZ (150 mg kg^{-1} body weight) into the tail vein and were used for experiments at four-six weeks after injection.

Diaphragm and *flexor digitorum brevis* (FDB) muscles were isolated from normal (body weight 35–42 g, feeding blood glucose level: 5.2–7.4 mM) and STZ-diabetic (body weight 27–37 g, feeding blood glucose level: 22.0–35.1 mM) mice. Blood samples were obtained from the orbital vein plexus. Blood glucose levels were measured by the glucose oxidase method on a glucose analyzer (Beckman, CA, U.S.A.).

Intracellular recordings

The left hemidiaphragm muscles were placed in a bath perfused with Krebs-Henseleit solution (KHS) saturated with 95% O_2 + 5% CO_2 at 35°C. The composition of the solution was as follows (mM): NaCl 137, KCl 2.5, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 15 and glucose 10. Intracellular recordings were performed with microelectrodes (5–25 MΩ) filled with 3 M KCl. The microelectrode was inserted near an endplate region such that the rising times of the miniature endplate potentials were 1.0 ms or less. ACh was applied by iontophoresis (rectangular current pulses with 2 ms duration at 5 or 10 Hz) from a microelectrode (100–200 MΩ) filled with 2 M ACh. To avoid leakage of ACh, a few nA of braking current was applied. The tip of the iontophoretic microelectrode was positioned to yield an 8 mV ACh potential with a rising time of below 20 ms. Experiments were conducted at the membrane potential of -65 ± 2 mV. The data were plotted on semilogarithmic coordinates, and the kinetics of biphasic onset of desensitization were analyzed. The slow time constant was calculated from a least-square line for the slow component. The fast time constant was obtained after subtraction of the slow component from the first point.

Single channel recordings

FDB muscles were isolated from the hind feet of mice. Dissection and enzyme treatment were carried out in KHS (changing from 5.9 mM to 2.5 mM KCl) saturated with 95% O_2 + 5% CO_2. The muscle tissue was incubated at 37°C for 75 min in KHS containing 0.2% collagenase, transferred to KHS without $CaCl_2$ containing 0.05% trypsin for 15 min, rinsed in KHS, and then triturated gently to separate out and obtain single cells. The single cells were pretreated with the protein kinase inhibitors for 1 h.

ACh (1 μM)-activated channel currents at the endplate of each muscle cell were recorded in KHS at 24–26°C by the cell-attached patch configuration (Hamill *et al.*, 1981) and a patch clamp system (Axopatch-1D; Axon Instruments, CA, U.S.A.). Details of the patch clamp experiments have already been described (Nojima *et al.*, 1992). Recordings of all patch currents, low-pass filtered at 2 kHz, were stored in a PCM data recorder (RP-880; NF Electric Instruments, Japan). The threshold for detecting opening and closing transitions was set at a current amplitude of 2 pA. Channel conductance was obtained from the slope of the current-voltage relationship. The mean open time was determined at the resting membrane potential. Channel opening frequency was measured as the number of events detected per 10 s. Channel currents were recorded continuously at the resting membrane potential for 20 min immediately after patch-seal formation. At the end of these experiments, the cells were impaled with the patch pipette, and the intracellular membrane potentials were measured directly.

Drugs

Acetylcholine chloride (Dai-ichi, Tokyo, Japan), collagenase (for cell dispersion; Wako Pure Chemical Industries, Osaka, Japan), trypsin (Type III) and streptozotocin (Sigma, St. Louis, MO, U.S.A.) were used. H-89 (N-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide), KN-62 (1-[N,O-*bis*(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) (kind gifts from Prof. H. Hidaka, Department of Pharmacology, Nagoya University School of Medicine, Nagoya, Japan) and staurosporine (Kyowa Medix, Tokyo, Japan) were dissolved in dimethylsulphoxide (DMSO) and diluted to final concentrations of 0.05% v/v DMSO. The concentration of solvent used did not affect resting membrane potential and ACh-activated channel activities.

Statistical analysis

Data are expressed as mean ± s.e. Student's unpaired *t* test, or one-way analysis of variance (ANOVA) followed by Scheffe multiple-comparison test were used to evaluate statistical differences between the means. $P<0.05$ or $P<0.01$ were used as the limit for statistical significance.

