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Enhancement by calcitonin gene-related peptide of non-contractile Ca^{2+}-induced nicotinic receptor desensitization at the mouse neuromuscular junction

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1 Nicotinic acetylcholine receptor (AChR)-operated non-contractile Ca^{2+} mobilization (unaccompanied by muscle contraction) depressed contractile Ca^{2+} mobilization (accompanied by muscle contraction) in mouse diaphragm muscles. In the process of nicotinic AChR desensitization, the enhancing role of calcitonin gene-related peptide (CGRP) on the non-contractile Ca^{2+}-induced depression of contractile Ca^{2+} mobilization was investigated by measurement of Ca^{2+}-aequorin luminescence in the presence of neostigmine (0.1 μM).

2 When the phrenic nerve was stimulated with paired pulses at 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 not at the longer pulse intervals.

3 The extent of depression of S_2 was enhanced when the duration of non-contractile Ca^{2+} mobilization was prolonged by CGRP (10 nM). However, CGRP failed to enhance the depression of S_2 when non-contractile Ca^{2+} mobilization was not observed at the low external Ca^{2+} concentration (1.3 mM).

4 The enhancing effect by CGRP on the depression of S_2 was counteracted by staurosporine (3 nM), a protein kinase-C inhibitor, despite prolongation of the duration of non-contractile Ca^{2+} mobilization.

5 When H-89 (1 μM), a protein kinase-A inhibitor, completely blocked non-contractile Ca^{2+} mobilization, the depression of S_2 was diminished. The prolongation of the duration of non-contractile Ca^{2+} mobilization by AA373 (300 μM), a protein kinase-A activator, enhanced the depression of S_2. The enhancing effect was observed neither with CGRP nor with AA373, in the presence of H-89 (0.1 μM).

6 These findings suggest that the CGRP mobilizes non-contractile Ca^{2+} through activation of protein kinase-A, which in turn may activate protein kinase-C, then enhance the desensitization of postsynaptic nicotinic AChRs at the neuromuscular junction.

Keywords: Nicotinic acetylcholine receptor; non-contractile calcium; calcitonin gene-related peptide; protein kinase-A; protein kinase-C; desensitization; neuromuscular postsynapse

Introduction

Non-contractile and contractile Ca^{2+} mobilization are generated simultaneously at the neuromuscular junction by nerve stimulation in the presence of low concentrations of anticholinesterase agents (Kimura *et al.*, 1989). Non-contractile Ca^{2+} mobilization depresses the contractile Ca^{2+} mobilization through protein kinase-C activation (Kimura *et al.*, 1995). Non-contractile Ca^{2+} is mobilized independently of contractile Ca^{2+}, 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 low concentrations of competitive nicotinic antagonists, (+)-tubocurarine and pancuronium that have no effect on contractile Ca^{2+} transients (Kimura *et al.*, 1989), (2) a high concentration (5 mM) of external Ca^{2+} increases non-contractile Ca^{2+} mobilization, but decreases contractile Ca^{2+} transients (Kimura *et al.*, 1989) and (3) non-contractile Ca^{2+} mobilization is not due to Ca^{2+} release from the sarcoplasmic reticulum (Kimura *et al.*, 1991a,b).

The neuropeptide calcitonin gene-related peptide (CGRP), which co-exists with ACh in the motor nerve endings (Takami *et al.*, 1985a; Matteoli *et al.*, 1988), enhances non-contractile Ca^{2+} mobilization through protein kinase-A activation within muscle cells (Kimura *et al.*, 1993). CGRP also enhances the nicotinic AChR desensitization via activation of protein kinase-A (Mulle *et al.*, 1988). We therefore suppose that CGRP may mobilize non-contractile Ca^{2+} by activating protein kinase-A, then prevent the nicotinic AChR from responding to excessive stimulation by accumulated ACh.

In the present study, we investigated the enhancing role of CGRP and protein kinase-A-related agents on the non-contractile Ca^{2+}-induced depression of contractile Ca^{2+} mobilization using paired-pulse stimulation to elucidate the involvement of CGRP in 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 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

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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 thermo-module (Komatsu Electronics, Kanagawa, Japan). The solution was equilibrated with 95% O_2 and 5% CO_2.

