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Diabetic state-induced rapid inactivation of noncontractile Ca^{2+} mobilization operated by nicotinic acetylcholine receptor in mouse diaphragm muscle

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1 Diabetic modifications of nicotinic receptor-operated noncontractile Ca^{2+} mobilization observed in the presence of anticholinesterase were investigated by measuring Ca^{2+}-aequorin luminescence in diaphragm muscles of mice with diabetes induced by injections of streptozotocin (150 mg kg^{-1}, bolus i.v.) and alloxan (85 mg kg^{-1}, bolus i.v.).

2 The diabetic state accelerated the decline of noncontractile Ca^{2+} transients without affecting their peak amplitude. Insulin treatment reversed this alteration.

3 The increase in contractile Ca^{2+} transients by cholinesterase inhibition was attenuated 0.6 fold and became resistant to changes in $[Ca^{2+}]_o$ in the diabetic state.

4 Changes in extracellular pH from 7.6 to 5.6 depressed the peak amplitude of noncontractile Ca^{2+} transients without affecting their duration, and enhanced the peak amplitude of contractile Ca^{2+} transients.

5 These results suggest that the inactivation process of noncontractile Ca^{2+} mobilization is promoted in diabetic muscles, presumably by desensitization of the nicotinic acetylcholine receptor.

Keywords: Diabetes mellitus, nicotinic acetylcholine receptor, noncontractile calcium, desensitization, neuromuscular junction

Introduction

Diabetes is associated with various biochemical (Flaim *et al.*, 1980; Ganguly *et al.*, 1986), morphological (Chao *et al.*, 1976), electrophysiological (Grossie, 1982), and mechanical defects in skeletal muscle (Paulus & Grossie, 1983). The regulating system for intracellular Ca^{2+} is also modified in the diabetic muscle: diabetes induces an increase in Ca^{2+} pump activity of the sarcoplasmic reticulum and sarcolemma (Ganguly *et al.*, 1986; Taira *et al.*, 1991), an increase in Na^+ -Ca^{2+} exchange activity (Taira *et al.*, 1991) and an increase in the number of Ca^{2+} channels in the sarcolemma (Lee & Dhalla, 1992). These observations led us to examine the influence of the diabetic state on the nicotinic acetylcholine receptor (AChR)-operated noncontractile Ca^{2+} mobilization at the neuromuscular junction.

Noncontractile Ca^{2+} transients are generated by the continued action of acetylcholine (ACh) accumulated in the presence of anticholinesterase (Kimura *et al.*, 1989). The noncontractile Ca^{2+} mobilization is not due to Ca^{2+} release from the sarcoplasmic reticulum but closely related to Ca^{2+} influx through nicotinic AChR-channels, although the mobilization is considered to be independent of a component of endplate current (Kimura, I. *et al.*, 1991; Kimura, M. *et al.*, 1991).

In the present study, we demonstrated that the receptor-operated noncontractile Ca^{2+} mobilization is inhibited in diabetic skeletal muscle. To investigate whether the modification is related to diabetic ketoacidosis, we further examined the influence of extracellular pH changes on noncontractile Ca^{2+} mobilization.

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Methods

Animals

Male ddY mice (7-9 weeks old, 35.0±0.8 g, blood glucose (BG) levels: 123±11, $n=9$) were used as normal mice. Streptozotocin (STZ, 150 mg kg^{-1}) or alloxan (85 mg kg^{-1}) in saline solution was injected in bolus form into the tail vein of 4-week-old male ddY mice. Four to five weeks after injection, a marked increase in BG levels was observed in STZ-diabetic (32.4±0.8 g, BG: 502±30 mg dl^{-1}, $n=9$) and alloxan-diabetic mice (29.4±1.0 g, BG: 545±18 mg dl^{-1}, $n=8$). Blood samples were obtained from the orbital vein plexus. BG levels were measured by the glucose oxidase method on a glucose analyzer (Beckman, CA, U.S.A.). The animals in the insulin-treated diabetic group received 100 u kg^{-1} s.c. of insulin daily for 8 days before being killed at 8 weeks.

