Postsynaptic nicotinic receptor desensitized by non-contractile Ca^{2+} mobilization via protein kinase-C activation at the mouse neuromuscular junction

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1 Non-contractile Ca^{2+} mobilization (unaccompanied by muscle contraction) was initiated by nerve stimulation in the presence of neostigmine (more than $0.03 \mu\text{M}$) at the endplate region of mouse diaphragm muscles. In the process of nicotinic receptor desensitization, the depressant effect of non-contractile Ca^{2+} on contractile Ca^{2+} mobilization was investigated by measurement of Ca^{2+} -aequorin luminescence.

2 When the phrenic nerve was stimulated with paired pulses having intervals of 150, 300, 600, 1000 and 2000 ms, contractile Ca^{2+} transients were elicited during the generation of non-contractile Ca^{2+} mobilization. The amplitude of the contractile Ca^{2+} transients elicited by the second pulse (S_2) was depressed at the shorter pulse intervals, but recovered to the initial contractile response (S_1) at longer pulse intervals.

3 The extent of depression of S_2 was enhanced by increasing the concentration of neostigmine (0.03 to $0.3 \mu\text{M}$). When a low concentration ($0.05 \mu\text{M}$) of pancuronium, a competitive nicotinic antagonist, completely blocked non-contractile Ca^{2+} mobilization, the depression of S_2 was diminished.

4 The depression of S_2 was enhanced when the peak amplitude of non-contractile Ca^{2+} mobilization was raised by increasing the external Ca^{2+} concentration from 1.3 to 5 mM .

5 Staurosporine (10 nM), a protein kinase-C inhibitor, diminished the depression of S_2 despite large amounts of non-contractile Ca^{2+} mobilization. The diminishing effect of staurosporine was counteracted by TPA ($0.1 \mu\text{M}$), a protein kinase-C activator.

6 These findings suggest that non-contractile Ca^{2+} mobilization may enhance the desensitization of the postsynaptic nicotinic receptor via activation of protein kinase-C at the neuromuscular junction.

Keywords: Nicotinic acetylcholine receptor; non-contractile calcium; protein kinase-C; desensitization; neuromuscular post-synapse

Introduction

We previously reported that contractile and non-contractile Ca^{2+} mobilization is generated simultaneously at the neuromuscular junction by nerve stimulation in the presence of anticholinesterase agents (Kimura *et al.*, 1989). The mechanism of non-contractile Ca^{2+} mobilization is completely different from that of contractile Ca^{2+} mobilization, because (1) non-contractile Ca^{2+} mobilization requires the prolonged activation of nicotinic acetylcholine receptors (AChRs) as a result of accumulating acetylcholine (ACh) in the synaptic cleft and is specifically blocked by competitive nicotinic antagonists such as (+)-tubocurarine and pancuronium at low concentrations that have no effect on contractile Ca^{2+} transients (Kimura *et al.*, 1989); (2) a higher concentration (5 mM) of external Ca^{2+} increases non-contractile Ca^{2+} mobilization, but decreases contractile Ca^{2+} transients (Kimura *et al.*, 1989); (3) non-contractile Ca^{2+} mobilization is not due to Ca^{2+} release from sarcoplasmic reticulum (SR) (Kimura *et al.*, 1991a,b) and (4) activation of protein kinase-A within muscle cells is essential to mobilize non-contractile Ca^{2+} (Kimura *et al.*, 1993).