Results

Acceleration of nicotinic ACh receptor desensitization in the muscle of diabetic mice

To elucidate whether the desensitization of nicotinic AChR was accelerated in the diabetic state, the decrease in the trains of ACh potentials was compared between normal and diabetic muscles. When ACh potentials were evoked at 10 Hz, the amplitude of the ACh potentials decreased with time (Figure 1a), but declined to a greater extent in the diabetic muscle than in normal muscle, demonstrating that the desensitization of nicotinic AChR readily develops in diabetic muscle. The decline in the ACh potentials evoked at 5 and 10 Hz were frequency-dependent in normal muscle (Figure 1b). The diabetic state failed to accelerate significantly the desensitization produced at 5 Hz.

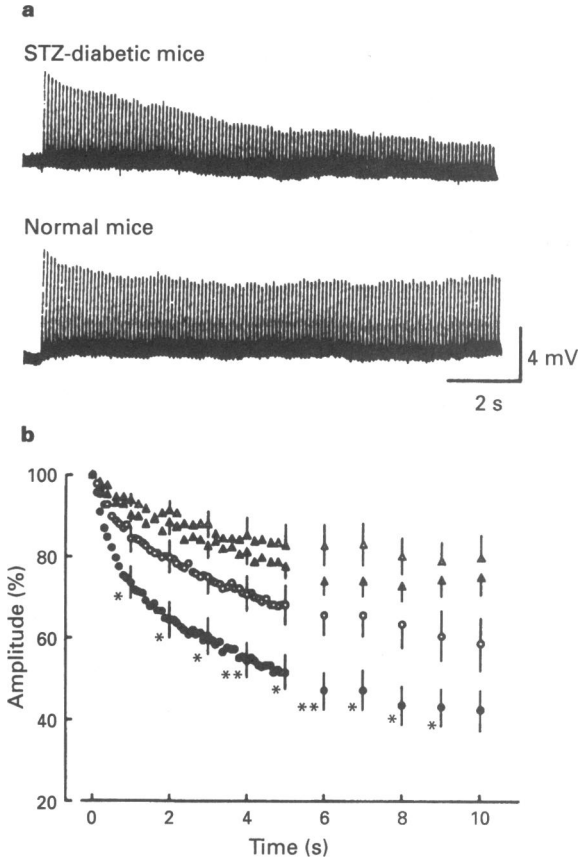

Figure 1 Desensitization in the trains of acetylcholine (ACh) potentials in normal and streptozotocin (STZ)-diabetic diaphragm muscles of mice. The amplitude of the initial ACh potential was 8 mV, and the potentials were evoked at 10 Hz. (a) Typical records showing desensitization in diabetic (upper) and normal muscle (lower). (b) Time courses of declines in the amplitude of ACh potentials evoked at 5 Hz (△) and 10 Hz (○) in normal muscle, and those evoked at 5 Hz (▲) and 10 Hz (●) in diabetic muscle. Amplitude is expressed as a percentage of the initial ACh potential (approximately 8 mV). Each point represents mean of 7–16 muscles. Bars show s.e. values at every second. $*P<0.05$, $**P<0.01$; compared with normal muscle by unpaired *t* test.

The desensitization occurred with fast and slow components, since these desensitization curves can be plotted in two exponential phases. In the ACh potentials evoked at 10 Hz, the fast time constant in diabetic muscles (0.5 ± 0.1 s, $n=13$) was not significantly different from that in normal muscles (0.5 ± 0.1 s, $n=11$), whereas the slow time constant in diabetic muscles (12 ± 1 s) was smaller than that in normal muscles (20 ± 3 s; $P<0.05$, determined by one-way ANOVA). The results indicate that the diabetic state affected the slow component of nicotinic AChR desensitization.

Extracellular calcium-dependence of nicotinic ACh receptor desensitization in normal and diabetic muscle cells

ACh-activated channel currents were recorded with a patch pipette containing various concentrations of Ca^{2+} (0, 2.5, and 5 mM) in normal and STZ-diabetic muscle cells (Table 1). The rate of the time-dependent decrease in channel opening frequency was enhanced in both normal and diabetic cells in an extracellular calcium concentration-dependent manner (Figure 2). In the presence of 2.5 mM Ca^{2+}, the ratio of channel opening frequencies at 13–16 min to between 0–10 s after membrane seal was 27 ± 3% ($n=9$) in STZ-diabetic muscle cells (Table 1). This value was significantly less than 53 ± 3% ($n=10$) obtained in normal muscle cells. In neither Ca^{2+}-free