Measurements of twitch tension

The diabetic and normal control mice were decapitated and exsanguinated. A segment of right phrenic nerve-diaphragm muscle was isolated and fixed in a chamber. The resting tension was adjusted to 200 mg. Modified Krebs solution (mM: NaCl 122, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5 and glucose 11.5) was equilibrated with 95% O_2 and 5% CO_2, maintained at 36°C, and perfused through the chamber. The phrenic nerve was stimulated at 0.1 Hz with supramaximal square pulses (0.6-1 V) of 0.1 ms duration. Isometric tension was recorded on a polygraph (San-ei, Tokyo, Japan) coupled to a carrier amplifier (Nihon Kohden, Tokyo, Japan) via an isometric transducer (Nihon Kohden).

Measurement of Ca^{2+} transients

We adopted the same procedures to measure Ca^{2+} -aequorin luminescence (Ca^{2+} transients) as in previous studies (Kimura *et al.*, 1990b). The aequorin solution (1 mg ml^{-1}) was pressure-injected by nitrogen gas at 5 to 6 atm and 6 to 8 atm for

2 s through a micropipette into the myoplasm in the endplate regions of muscle isolated from normal and diabetic mice, respectively. Ca^{2+} transients emitted from the injected area were measured with a photon counter (Hamamatsu Photonics, Shizuoka, Japan) and a photomultiplier tube (Hamamatsu Photonics) attached to an acrylic optical fibre (Ryo-mi Plastics, Toyama, Japan). To avoid qualitative changes in response due to the amount loaded, a similar amount of aequorin solution was usually injected into 30 to 50 fibres of the normal, STZ- and alloxan-diabetic muscle. Peak amplitudes of Ca^{2+} transients in each muscle were 6.9 ± 1.1 ($n=9$), 6.1 ± 1.0 ($n=9$) and 5.2 ± 0.5 kilocounts per second (kcps) ($n=8$), respectively. The signals were averaged 30 times by a signal processor (Sanei) to improve the signal-to-noise ratio.

Experimental protocol and analysis

Neostigmine (0.3 μM) was applied for 15 min, then the external Ca^{2+} concentration ($[Ca^{2+}]_o$) was changed at 10-min intervals in the presence of neostigmine. For the pH studies, a muscle segment was preincubated at the test pH for 15 min, then the neostigmine solution at the same pH was applied for 15 min. The averaged records for 30 signals of Ca^{2+} transients obtained during the last 5 min were analyzed. The averaged signals were vertically expanded by using the Y-range of the signal processor to determine the peak points of noncontractile Ca^{2+} transients. The normalization procedures were as follows. The peak amplitude of Ca^{2+} transients was expressed as a percentage of the averaged control values for 30 contractile Ca^{2+} transients obtained in the absence of neostigmine, at normal $[Ca^{2+}]_o$ (2.5 mM) and normal pH (7.6) of modified Krebs solution. The duration of noncontractile Ca^{2+} transients consists of rise time (T_1) and decay time (T_2). Rise time represents time to peak, and decay time represents the period from peak to e^{-1} amplitude of the noncontractile Ca^{2+} transients.

Drugs and solutions

Aequorin (Wako Pure Chemical, Osaka, Japan), alloxan monohydrate (Nacalai Tesque, Kyoto, Japan), insulin (ultralente: bovine 40 u ml^{-1}; Novo, Denmark), 2-[*N*-morpholino] ethanesulphonic acid (Mes; Sigma Chemical, St. Louis, MO, U.S.A.), neostigmine methylsulphate (Sigma) and streptozotocin (Sigma) were used.

The pH was changed by adding Mes (5 mM) to modified Krebs solution, and was adjusted with HCl or NaOH.

Statistical analysis

Data are expressed as means $\pm$ s.e. One-way analysis of variance (ANOVA) and Scheffe multiple-comparison test were used to evaluate statistical differences between the means. Only the statistical differences of the data in Figure 2 were determined by Student's unpaired *t* test, since numbers of mice used were quite different between the insulin-treated and non-treated experiments. $P<0.05$ and $P<0.01$ were used as the limit for statistical significance.

Results

Inhibitory influence of STZ- and alloxan-diabetes on Ca^{2+} mobilization

Diabetic state-induced alterations of Ca^{2+} transients and twitch tension in the presence of neostigmine (0.3 μM) were examined in STZ- and alloxan-diabetic diaphragm muscles. Although contractile Ca^{2+} transients and twitch tension were increased by treatment with neostigmine in the diabetic muscles, the increase in contractile transients was attenuated to 0.6 fold in either type of diabetic muscle, compared with that in normal muscle (Figures 1, 2a). Likewise, twitch tension was attenuated to 0.8 fold in both diabetic states (Figure 3c: $[Ca^{2+}]_o$ = 2.5 mM). Daily injection for the last 8 days with insulin (100 u kg^{-1} s.c.) in the STZ-diabetic mouse (7 weeks old) completely reversed the plasma glucose level (91 ± 16 mg dl^{-1}, $n=4$), and partially prevented the diabetic state-induced attenuation of contractile Ca^{2+} transients (Figure 2a).