Non-contractile Ca^{2+} is not linked to muscle contraction despite the fact that much larger amounts of Ca^{2+} are mobilized than during contractile Ca^{2+} mobilization. Non-contractile Ca^{2+} is mobilized under desensitizing conditions, since desensitization to nerve stimulation readily occurs after inhibiting cholinesterase (Magleby & Pallotta, 1981). The increase in intracellular Ca^{2+} accelerates the rate of nicotinic AChR desensitization in skeletal muscles (Manthey, 1970;

Nastuk & Parsons, 1970; Miledi, 1980). We therefore suspect that non-contractile Ca^{2+} may prevent the nicotinic AChR from responding to excessive stimulation by accumulated ACh. The functional role of non-contractile Ca^{2+} , however, has not been established. In the present study, we investigated the depressant effect of non-contractile Ca^{2+} on contractile Ca^{2+} mobilization using paired-pulse stimulation to elucidate the functional role of non-contractile Ca^{2+} mobilization in the process of nicotinic AChR desensitization.

Methods

Muscle preparations

Male ddY mice (7–9 weeks old, 28–42 g) were killed by decapitation. The right phrenic nerve-diaphragm muscle with the tendon was isolated, and cut into a strip 10 mm wide. The corner of a muscle strip was fixed with pins on rubber plates in a chamber. 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 perfused through the chamber at a rate of 1.5 ml min^{-1} . The tendon was tied with silk thread and connected to an isometric transducer (Nihon Kohden, Tokyo, Japan), and the resting tension was adjusted to 200 mg. The bath solution was maintained at 36°C by a heated copper plate under the chamber regulated by a thermomodule (Komatsu Electronics, Kanagawa, Japan). The solution was equilibrated with 95% O_2 and 5% CO_2 .

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Measurement of intracellular Ca^{2+} transients

We adopted the same procedures for measuring Ca^{2+} -aequorin luminescence (Ca^{2+} transients) as in the previous paper (Kimura *et al.*, 1990). The aequorin solution (1 mg ml^{-1}) was expelled from the micropipette into the end-plate region of 40 to 50 muscle fibres by 2 s nitrogen gas pressure pulses of $5\text{--}6 \text{ kg cm}^{-2}$ delivered by a pressure system having two pressure valves (J262D23, Asco, Toyama, Japan) operated electromagnetically. Ca^{2+} transients emitted from the aequorin-injected area were amplified with a photomultiplier tube (Hamamatsu Photonics, Shizuoka, Japan) and measured with a photon counter (Hamamatsu Photonics). The open gate time of the photon counter was set at 10 ms and the closed gate time at $20 \mu\text{s}$. To decrease photon loss, one end of an acrylic optical fibre (Ryo-mi Plastics, Toyama, Japan) was placed near the injected area, 0.5 mm away from the surface of the muscle, and the other end attached to the photomultiplier tube. When the phrenic nerve was stimulated supramaximally ($0.4\text{--}0.8 \text{ V}$) at 0.1 Hz with a 0.1 ms duration square pulse via a pair of platinum electrodes placed 1 mm apart, Ca^{2+} transients and twitch tension were recorded simultaneously. The signals were averaged with a signal processor (San-ei, Tokyo, Japan) to improve the signal-to-noise ratio. The records for 30 Ca^{2+} transient signals obtained 5 to 10 min after the generation of non-contractile Ca^{2+} mobilization were analysed. The normalization procedures were as follows. The peak amplitude of both non-contractile and contractile Ca^{2+} transients was expressed as a percentage of contractile Ca^{2+} transients obtained during the 5 to 0 min before neostigmine was applied. The duration of non-contractile Ca^{2+} mobilization was determined at e^{-1} amplitude of the signal.

Analysis of paired-pulse stimulation data

The phrenic nerve was stimulated with paired-pulses having intervals of 150, 300, 600, 1000 and 2000 ms, 5 min after the generation of non-contractile Ca^{2+} mobilization by application of neostigmine. The ratio (S_2/S_1) of the peak amplitude of contractile Ca^{2+} transients elicited by the second pulse (S_2) to that by the first pulse (S_1) was used to normalize the changes in S_2 . S_2 was calculated as the difference between the amplitude of non-contractile Ca^{2+} mobilization and the actually measured peak amplitude of contractile Ca^{2+} transients. A decrease in the S_2/S_1 value indicates the increased depression of S_2 .