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 a micropipette into the end-plate region of 40 to 50 muscle fibres by 2-s pulse of nitrogen gas pressure (5–6 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 μs. 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 was attached to the photomultiplier tube. When the phrenic nerve was stimulated supramaximally (0.4–0.8 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 15 to 20 min after the application of neostigmine (0.1 μM) were analyzed. The peak amplitudes of both non-contractile and contractile Ca^{2+} transients were 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 at intervals of 150, 300, 600, 1000 and 2000 ms, 15 min after the 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 (Wako Pure Chemical, Osaka, Japan) was dissolved in Ca^{2+}-free distilled water containing 5 μM EDTA. AA373 (3-(2′-hydroxy-4′,5′-diethoxybenzoyl) propionic acid) (Takeda Chemical, Osaka), H-85 (*N*-[2-(*N*-formyl-*p*-chlorocinnamylamino)ethyl]-5-isoquinolinesulphonamide), H-89 (*N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulphonamide) (0.001–0.01% dimethyl sulphoxide solution; provided by Prof. H. Hidaka, Department of Pharmacology, Nagoya University School of Medicine), neostigmine methylsulphate (Sigma Chemical, MO, U.S.A.) and staurosporine (0.003% *N*,*N*-dimethylformamide solution; Kyowa Medix, Tokyo, Japan) were bath-applied.

Statistical analysis

Data are expressed as means ± s.e.mean. One-way analysis of variance (ANOVA) and the Scheffe multiple-comparison test were used to determine statistical significances at *P* levels of 0.05 or 0.01.

Results

CGRP enhances the depression of contractile Ca^{2+} transients elicited by the second pulse

The phrenic nerve was stimulated with paired pulses having intervals of 150, 300, 600, 1000 and 2000 ms in the presence of neostigmine (0.1 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 not at longer pulse intervals as non-contractile Ca^{2+} mobilization decreased (Figure 1a). Twitch tension was depressed in the same manner as the change in contractile Ca^{2+} transients. The amplitude of the first twitch tension was 129 ± 3% (n = 39) for the response before the application of neostigmine, and those of the second twitch tension were 96 ± 6% (n = 8), 103 ± 3% (n = 8), 119 ± 4% (n = 8), 122 ± 2% (n = 8) and 128 ± 1% (n = 7) at pulse intervals of 150, 300, 600, 1000 and 2000 ms respectively. To investigate the effect of CGRP on the depression of contractile Ca^{2+} transients, after pretreatment for 15 min with CGRP (10 nM), neostigmine (0.1 μM) was applied in combination with CGRP, and the preparation was then stimulated with paired pulses. CGRP prolonged the duration of non-contractile Ca^{2+} mobilization (Figure 4), but did not affect contractile Ca^{2+} transients elicited by the first pulse, as previously reported (Kimura *et al.*, 1993), whereas, contractile Ca^{2+} transients elicited by the second pulse were more depressed by CGRP (Figure 1a). 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 elicited by the first pulse (S_1) was calculated. The S_2/S_1 values at 600 and 1000 ms intervals were significantly decreased by CGRP (Figure 1b). Very little decrease in S_2/S_1 was observed in the presence of CGRP without neostigmine (S_2/