Table 1 Extracellular calcium dependence on resting membrane potentials (RMP) and acetylcholine (ACh)-activated channel currents in normal and streptozotocin (STZ)-diabetic skeletal muscle cells

Conc. of Ca^{2+} (mM)	n	*RMP* (mV)	*Conductance* (pS)	*Mean open time* (ms)	*Opening frequency* (Hz) *0–10 s (A)*	*13–16 min (B)*	*(B)/(A) (%)*
Normal							
0	7	−76±2	80±2	0.44±0.01	11.0±2.1	7.6±1.2	72±3
2.5	10	−76±1	64±1	1.19±0.03	10.2±0.6	5.2±0.3	53±3
5	7	−74±2	65±1	1.35±0.03	8.7±0.8	2.1±0.3	25±4
STZ							
0	8	−75±2	79±1	0.47±0.01	9.6±0.8	6.8±0.7	72±4
2.5	9	−74±1	67±1	1.13±0.03	9.6±0.7	2.4±0.2	27±3**
5	8	−76±2	67±2	1.28±0.03	7.8±0.7	1.3±0.2	17±2

ACh (1 μM)-activated channel currents were recorded continuously at the endplate immediately after seal formation. A patch pipette contained various concentrations of Ca^{2+} (0, 2.5 and 5 mM). Mean open time was estimated from the currents recorded at RMP. Significant differences (**$P<0.01$) were detected by an unpaired t test between normal and STZ. Each value represents mean±s.e. (n: number of observations).

nor 5 mM Ca^{2+} were there significant differences between the values in normal and diabetic cells. No significant differences between the values in normal and diabetic cells were observed for initial channel opening frequency estimated at 0–10 s after membrane seal, channel conductance and mean open time of channel currents, and resting membrane potential in 0, 2.5, and 5 mM Ca^{2+}.

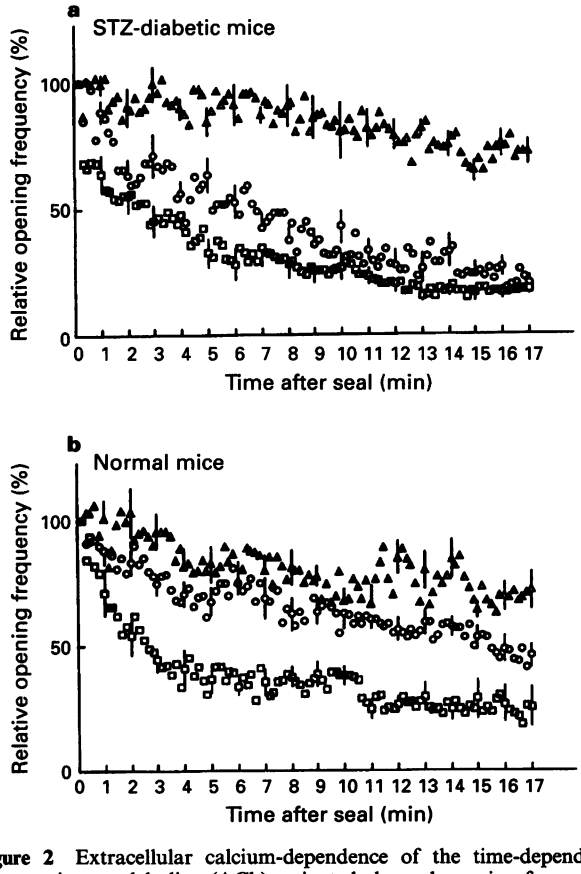

Figure 2 Extracellular calcium-dependence of the time-dependent decrease in acetylcholine (ACh)-activated channel opening frequency in normal (b) and streptozotocin (STZ)-diabetic skeletal muscle cells (a) of mice. A patch pipette contained ACh (1 μM) with various concentrations of Ca^{2+} (△ 0 mM $CaCl_2$+1 mM EGTA; ○ 2.5 mM $CaCl_2$; □ 5 mM $CaCl_2$). Relative channel opening frequency was expressed as a percentage of initial frequency in each condition. Each point represents the mean of 7–10 patches in normal mice and 8–9 patches in STZ-diabetic mice. Bars show s.e. values at every minute. Results of data analysis are shown in Table 1.