Drugs and solutions

Aequorin (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, Wako Pure Chemical, Osaka, Japan) was dissolved in Ca^{2+} -free distilled water containing EDTA ($5 \mu\text{M}$). Caffeine (Nacalai Tesque, Tokyo, Japan), H-7 (Seikagaku, Tokyo, Japan), neostigmine methylsulphate (Sigma Chemical, MO, U.S.A.), 12-O-tetradecanoylphorbol 13-acetate (TPA, 0.01% N,N-dimethylformamide solution; Sigma), pancuronium bromide (Sankyo, Tokyo, Japan) and staurosporine (0.003–0.01% N,N-dimethylformamide solution; Kyowa Medix, Tokyo, Japan) were bath-applied.

Statistical analysis

Data are expressed as means $\pm$ s.e. One-way analysis of variance (ANOVA) with the Scheffe multiple-comparison test was used to determine statistical significances at P levels of 0.05 or 0.01.

Results

Neostigmine concentration-dependently increases contractile and non-contractile Ca^{2+} transients

To investigate the relationship between the amounts of ACh accumulated in the synaptic cleft and non-contractile Ca^{2+}

mobilization, the muscle preparations were exposed to various concentrations of an anticholinesterase agent, neostigmine. Non-contractile Ca^{2+} mobilization was initiated by nerve stimulation in the presence of neostigmine (more than $0.03 \mu\text{M}$) (Figure 1a). The peak amplitude of non-contractile

Figure 1 Changes in contractile and non-contractile Ca^{2+} transients in diaphragm muscles of mice in response to increasing neostigmine concentrations. (a) Typical traces of averaged records for 30 signals of Ca^{2+} transients during the 5 to 10 min after generation of non-contractile Ca^{2+} transients by application of neostigmine (0.03–1 μM). The large, rapid increase in Ca^{2+} represents the contractile transients, and the slower prolonged increase represents the non-contractile transients. Data obtained from separate experiments have been normalized and superimposed. The ordinate calibration bar represents 50% of the amplitude of contractile Ca^{2+} transients obtained 5 to 0 min before the application of neostigmine. Log concentration-response curves for the effect of neostigmine on the peak amplitude of contractile Ca^{2+} transients (b), and on the peak amplitude (c) and duration (d) of non-contractile Ca^{2+} transients are shown. Data are means $\pm$ s.e. of 5 to 10 separate experiments.

Ca^{2+} mobilization yielded a maximum response at 0.1–0.3 μM (Figure 1c). The duration of non-contractile Ca^{2+} mobilization was concentration-dependently prolonged by neostigmine (0.03–0.3 μM) (Figure 1d). At a higher neostigmine concentration (1 μM), the peak amplitude of non-contractile Ca^{2+} mobilization was slightly decreased, but its duration was even more prolonged. The total amount of non-contractile Ca^{2+} mobilization was the same at both the 0.3 μM and 1 μM concentrations (data not shown).

The peak amplitude of contractile Ca^{2+} transients was increased by application of neostigmine (0.03–0.3 μM) (Figure 1b). This augmenting effect of neostigmine on the peak amplitude tended to decrease at 1 μM . No changes in the duration of contractile Ca^{2+} transients was observed (data not shown).

Non-contractile Ca^{2+} depresses contractile Ca^{2+} transients elicited by the second pulse