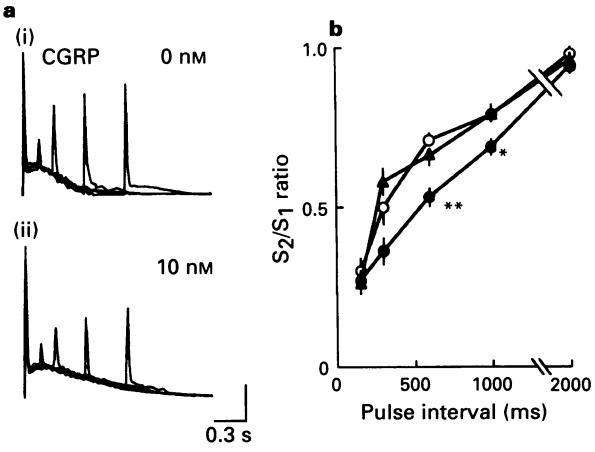

Figure 1 Enhancement of non-contractile Ca^{2+}-induced depression of the second contractile Ca^{2+} transients by calcitonin gene-related peptide (CGRP) in mouse diaphragm muscles with neostigmine (0.1 μM). CGRP (10 nM) and H-89 (0.1 μM) were added 15 min before the application of neostigmine and CGRP, respectively. (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 15 to 20 min after the application of neostigmine in the absence (i) and presence of CGRP (ii). 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 neostigmine. (b) Effects of CGRP (●: 10 nM) alone and in the presence of H-89 (▲: 0.1 μM) 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 (0.1 μM). Data shown are means ± s.e.mean of 6 to 8 separate experiments. $*P < 0.05$ and $**P < 0.01$; significantly different from the control response with neostigmine alone (○) at each pulse interval based on one-way ANOVA.

$S_1 = 0.96 \pm 0.03$ at a pulse interval of 150 ms, $n = 5$). A low concentration of H-89 (0.1 μM), a selective inhibitor of protein kinase-A (Chijiwa *et al.*, 1990) which had effects on neither non-contractile nor contractile Ca^{2+} transients alone, diminished both the prolonging effect on the duration of non-contractile Ca^{2+} mobilization (Figure 4) and the enhancing effect on the decrease of S_2/S_1 values, caused by 10 nM CGRP (Figure 1b).

CGRP enhances the depression of contractile Ca^{2+} transients via non-contractile Ca^{2+} mobilization which activates protein kinase-C

To investigate the involvement of non-contractile Ca^{2+} mobilization in CGRP-induced enhancement of the depression of contractile Ca^{2+} mobilization, the effect of CGRP on S_2/S_1 values was examined in the absence of non-contractile Ca^{2+} mobilization. Simultaneously with exposure of the preparation to neostigmine (0.1 μM), the concentration of external Ca^{2+} was reduced from 2.5 mM to 1.3 mM after pretreatment for 15 min with CGRP (10 nM). When non-contractile Ca^{2+} mobilization was not observed at the low concentration (1.3 mM) of external Ca^{2+}, CGRP (10 nM) did not affect the S_2/S_1 values (Figure 2a,b), indicating that CGRP failed to enhance the depression of contractile Ca^{2+} mobilization in the absence of non-contractile Ca^{2+} mobilization. Thus, CGRP depresses contractile Ca^{2+} mobilization through non-contractile Ca^{2+} mobilization.

The relationship between non-contractile Ca^{2+} mobilization and CGRP-enhanced depression of contractile Ca^{2+} mobilization was investigated further. The effect of staurosporine (3 nM), a protein kinase-C inhibitor, was examined on CGRP-induced depression of contractile Ca^{2+} transients, since non-contractile Ca^{2+} mobilization promote the depression of contractile Ca^{2+} mobilization via activation of protein kinase-C (Kimura *et al.*, 1995). Staurosporine counteracted CGRP (10 nM)-enhanced depression of contractile transients elicited by the second pulse (Figure 3a,b), despite having no effect on the prolongation of duration on non-contractile Ca^{2+} mobilization by 10 nM CGRP (Figure 4). Staurosporine at this concentration (3 nM) had no effect on the S_2/S_1 values in the absence of CGRP (data not shown). These findings indicate that CGRP may enhance the depression of contractile Ca^{2+} mobilization via non-contractile Ca^{2+} mobilization which activates the protein kinase-C.