Recovery effect of staurosporine on the diabetic state-augmented desensitization of nicotinic ACh receptor

In the presence of 2.5 mM Ca^{2+}, pretreatment with 10 nM staurosporine (a PK-C inhibitor, Tamaoki *et al.*, 1986) significantly restored the diabetic state-induced accelerated decrease in channel opening frequency (from 27% to 42% in relative opening frequency) (Figure 3 and Table 2). Pretreatment with 3 μM H-89 (a PK-A inhibitor, Chijiwa *et al.*, 1990) and 5 μM KN-62 (a calmodulin kinase II inhibitor, Tokumitsu *et al.*, 1990) had no effect on the time-dependent change in diabetic cells. In normal muscle cells, the rate of the time-dependent decrease in the channel opening frequency did not change with pretreatment of these protein kinase inhibitors. These inhibitors did not affect the values of initial opening frequency, conductance, mean open time of channel currents, or resting membrane potentials in either diabetic or normal cells (data not shown).

Discussion

We demonstrated that the diabetic state accelerated the progression of agonist-induced desensitization of nicotinic AChR at the endplate. The decline in trains of ACh potentials evoked at high frequency was due to desensitization of nicotinic AChR (Anwyl & Narahashi, 1980), and this desensitization develops with two components at the frog neuromuscular junction (Feltz & Trautmann, 1982). In this study, the decline in ACh potentials was also observed to occur in two phases in mouse diaphragm muscle, and only the slow component of desensitization was frequency-dependently enhanced in the diabetic state. Intracellular Ca^{2+} binds directly to the nicotinic AChR (Chang & Neumann, 1976) and/or to internal sites of the post-junctional membrane (Nastuk & Parsons, 1970), thereby accelerating the slow component of desensitization of the receptor (Chesnut, 1983). We propose that the diabetic state-induced acceleration of the nicotinic AChR desensitization may be caused by the localized increase of $[Ca^{2+}]_i$ at the internal surface of the muscle membrane.

Several findings partly support our view that the resting $[Ca^{2+}]_i$ may rise during the diabetic state: (1) the amount of 92K dalton protein is decreased due to degradation by the increased activity of calpain (Ca^{2+}-activated neutral protease) in hind-limb skeletal muscle of diabetic mice (Kobayashi *et al.*, 1989); (2) the Ca^{2+} content of gastrocnemius muscle is increased in alloxan-diabetic mice (Nakagawa *et al.*, 1989), and (3) intracellular Ca^{2+} mobilization evoked by direct stimulation of alloxan-diabetic diaphragm muscle becomes insensitive to external Ca^{2+}, but sensitivity to external Ca^{2+} can be restored by intracellular injection of the Ca^{2+} chelator EGTA into the diabetic muscle fibres (Kimura *et al.*, 1990).