To elucidate the effect of non-contractile Ca^{2+} on contractile Ca^{2+} mobilization, the phrenic nerve was stimulated with paired pulses with intervals of 150, 300, 600, 1000 and 2000 ms in the presence of neostigmine (0.03 μM). The amplitudes of the contractile Ca^{2+} transients elicited by the second pulse were depressed at shorter pulse intervals as non-contractile Ca^{2+} mobilization increased, but were restored to the initial contractile response at longer pulse intervals as non-contractile Ca^{2+} mobilization decreased (Figure 2a). Twitch tension was depressed in the same manner as the change in contractile Ca^{2+} transients. The amplitude of the first twitch tension was $118 \pm 2\%$ ($n = 29$) for the response before application of neostigmine, and those of the second twitch tension were $96 \pm 3\%$ ($n = 7$), $102 \pm 4\%$ ($n = 7$), $103 \pm 3\%$ ($n = 9$) and $112 \pm 4\%$ ($n = 6$) at pulse intervals of 150, 300, 600 and 1000 ms respectively. When the concentration of neostigmine was increased to 0.1 μM , contractile Ca^{2+} transients elicited by the second pulse were more depressed (Figure 2a), whereas non-contractile Ca^{2+} mobilization was larger as stated above. The blocking action of neostigmine on nicotinic AChR-channels may be ruled out, since low concentrations of neostigmine used in this study have no effect on mouse endplate channels (Chang *et al.*, 1987). Thus, the depression of contractile transients may depend on the amount of ACh accumulated in the synaptic cleft, suggesting that these contractile response phenomena may be caused by nicotinic AChR desensitization.

To evaluate the extent of depression of contractile Ca^{2+} transients quantitatively, the ratio (S_2/S_1) of the peak amplitude of the contractile response elicited by the second pulse (S_2) to that by the first pulse (S_1) was calculated. The S_2/S_1 values were decreased both by shortening the pulse intervals and by increasing the concentrations of neostigmine (Figure 2b). These findings suggest that the decrease in S_2/S_1 may be correlated with the increase in peak amplitude of non-contractile Ca^{2+} mobilization.

In the absence of neostigmine, when no non-contractile Ca^{2+} mobilization was generated, all of the contractile Ca^{2+} transients elicited by paired-pulse stimulation were almost constant, demonstrating that the contractile response was unaffected by paired pulses with these intervals (Figure 3a). Caffeine (5 mM), a promoter of Ca^{2+} release from the SR, increased contractile Ca^{2+} transients to the same extent as neostigmine; however, the second paired-pulse contractile Ca^{2+} transients were hardly depressed at all (Figure 3b). These findings indicate that there is little interaction between two successive contractile Ca^{2+} transients.

To determine whether non-contractile Ca^{2+} depresses contractile Ca^{2+} transients, the effect of blocking non-contractile Ca^{2+} mobilization on S_2/S_1 values was investigated. After pretreatment for 30 min with pancuronium (0.05 μM), a competitive nicotinic antagonist, neostigmine (0.3 μM) was applied in combination with pancuronium, and the preparation was then stimulated with paired pulses. Non-contractile Ca^{2+}

mobilization was blocked by pancuronium at the low concentration with no effect on contractile Ca^{2+} mobilization elicited by the first pulse, as previously reported (Kimura *et al.*, 1989). All of the S_2/S_1 values of contractile transients examined at 150, 300, 600 and 1000 ms intervals were significantly increased by pancuronium (Figure 4), indicating that blockade of non-contractile Ca^{2+} mobilization caused

Figure 2 Neostigmine concentration-dependent depression of contractile Ca^{2+} transients elicited during generation of non-contractile Ca^{2+} transients in mouse diaphragm muscles. (a) Typical traces of averaged records for 30 Ca^{2+} transient signals elicited by paired-pulse nerve stimulation with intervals of 150, 300, 600 and 1000 ms during the 8 to 13 min after application of 0.03 μM (above) and 0.1 μM neostigmine (below). Data obtained from separate experiments have been normalized and superimposed. The ordinate calibration bar represents 50% of the amplitude of contractile Ca^{2+} transients before the application of neostigmine. (b) Effects of neostigmine (●: 0.03 μM , ▼: 0.06 μM , ■: 0.1 μM , ▲: 0.3 μM) on the time course of the ratio of contractile Ca^{2+} transients elicited by the second pulse (S_2) to those elicited by the first pulse (S_1). The data shown are means $\pm$ s.e. of 5 to 9 separate experiments. * $P < 0.05$, ** $P < 0.01$; significantly different from the response to neostigmine (0.03 μM) at each pulse interval based on one-way ANOVA.

the decrease in S_2/S_1 to diminish. Very little decrease in S_2/S_1 was observed in the presence of pancuronium without neostigmine ($S_2/S_1 = 0.95 \pm 0.03$ at a pulse interval of 150 ms, $n = 5$). Thus, non-contractile Ca^{2+} may depress contractile Ca^{2+} mobilization.