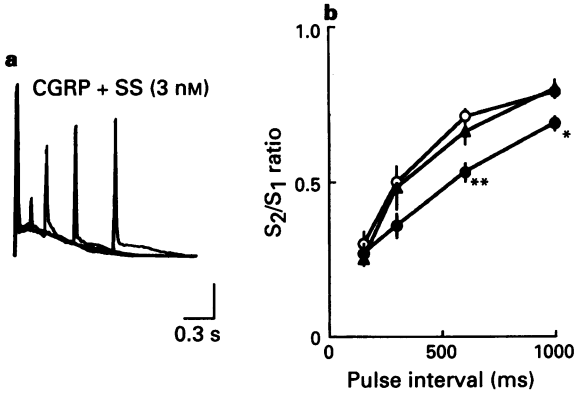

Figure 3 Involvement of protein kinase-C in CGRP-induced enhancement of the depression of the second contractile Ca^{2+} transients. CGRP (10 nM) and staurosporine (3 nM) were added 15 min before the application of neostigmine (0.1 μM) and CGRP, respectively. (a) Typical traces of averaged records for 30 Ca^{2+} transient signals in response to paired-pulse stimulation with intervals of 150, 300, 600 and 1000 ms during the 15 to 20 min after the 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 neostigmine. (b) Effects of CGRP (●: 10 nM) alone and in the presence of staurosporine (▲: 3 nM) on the time course of the ratio of S_2 to S_1 with neostigmine. Data shown are means ± s.e.mean of 6 to 8 separate experiments. $^*P<0.05$ and $^{**}P<0.01$; significantly different from the control response with neostigmine alone (○) at each pulse intervals based on one-way ANOVA.

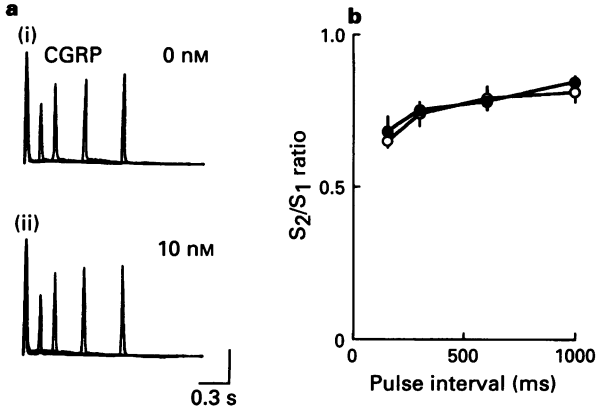

Figure 2 Requirement of non-contractile Ca^{2+} mobilization for the CGRP-enhanced depression of the second contractile Ca^{2+} transients. (a) Typical traces of averaged records for 30 Ca^{2+} transient signals in response to paired-pulse nerve stimulation with intervals of 150, 300, 600 and 1000 ms during the 15 to 20 min after the application of neostigmine (0.1 μM) at low concentration (1.3 mM) of external Ca^{2+} in the absence (i) and presence of CGRP (10 nM, ii). 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 neostigmine. (b) The time course of the ratio of S_2 to S_1 with neostigmine (0.1 μM) in the absence (○) and presence of CGRP (●: 10 nM) at the low concentration (1.3 mM) of external Ca^{2+}. Data shown are means ± s.e.mean of 6 separate experiments.