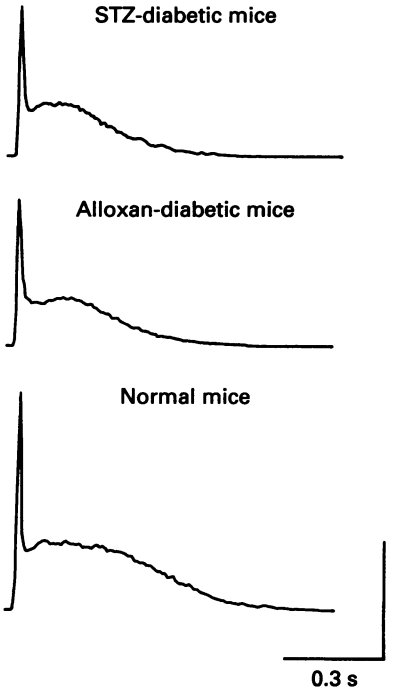

Figure 1 Typical traces of Ca^{2+} transients elicited by nerve stimulation in the presence of 0.3 μM neostigmine in the diabetic and normal muscles. These traces are averaged records for 30 signals of Ca^{2+} transients 10–15 min after application of neostigmine to diaphragm muscles isolated from STZ-diabetic (upper), alloxan-diabetic (middle) and normal mice (lower). The large, rapid increase in Ca^{2+} represents the contractile transients, and the slower prolonged increase represents the noncontractile transients. Ordinates calibration bar represents 100% amplitude of contractile Ca^{2+} transients before application of neostigmine. Similar results were obtained in 8 to 9 separate experiments.

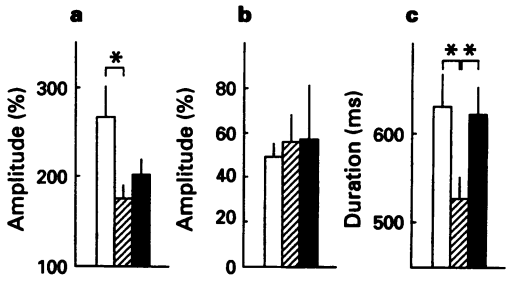

Figure 2 Diabetic modifications of contractile and noncontractile Ca^{2+} mobilization in the presence of 0.3 μM neostigmine. One group of the STZ-diabetic mice was given subcutaneous injections of 100 u kg^{-1} insulin (ultralente) daily for the last week before death at 8 weeks. (a) Contractile Ca^{2+} transients in the diaphragm muscle of normal (open column), STZ-diabetic (hatched column) and insulin-treated diabetic mice (solid column). (b and c) Peak amplitude and duration, of noncontractile Ca^{2+} transients, respectively. Peak amplitude of either type of Ca^{2+} transients is expressed as a percentage of 30 contractile Ca^{2+} transients obtained 5–0 min before the application of neostigmine. The data are means $\pm$ s.e. of 30 Ca^{2+} transient signals obtained 10–15 min after the neostigmine application in 4 to 12 separate experiments. *$P<0.05$; significantly different from the values in normal muscles, based on Student's *t* test.

Noncontractile Ca^{2+} transients were generated 4.5 ± 0.4 min ($n=11$) after neostigmine was applied to the normal muscle preparations. In diabetic muscles, the beginning of the mobilization of noncontractile transients was more rapid: lag times in STZ- and alloxan-diabetic muscles were significantly decreased to 2.7 ± 0.3 min ($n=9$, $P<0.01$) and 3.3 ± 0.2 min ($n=14$, $P<0.05$), respectively. Moreover, the duration of noncontractile Ca^{2+} transients was shortened 0.8 fold in either type of diabetic muscle (Figures 1, 2c), whereas the peak amplitude of noncontractile transients was not affected (Figure 2b). The mode of shortening of noncontractile Ca^{2+} transients by the diabetic state was further analyzed in terms of rise time (T_1) and decay time (T_2, see methods), the components of total duration. T_1 of noncontractile transients observed in the normal state (151 ± 10 ms, $n=10$) was equal to that in the STZ-diabetic state (121 ± 10, $n=9$) and in the al-