The depression of contractile Ca^{2+} transients by non-contractile Ca^{2+} is dependent on the external Ca^{2+} concentration

The relationship between the extent of changes in S_2/S_1 and the amounts of non-contractile Ca^{2+} mobilized was further investigated. Simultaneously with exposure of the preparation to neostigmine ($0.3 \mu\text{M}$), the $[\text{Ca}^{2+}]_o$ was changed either to 1.3 mM or to 5 mM. The peak amplitude of non-contractile Ca^{2+} mobilization depends on the external Ca^{2+} concentration (1.3–5 mM), as shown previously (Kimura *et al.*, 1989). When non-contractile Ca^{2+} mobilization was reduced by decreasing $[\text{Ca}^{2+}]_o$ from 2.5 to 1.3 mM, S_2 was higher than under the normal conditions (2.5 mM $[\text{Ca}^{2+}]_o$), despite using the same pulse intervals (Figure 5a). In contrast, increasing non-contractile Ca^{2+} mobilization by raising $[\text{Ca}^{2+}]_o$ to 5 mM caused a decrease in the S_2 of the contractile transients. The time course of S_2/S_1 revealed different patterns at various concentrations of $[\text{Ca}^{2+}]_o$ (Figure 5b). The largest increase in the difference between the peak amplitudes of non-contractile Ca^{2+} mobilization at each $[\text{Ca}^{2+}]_o$ was observed at a pulse interval of 300 ms, where the peak amplitudes were $7 \pm 3\%$, $32 \pm 8\%$ and $63 \pm 8\%$ for contractile Ca^{2+} transients before application of neostigmine at 1.3 mM, 2.5 mM and 5 mM $[\text{Ca}^{2+}]_o$, respectively. The S_2/S_1 value decreased from 0.79 to 0.24 when the $[\text{Ca}^{2+}]_o$ was raised from 1.3 to 5 mM (Figure 5c). Thus, the decrease in the S_2/S_1 value of contractile Ca^{2+} transients was dependent on the peak amplitude of non-contractile Ca^{2+} mobilization, which was regulated by $[\text{Ca}^{2+}]_o$.

Figure 3 Negligible interaction between two successive contractile Ca^{2+} transients elicited by paired-pulse nerve stimulation with (b) and without 5 mM caffeine (a). Typical traces of averaged records for 30 contractile Ca^{2+} transient signals elicited by paired-pulse stimulation with intervals of 150, 300, 600 and 1000 ms in the absence of neostigmine, showing no influence of the increase in the first contractile Ca^{2+} transients (S_1) on the second contractile transients (S_2). Data obtained from separate experiments have been normalized and superimposed. The ordinate calibration bar represents 50% of the amplitude of the contractile Ca^{2+} transients before the application of caffeine.

Non-contractile Ca^{2+} may depress contractile Ca^{2+} transients via activation of protein kinase-C

To investigate the involvement of protein kinase-C in non-contractile Ca^{2+} -induced depression of contractile Ca^{2+} mobilization, staurosporine (10 nM), a protein kinase-C inhibitor, was added 30 min before the addition of neostig-