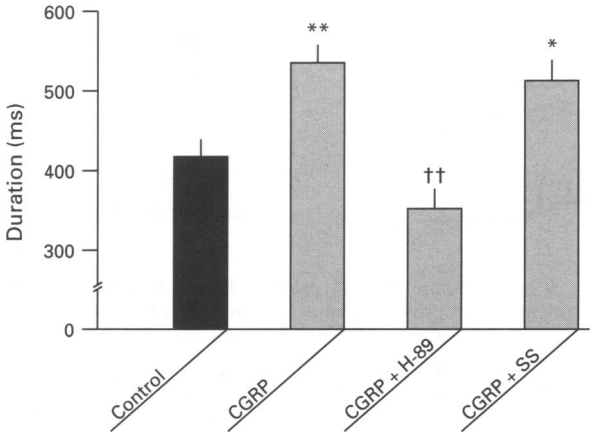

Figure 4 Prolongation of the duration of non-contractile Ca^{2+} mobilization by CGRP and involvement of protein kinase-A but not protein kinase-C in mouse diaphragm muscles with neostigmine (0.1 μM). CGRP (10 nM) was added 15 min before the application of neostigmine. H-89 (0.1 μM) and staurosporine (SS: 3 nM) were added 15 min before the addition of CGRP. The duration of non-contractile Ca^{2+} mobilization was determined at e^{-1} amplitude of the signal. Data shown are means ± s.e.mean of 6 to 8 separate experiments. Significantly different from the response to neostigmine alone ($^*P<0.05$ and $^{**}P<0.01$) and from the effect of CGRP ($\dagger\dagger P<0.01$) at each pulse interval based on one-way ANOVA.

Activation of protein kinase-A enhances the depression of contractile Ca^{2+} transients by non-contractile Ca^{2+}

To investigate the involvement of protein kinase-A in the non-contractile Ca^{2+}-induced depression of contractile Ca^{2+} mobilization, H-89 (1 μM) was added 30 min before the application of neostigmine (0.1 μM). Non-contractile Ca^{2+} mobilization was blocked by H-89 (1 μM) with no effect on contractile Ca^{2+} mobilization elicited by the first pulse, as previously reported (Kimura *et al.*, 1993). The S_2/S_1 values of contractile Ca^{2+} transients examined at 150 and 300 ms intervals were significantly increased by H-89, whereas H-85 (1 μM), a non-effective analogue of H-89, had effects neither on non-contractile Ca^{2+} mobilization nor on the S_2/S_1 values (Figure 5a, b). These results demonstrate that inhibition of protein kinase-A decreases both non-contractile Ca^{2+} mobilization and the depression of contractile Ca^{2+} transients elicited by the second pulse.

The activation of protein kinase-A prolongs the duration of non-contractile Ca^{2+} mobilization, as shown previously (Kimura *et al.*, 1993). After pretreatment for 30 min with AA373 (300 μM), a protein kinase-A activator (Kimura *et al.*, 1977), neostigmine (0.1 μM) was applied in combination with AA373, and the preparation was then stimulated with paired pulses. The duration of non-contractile Ca^{2+} mobilization was prolonged by AA373 (300 μM) (521 ± 29 ms, $n=8$; $P<0.01$), without affecting the peak amplitude of both non-contractile and contractile Ca^{2+} transients elicited by the first pulse (Figure 5a). The S_2/S_1 values at 300, 600 and 1000 ms intervals were significantly decreased by AA373 (Figure 5b). AA373 failed to prolong the duration of non-contractile Ca^{2+} mobilization, and to decrease the S_2/S_1 values in the presence of H-89 (0.1 μM) (data not shown). Hardly any decrease in S_2/S_1 was observed in the presence of AA373 or H-89 without neostigmine ($S_2/S_1 = 0.95 \pm 0.04$ and 0.96 ± 0.03 at a pulse interval of 150 ms, $n=4$ respectively). AA373 (300 μM) did not affect the S_2/S_1 values at low external Ca^{2+} concentration (1.3 mM), where non-contractile Ca^{2+} mobilization does not occur, despite the presence of neostigmine (0.1 μM) (data not shown). These results indicate that the influence of protein kinase-A activation is similar to that of CGRP application.