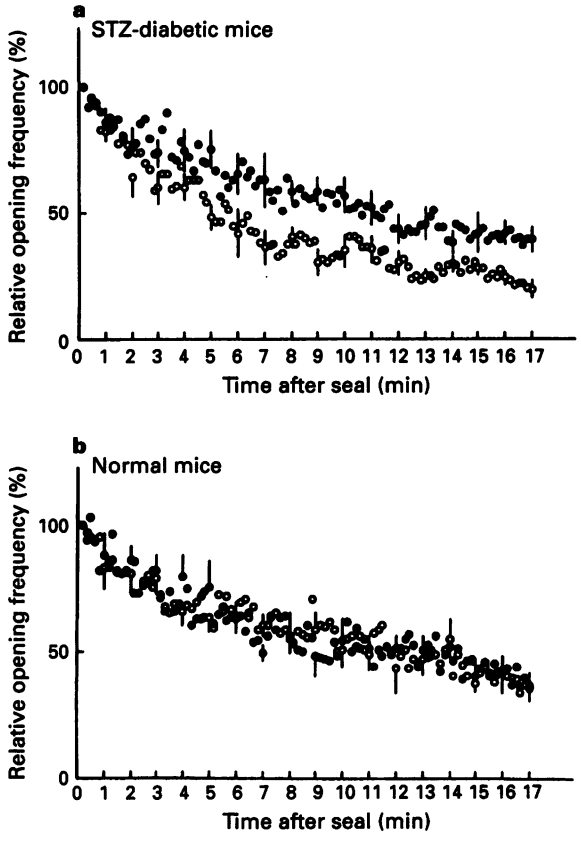

Figure 3 Restoring effect by staurosporine on the time-dependent decrease in acetylcholine (ACh)-activated channel opening frequency in (a) streptozotocin (STZ)-diabetic skeletal muscle cells; (b) normal cells. ACh (1 μM)-activated channel currents were recorded continuously after 1 h pretreatment with (●) or without (○) 10 nM staurosporine in 2.5 mM Ca^{2+}. Relative channel opening frequency was expressed as a percentage of initial frequency in each condition. Each point represents mean of 6 patches in normal mice and 7–8 patches in STZ-diabetic mice. Bars show s.e. values at every minute. Results of data analysis are shown in Table 2.

Table 2 Effects of protein kinase inhibitors on acetylcholine (ACh)-activated channel opening frequency in normal and streptozotocin (STZ)-diabetic skeletal muscle cells

Treatment	n	*Relative opening frequency (%) 0–3 min*	*13–16 min*	
Normal				
Vehicle (0.05% DMSO)	7	83±5	46±5	
Staurosporine (10 nM)	6	85±3	46±4	
H-89 (3 μM)	7	83±5	39±5	**
KN-62 (5 μM)	5	84±4	42±3	
STZ-diabetic				
Vehicle (0.05% DMSO)	7	79±4	27±2	
Staurosporine (10 nM)	8	85±5	42±5	*
H-89 (3 μM)	7	69±6	31±3	
KN-62 (5 μM)	7	72±5	23±4	

ACh (1 μM)-activated channel currents were recorded continuously at the endplate in the presence of 2.5 mM $CaCl_2$. The single cells were pretreated with protein kinase inhibitors for 1 h. Relative channel opening frequency is expressed as a percentage of initial frequency in each condition. Significant differences: $*P<0.05$ and $**P<0.01$ were detected by an unpaired t test. Each value represents mean ± s.e. (n: number of observations).

The development of desensitization of the nicotinic AChR channel has been demonstrated by the time-dependent decrease in channel opening frequency using patch clamp technique (Dionne, 1989; Tattersall, 1990; Nojima *et al.*, 1994). In the present study, we found that the time-dependent decrease in channel opening frequency of STZ-diabetic muscle cells was greatly accelerated compared with normal cells in 2.5 mM Ca^{2+}. The decrease may be already refractory at 2.5 mM Ca^{2+} in the diabetic state, but the higher concentration of 5 mM Ca^{2+} may be required in the normal state. Extracellular calcium-dependence of nicotinic AChR desensitization was altered steeply in the diabetic state.

The enhanced rate of the time-dependent decrease in channel opening frequency was prevented by pretreatment with a PK-C inhibitor, but not by pretreatment with a PK-A inhibitor or a calmodulin kinase II inhibitor. These results demonstrate that the desensitization of nicotinic AChR channels in the diabetic state was accelerated by activation of PK-C. In glomeruli isolated from the STZ-diabetic rat, PK-C activation is demonstrated by an increase in the amount of enzyme associated with the membrane fraction (Craven & DeRubertis, 1989). The inhibition of PK-C prevents the decrease in Na^+ and K^+-ATPase activity in the sciatic nerve of alloxan-induced diabetic mice (Hermenegildo *et al.*, 1992). Therefore, the abnormal activation of PK-C may play a key role in the dysfunction of the nicotinic AChR channel in skeletal muscle cells of diabetic mice. Increasing the Ca^{2+} concentrations in a patch pipette reduced the conductance of ACh-activated channel currents and prolonged their mean open time, as shown in previous experiments (Nojima *et al.*, 1992). This modulation of channel activities may be caused by Ca^{2+} influx through the nicotinic AChR channels. The level of 1,2-diacylglycerol, an endogenous activator of PK-C, is increased in the small intestines of STZ-diabetic rats (Wali *et al.*, 1990), which may be involved in the accelerated process of nicotinic AChR desensitization through activation of PK-C. In this study, we showed that the progressive decrease in channel opening frequency of STZ-diabetic muscle cells may be caused by Ca^{2+} influx through the activated nicotinic AChR channels, because this event was shown to be dependent upon Ca^{2+} in the extracellular space.

In the diabetic state, nicotinic AChR channels may be desensitized readily by activation of the PK-C phosphorylation system, and this event may be caused by an increase in the amount of intracellular Ca^{2+}.

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