Figure 4 Influence of pancuronium-induced blockade of non-contractile Ca^{2+} transients on contractile Ca^{2+} transients elicited by the second pulse: evidence for the depressant effect of non-contractile Ca^{2+} . Pancuronium ($0.05 \mu\text{M}$) was added 30 min before neostigmine ($0.3 \mu\text{M}$). (a) Typical traces of averaged records for 30 Ca^{2+} transient signals in response to paired-pulse nerve stimulation with intervals of 150, 300, 600 and 1000 ms during the 8 to 13 min after application of neostigmine in the absence (above) and presence of pancuronium (below). Data obtained from separate experiments have been normalized and superimposed. The ordinate calibration bar represents 50% of the amplitude of the contractile Ca^{2+} transients before the application of neostigmine. (b) Effects of pancuronium (●: $0.05 \mu\text{M}$) on the time course of the ratio of the second contractile Ca^{2+} transients (S_2) to the first contractile transients (S_1) in the presence of neostigmine ($0.3 \mu\text{M}$). Data shown are means $\pm$ s.e. of 4 to 5 separate experiments. ** $P < 0.01$; significantly different from the control response with neostigmine alone (○) at each pulse interval based on one-way ANOVA.

Figure 5 External Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$)-dependency of the depressant effect of non-contractile Ca^{2+} on contractile Ca^{2+} transients elicited by the second pulse. (a) Typical traces of averaged records for 30 Ca^{2+} transient signals in response to paired-pulse stimulation with intervals of 150, 300, 600 and 1000 ms during the 8 to 13 min after application of $0.3 \mu\text{M}$ neostigmine at low (1.3 mM, above) and high $[\text{Ca}^{2+}]_o$ (5 mM, below). Data obtained from separate experiments have been normalized and superimposed. The ordinate calibration bar represents 50% of the amplitude of the contractile Ca^{2+} transients obtained before the application of neostigmine. Typical traces at normal $[\text{Ca}^{2+}]_o$ (2.5 mM) are shown in Figure 4a (above). (b) Influence of $[\text{Ca}^{2+}]_o$ (●: 1.3 mM, ○: 2.5 mM, ▲: 5 mM) on the time course of the ratio of the second contractile Ca^{2+} transients (S_2) to the first contractile transients (S_1) in the presence of neostigmine ($0.3 \mu\text{M}$). Data shown are means $\pm$ s.e. of 5 to 7 separate experiments. * $P < 0.05$, ** $P < 0.01$; significantly different from the responses with neostigmine at normal $[\text{Ca}^{2+}]_o$ (2.5 mM) at each pulse interval based on one-way ANOVA. (c) Log concentration-response curves of $[\text{Ca}^{2+}]_o$ on the S_2/S_1 ratio obtained at a pulse interval of 300 ms (●) and on the peak amplitude of the non-contractile Ca^{2+} transients elicited by the first pulse (▲).

mine ($0.3 \mu\text{M}$). Staurosporine (10 nM) diminished the depression of the contractile Ca^{2+} transients elicited by the second pulse, despite having no effect on non-contractile Ca^{2+} mobilization (Figure 6a). The S_2/S_1 values at 150, 600 and 1000 ms intervals were significantly higher in the presence of staurosporine than those in its absence (Figure 6b). H-7 ($10 \mu\text{M}$), a protein kinase-C inhibitor, also increased S_2/S_1 at 150, 600 and 1000 ms intervals, despite large amounts of non-contractile Ca^{2+} mobilization (data not shown). Blocking actions on activated nicotinic channels may be ruled out, since H-7 has no effect on endplate channels at this concentration (Reuhl *et al.*, 1992). Moreover, TPA, a specific activator of protein kinase-C, inhibited the diminishing effect of staurosporine on the depression of contractile transients (Figure 6b) at the lower concentration ($0.1 \mu\text{M}$), but did not affect either type of Ca^{2+} transients alone. These findings indicate that non-contractile Ca^{2+} may depress contractile Ca^{2+} mobilization via activation of protein kinase-C.