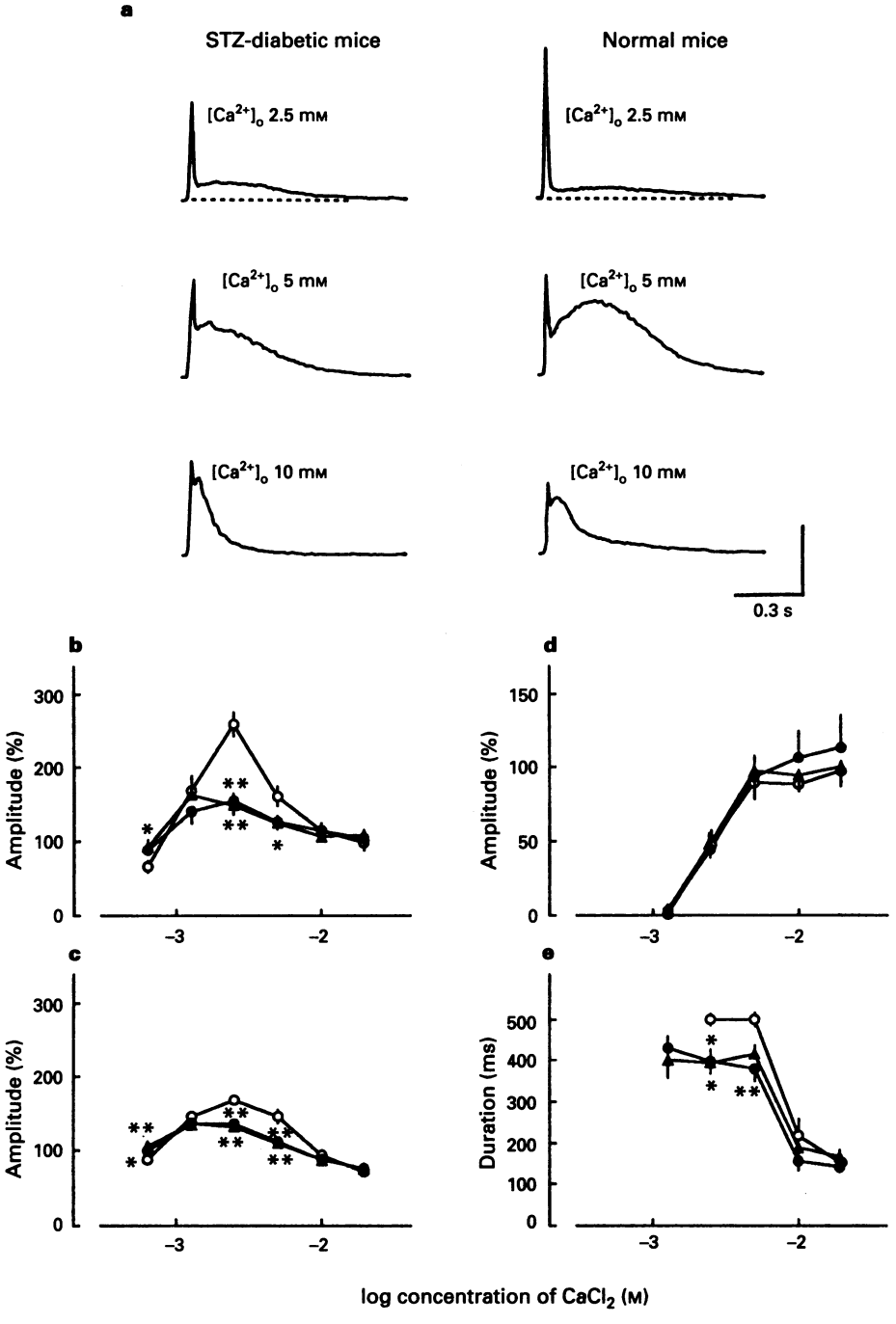

Figure 3 $[Ca^{2+}]_o$-dependence of Ca^{2+} transients and twitch tension in the presence of 0.3 μM neostigmine in diaphragm muscles of diabetic and normal mice. Neostigmine was applied for 15 min, then $[Ca^{2+}]_o$ was changed at 10 min intervals. (a) Typical traces of averaged records for 30 signals of Ca^{2+} transients in the STZ-diabetic state (left) and normal state (right). Upper: response in normal Krebs $[Ca^{2+}]_o$ (2.5 mM) after application of neostigmine; middle: response at the 2 fold higher $[Ca^{2+}]_o$ (5 mM), and lower: response at the 4 fold higher $[Ca^{2+}]_o$ (10 mM). Ordinates calibration bar represents 5 kcps. Log concentration-response curves for $CaCl_2$ on peak amplitude of (b) contractile Ca^{2+} transients, (c) twitch tension, (d) noncontractile Ca^{2+} transients, and (e) on duration of noncontractile transients observed in the presence of neostigmine in normal (○), STZ-diabetic (●), and alloxan-diabetic state (▲). Peak amplitudes of contractile and noncontractile Ca^{2+} transients are expressed as percentages of contractile Ca^{2+} transients obtained before the application of neostigmine. Peak amplitude of twitch tension is expressed as percentage of its own response before neostigmine application. The data given are means ± s.e. of 8 to 10 separate experiments. $^*P<0.05$; $^{**}P<0.01$, significantly different from the values in normal muscles, based on one-way ANOVA (Scheffe multiple-comparison test).

loxan-diabetic state (147±14 ms, $n=8$). On the other hand, T_2 was markedly decreased by the alloxan-diabetic state (245±19 ms, $n=8$, $P<0.01$), from 346±14 ms ($n=10$) in the normal state, although T_2 was slightly decreased in the STZ-diabetic state (276±26 ms, $n=9$). The decrease in T_2 indicates that the decline of noncontractile Ca^{2+} transients was accelerated in the diabetic state. Insulin injection into STZ-diabetic mouse reversed the duration of noncontractile Ca^{2+} transients to the normal level (Figure 2c).

Diabetic modifications of $[Ca^{2+}]_o$-dependence of contractile Ca^{2+} transients, noncontractile Ca^{2+} transients and twitch tension were investigated quantitatively, by changing $[Ca^{2+}]_o$ from 2.5 mM (normal $[Ca^{2+}]_o$). In normal muscles, optimal $[Ca^{2+}]_o$ was 2.5 mM for contractile Ca^{2+} transients and twitch tension (Figures 3b,c): these contractile responses were decreased either by lower $[Ca^{2+}]_o$ (0.63–1.3 mM) or by higher $[Ca^{2+}]_o$ (5–20 mM). In alloxan-diabetic muscles, the increases in the contractile responses were attenuated at 2.5–5 mM $[Ca^{2+}]_o$, whereas they were rather enhanced at 0.63 mM, compared with those in normal muscles. Similar $[Ca^{2+}]_o$ -response curves for contractile Ca^{2+} transients and twitch tension were also obtained in STZ-diabetic muscle. Consequently, these contractile responses appeared to become resistant or insensitive to the changes in $[Ca^{2+}]_o$ in the diabetic state. On the other hand, the optimal $[Ca^{2+}]_o$ for noncontractile Ca^{2+} transients was 5 mM in both the normal and diabetic state (Figure 3a): the peak amplitude of noncontractile transients was increased 2 fold by higher $[Ca^{2+}]_o$ (5-20 mM) and was decreased by lower $[Ca^{2+}]_o$ (1.3 mM), compared with the amplitude at normal $[Ca^{2+}]_o$ of the nutrient solution (2.5 mM) (Figure 3d). The duration of noncontractile transients was unchanged by 2.5–5 mM $[Ca^{2+}]_o$ and shortened by 10-20 mM $[Ca^{2+}]_o$ in both the normal and diabetic muscles (Figure 3e), although the duration at 2.5–5 mM $[Ca^{2+}]_o$ was shortened by 20% in both types of diabetic muscles compared with normal muscle. These results indicate that $[Ca^{2+}]_o$ -dependence of peak amplitude and duration of noncontractile Ca^{2+} transients was not affected by the diabetic state.