Figure 6 Involvement of protein kinase-C in non-contractile Ca^{2+} -induced depression of contractile Ca^{2+} transients. Staurosporine (10 nM) was added 30 min before neostigmine ($0.3 \mu\text{M}$). TPA ($0.1 \mu\text{M}$) was added 10 min before the addition of staurosporine. (a) Typical traces of averaged records for 30 Ca^{2+} transient signals in response to paired-pulse stimulation with intervals of 150, 300, 600 and 1000 ms during the 8 to 13 min after application of neostigmine in the presence of staurosporine. Data obtained from separate experiments have been normalized and superimposed. The ordinate calibration bar represents 50% of the amplitude of the contractile Ca^{2+} transients before the application of neostigmine. Typical traces of control responses in the presence of neostigmine alone are shown in Figure 4a (above). (b) Effects of staurosporine (●: 10 nM) alone and in the presence of TPA (▲: $0.1 \mu\text{M}$) on the time course of the ratio of S_2 to S_1 in the presence of neostigmine. Data shown are means $\pm$ s.e. of 3 to 7 separate experiments. * $P < 0.05$, ** $P < 0.01$; significantly different from the control response with neostigmine alone (○) at each pulse interval based on one-way ANOVA.

Discussion

Nicotinic AChR desensitization readily develops as a result of the accumulation of nerve-released ACh at the neuromuscular junction after cholinesterase inhibition. Non-contractile Ca^{2+} mobilization occurs via the nicotinic AChR only under such desensitizing conditions and depends on the amount of ACh accumulated in the synaptic cleft (Kimura *et al.*, 1989; 1993). These findings led us to examine the relationship between non-contractile Ca^{2+} mobilization and the desensitization using paired-pulse stimulation of a motor nerve.

There is no direct causal relationship between contractile and non-contractile Ca^{2+} mobilization, since geographotoxin II, a skeletal muscle Na^+ channel blocker (Cruz *et al.*, 1985; Ohizumi *et al.*, 1986), selectively blocked contractile Ca^{2+} transients without affecting non-contractile Ca^{2+} mobilization (Kimura *et al.*, 1991b), and caffeine (5 mM) increased contractile Ca^{2+} transients but decreased non-contractile Ca^{2+} mobilization (Kimura *et al.*, 1991a). Nevertheless, the contractile Ca^{2+} transients elicited by the second pulse (S_2) during the generation of non-contractile Ca^{2+} mobilization were depressed at shorter pulse intervals, but were restored to the initial contractile response (S_1) at longer pulse intervals. The rapid depression of contractile Ca^{2+} transients followed by gradual recovery to initial values is a phenomenon quite similar to the time course of the nicotinic AChR desensitization, measured by paired pulses of ACh using the patch clamp technique (Franke *et al.*, 1992; Bufler *et al.*, 1993). Moreover, the extent of the depression of S_2 , indicated by the decrease in S_2/S_1 , was enhanced by increasing concentrations of neostigmine. S_2/S_1 did not decrease in the absence of neostigmine. These changes in contractile transients were also considered to be consistent with concentration-dependent agonist enhancement of both the rate and extent of the nicotinic AChR desensitization (Adams, 1975; Chesnut, 1983; Dilger & Liu, 1992). Thus, the decrease in S_2/S_1 observed in this study appears to reflect nicotinic AChR desensitization.

The extent of depression of contractile Ca^{2+} transients was dependent on the peak amplitude of non-contractile Ca^{2+} mobilization regulated by $[\text{Ca}^{2+}]_o$. The development of nicotinic AChR desensitization is enhanced by increasing $[\text{Ca}^{2+}]_o$ (Manthey, 1966; 1970; Nastuk & Parsons, 1970; Nojima *et al.*, 1992), a consequence of accumulation of Ca^{2+} at the inner surface of the sarcolemmal membrane due to Ca^{2+} influx via the nicotinic AChR (Miledi, 1980; Chesnut, 1983). Intracellular Ca^{2+} release from the SR, however, is unrelated to desensitization of nicotinic AChR since caffeine does not affect the rate of desensitization (Magazanik & Vyskočil, 1970). Non-contractile Ca^{2+} may stabilize nicotinic AChR in refractory states and protect it against over-excitation at the motor endplate, depressing contractile Ca^{2+} mobilization.