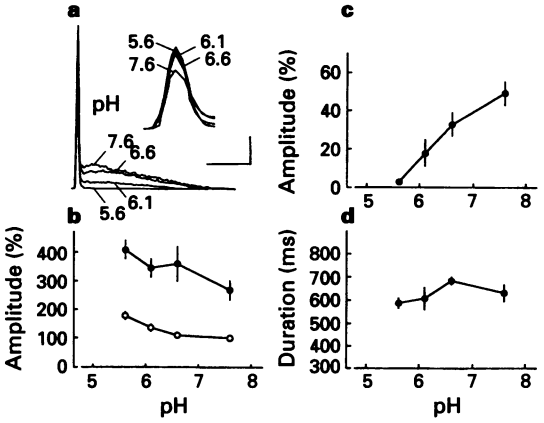

Figure 4 pH-dependence of contractile and noncontractile Ca^{2+} mobilization in normal diaphragm muscle treated with 0.3 μM neostigmine. Extracellular pH was changed 15 min before the application of neostigmine. (a) Typical traces of averaged records for 30 signals of Ca^{2+} transients at acidic pH. The data obtained from separate preparations were normalized and superimposed, using a digitizer (Graphtec, Tokyo, Japan). Abscissa and ordinate scale calibration bars represent 300 ms and 50% amplitude of contractile Ca^{2+} transients obtained before neostigmine exposure, respectively. Inset: the contractile component of typical traces. The traces of Ca^{2+} transients were expanded 10 fold along the time axis, to demonstrate clearly the changes in the contractile Ca^{2+} transients induced by acidic pH. In this case, the abscissa and ordinate calibration bars indicate 30 ms and 100% amplitude, respectively. (b) Enhancing effects of pH on contractile Ca^{2+} transients before the application of neostigmine (○), and on the increase in the contractile Ca^{2+} transients by the application of neostigmine (●). (c and d) Effects of pH on peak amplitude and duration, respectively of noncontractile Ca^{2+} transients in the presence of neostigmine. Peak amplitudes of both types of Ca^{2+} transients are expressed as percentages of 30 contractile Ca^{2+} transients obtained 5–0 min before the changes in extracellular pH. The data given are means±s.e. of 4 to 9 separate experiments.

Reverse effects of acidic pH on noncontractile and contractile Ca^{2+} mobilization

Effects of acidic pH on Ca^{2+} mobilization were examined in normal muscles, for comparison with diabetic modifications. Before the application of neostigmine, contractile Ca^{2+} transients were potentiated by treating with acidic pH solution. The potentiation was dependent on the reduction of pH from 7.6 (normal pH of nutrient solution) to 5.6 (Figure 4b). Twitch tension was not altered under such conditions (data not shown).

After the application of neostigmine (0.3 μM), contractile and noncontractile Ca^{2+} transients were modified by the pH as shown in Figure 4a. The contractile Ca^{2+} transients were enhanced by acidic pH (5.6–6.6) (Figure 4b), compared with that normally observed at pH 7.6. However, the extent of increase in contractile Ca^{2+} transients induced by neostigmine application was rather diminished at the lower pH (5.6–6.1) as observed in the diabetic state, since these pH levels potentiated the contractile transients before neostigmine application. In contrast, the peak amplitude of noncontractile Ca^{2+} transients was reversibly depressed when extracellular pH was reduced from 7.6 to 5.6 (Figure 4c). This pH dependence had a pK of 6.3. The duration of noncontractile Ca^{2+} transients was not affected by the acidic pH (Figure 4d).

Discussion

Noncontractile Ca^{2+} mobilization requires the prolonged action of ACh accumulated in the synaptic cleft of nicotinic AChR (Kimura *et al.*, 1989). Desensitization of nicotinic AChR can readily occur to nerve stimulation under such conditions (Magleby & Pallotta, 1981). Our recent study has shown that the diabetic state greatly accelerated the decline in trains of ACh potentials evoked iontophoretically at high frequency and the time-dependent decrease in the AChR-channel opening frequency, indicating the enhancement of AChR desensitization by diabetes (Nojima *et al.*, 1995). We have shown here that the duration, especially the decay time (duration of inactivation phase), of noncontractile Ca^{2+} transients was shortened in the diabetic state, although their peak amplitude was not affected. Nicotinic AChR closed channel blockers are reported to shorten the duration of noncontractile Ca^{2+} transients by enhancing the receptor desensitization (Kimura, M. *et al.*, 1991). In addition, the decay time of the noncontractile Ca^{2+} transients is selectively shortened by low concentrations of competitive nicotinic antagonists, and reflects the period of functionally available state of nicotinic AChR operating the noncontractile Ca^{2+} mobilization (Tsuneki *et al.*, 1994). Therefore, the diabetic state-induced shortening of the decay time is considered to be caused by promoting the inactivation process of nicotinic AChR. We suggest that the desensitization of nicotinic AChR, enhanced in the diabetic state, may account for the acceleration of the decline of noncontractile Ca^{2+} transients.