The depolarization of postsynaptic membranes is prolonged by the persistent action of ACh accumulated after cholinesterase inhibition. However, the depression of contractile Ca^{2+} transients observed in this study is not involved in the depolarizing blockade of nicotinic AChR (Burns & Paton, 1951), since the recovery time from the depression of contractile Ca^{2+} transients based on the second pulse is 10–20 fold longer than the duration of the prolonged endplate potential in the presence of anticholinesterase agents.

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- S_2 was hardly depressed at all when S_1 was increased by caffeine in the absence of neostigmine, although complete recovery of Ca^{2+} content in the SR has been reported to require a few seconds (Somlyo *et al.*, 1985). Therefore, the depression of the second contractile Ca^{2+} transients by paired-pulse stimulation may not be due to the inactivation or depletion caused by the first Ca^{2+} release from the SR (Schneider & Simon, 1988).
- When non-contractile Ca^{2+} mobilization was depressed either by 1.3 mM $[\text{Ca}^{2+}]_o$ or by 0.05 μM pancuronium in the presence of neostigmine, the decrease in S_2/S_1 was greatly reduced (Figures 4b and 5b). However, part of the decrease still remained. This remaining part may reflect the direct desensitizing effect of ACh accumulated after the cholinesterase inhibition, since the nicotinic agonist can directly cause nicotinic AChR desensitization even in the absence of external Ca^{2+} (Nelson *et al.*, 1980).
- We have previously reported that TPA (0.3–1 μM), a protein kinase-C activator, depresses contractile Ca^{2+} transients elicited by single-pulse nerve stimulation in mouse diaphragm muscle (Kimura *et al.*, 1993). After pretreatment with protein kinase-C inhibitors, even large amounts of non-contractile Ca^{2+} failed to depress the second contractile Ca^{2+} transients elicited by paired-pulse stimulation. Protein kinase-C is translocated from the cytosol to plasma membranes in a Ca^{2+} -dependent manner (Kraft & Anderson, 1983; Melloni *et al.*, 1985). Activation of nicotinic AChR causes translocation of protein kinase-C to postsynaptic membranes, thereby activating the protein kinase in skeletal muscle (Eusebi *et al.*, 1987). The agonist-induced phosphorylation of nicotinic AChR in rat myotubes is dependent on external Ca^{2+} and is abolished by nicotinic AChR-channel blockers, suggesting that Ca^{2+} influx via the nicotinic AChR-channel is crucial to the activation of protein kinase-C (Miles *et al.*, 1994). The protein kinase-C which is activated via the nicotinic AChR has been shown to enhance the receptor desensitization (Eusebi *et al.*, 1987). The results of the present study suggest that nicotinic AChR-operated non-contractile Ca^{2+} may desensitize the receptor via activation of protein kinase-C within skeletal muscle cells. The depressant effect of protein kinase-C on the second contractile Ca^{2+} transients elicited by paired-pulse stimulation may not be due to a decrease in ACh release from motor nerve terminals, since the activator of protein kinase-C instead increases ACh release (Eusebi *et al.*, 1986; Caratsch *et al.*, 1988). In addition, the skeletal muscle dihydropyridine-sensitive Ca^{2+} channels in the transverse-tubule membranes are activated by protein kinase-C phosphorylation (Chang *et al.*, 1991). Thus, we may also exclude any possible interaction between protein kinase-C and the muscular processes, such as modulation of Ca^{2+} release from SR.
- In conclusion, the non-contractile Ca^{2+} may enhance the desensitization of postsynaptic nicotinic AChRs via activation of protein kinase-C, depressing the contractile Ca^{2+} mobilization at the neuromuscular junction.

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