Since the diabetic modifications of noncontractile Ca^{2+} mobilization were reversible upon treatment of the diabetic mouse with insulin, they may be a consequence of hypoinsulinaemia and concomitant metabolic abnormalities. Some of these abnormalities caused an increase in Ca^{2+} -pump activity in the sarcoplasmic reticulum and sarcolemma of diabetic muscle (Ganguly *et al.*, 1986; Taira *et al.*, 1991). Therefore, the shortening effect of diabetes on the noncontractile Ca^{2+} transients may be in part due to activation of the Ca^{2+} -pump. Alternatively, the diabetic effect may be explained by a decrease in ACh release from the nerve terminal (Kimura *et al.*, 1993). Neither acetylcholinesterase nor pseudocholinesterase

activity is affected by the diabetic state in skeletal muscles (Matsui *et al.*, 1990), suggesting that the susceptibility to anticholinesterase may not be altered in the diabetic state.

We further demonstrated here that contractile Ca^{2+} transients were not markedly increased by neostigmine application to the diabetic muscle, and then became somewhat resistant to changes in $[Ca^{2+}]_o$, compared with those in the normal muscle. Intracellular Ca^{2+} mobilization evoked by direct stimulation of alloxan-diabetic diaphragm muscle was also shown to be insensitive to the $[Ca^{2+}]_o$ changes, and the sensitivity to $[Ca^{2+}]_o$ was restored by intracellular injection of the Ca^{2+} chelator EGTA, into the diabetic muscle fibres (Kimura *et al.*, 1990a). This raises the possibility that the resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) may be elevated in diabetic muscles. Intracellular Ca^{2+}, directly binding to the nicotinic AChR and/or to the internal sites of post-junctional membrane, enhances the receptor desensitization (Nastuk & Parsons, 1970; Chang & Neumann, 1976; Miledi, 1980). Hence, we suggest that the attenuation of contractile Ca^{2+} transients in the diabetic state may be related to the enhancement of the AChR desensitization via the elevation of resting $[Ca^{2+}]_i$. These diabetic alterations of contractile Ca^{2+} mobilization were not completely restored by treatment with insulin, despite the recovery of BG levels. Moreover, abnormalities in resting membrane potential and conductance do not return to normal levels with insulin treatment (Kimura *et al.*, 1988). Therefore, chronic diabetes may cause the secondary defects in the contractile Ca^{2+} mobilization system of skeletal muscle, in addition to the influence of hyperglycaemia.

Chronic diabetes is known to be accompanied by the ketoacidosis (Taylor & Agius, 1988). In the present study, the increase in contractile Ca^{2+} transients by neostigmine application was attenuated at acidic pH, although contractile Ca^{2+} transients were enhanced by acidic pH alone. The diabetic ketoacidosis may contribute to secondary defects in contractile Ca^{2+} mobilization, resulting in attenuation of the increase in contractile Ca^{2+} transients by cholinesterase inhibition in the diabetic state. In addition, the peak amplitude, but not the duration, of noncontractile Ca^{2+} transients was depressed by acidic pH, whereas their duration was shortened by diabetes. The depressant effect of the pH may be due to the inactivation of the nicotinic AChR-channel, because the changes in extracellular pH alter the gating and permeability properties of the nicotinic AChR-channel from *Torpedo* electroplax and mouse muscle cells: acidic pH decreases both the single channel conductance and the mean open time (Pappone & Barchfeld, 1990; Palma *et al.*, 1991), and accelerates the rate of AChR desensitization (Li & McNamee, 1992). Although the diabetic ketoacidosis cannot completely desensitize the nicotinic AChR to depress the peak amplitude of noncontractile Ca^{2+} transients because of its weak acidity, the chronic action of the ketoacidosis may partly contribute to the shortening of the noncontractile Ca^{2+} transients by converting the AChR into a readily desensitizable state.

In conclusion, the inactivation process of noncontractile Ca^{2+} mobilization is promoted in diabetic muscles, presumably by desensitization of the nicotinic acetylcholine receptor.

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