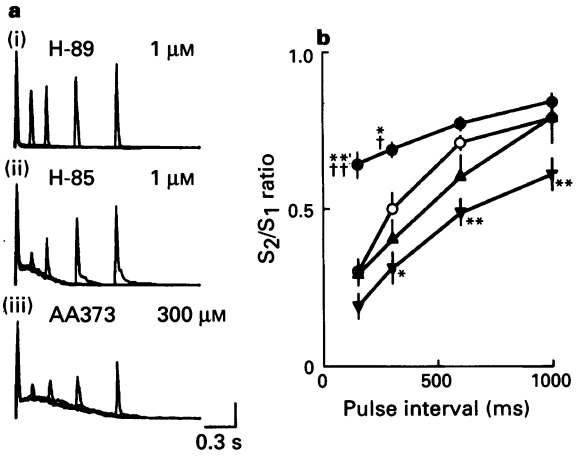

Figure 5 Suppression by a protein kinase-A inhibitor and enhancement by a protein kinase-A activator, of non-contractile Ca^{2+}-induced depression of the second contractile Ca^{2+} transients. H-89 (1 μM), H-85 (1 μM) and AA373 (300 μM) were added 30 min before the application of neostigmine (0.1 μM). (a) Typical traces of averaged records for 30 Ca^{2+} transient signals elicited by paired-pulse stimulation with intervals of 150, 300, 600 and 1000 ms during 15 to 20 min after the application of neostigmine in the presence of H-89 (i), H-85 (ii) and AA373 (iii). 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 neostigmine. (b) Effects of H-89 (●), H-85 (▲) and AA373 (▼) on the time course of the ratio of S_2 to S_1 in the presence of neostigmine. Data shown are means ± s.e.mean of 5 to 8 separate experiments. Significantly different from the control response with neostigmine alone (○) (*$P<0.05$ and **$P<0.01$) and from the effect of H-85 (†$P<0.05$ and ††$P<0.01$) at each pulse interval based on one-way ANOVA.

Discussion

Desensitization is a general phenomenon where prolonged exposure of a receptor to an agonist results in decreased responsiveness despite the continuing presence of the stimulus. Nicotinic AChR desensitization readily develops as a result of the accumulation of nerve-released ACh at the neuromuscular junction after cholinesterase inhibition (Magleby & Pallotta, 1981). Non-contractile Ca^{2+} mobilization occurs via the nicotinic AChR activation only under such desensitizing conditions and depends on the amount of ACh accumulated in the synaptic cleft (Kimura *et al.*, 1989; 1993; 1995). Non-contractile Ca^{2+} mobilization depresses the contractile Ca^{2+} mobilization elicited by the second pulse, when the phrenic nerve is stimulated with paired pulses in the presence of low concentrations of neostigmine (Kimura *et al.*, 1995). The depression is promoted by increasing non-contractile Ca^{2+} mobilization. Moreover, the non-contractile Ca^{2+}-induced depression is diminished by staurosporine (10 nM), a protein kinase-C inhibitor. We then consider that non-contractile Ca^{2+} mobilization may enhance the nicotinic AChR desensitization through activation of protein kinase-C.

The neuropeptide CGRP has been identified in the spinal cord of several vertebrate species and in the motor nerve endings of the rodent neuromuscular junction (Takami *et al.*, 1985b; Fontaine *et al.*, 1986; New & Mudge, 1986; Matteoli *et al.*, 1988). The CGRP binds to CGRP receptors located at the neuromuscular postsynapse (Jennings & Mudge, 1989; Poper & Micevych, 1989; Roa & Changeux, 1991). CGRP activates adenylate cyclase and induces localized increase in the concentration of intracellular adenosine 3′, 5′-cyclic monophosphate, leading to the activation of protein kinase-A, in endplate-rich regions of skeletal muscle (Kobayashi *et al.*, 1987; Matsumoto *et al.*, 1992). This peptide stimulates the biosynthesis of nicotinic AChR (Fontaine *et al.*, 1986; New & Mudge, 1986), and prolongs AChR channel open time (Lu *et al.*, 1993; Owens & Kullberg, 1993) in cultured myotubes. Recently, we reported that CGRP prolonged the duration of non-contractile Ca^{2+} mobilization via activation of protein kinase-A, and that $CGRP_{8-37}$, a competitive CGRP antagonist, shortened the duration (Kimura *et al.*, 1993). Therefore, these data suggest that endogenious CGRP may promote non-contractile Ca^{2+} mobilization via the activation of protein kinase-A.

The phosphorylation of the nicotinic AChR modulates the function of its channel (Huganir & Greengard, 1983; Huganir *et al.*, 1986; Hopfield *et al.*, 1988). The activation of protein kinase-A accelerates the rate of nicotinic AChR desensitization (Albuquerque *et al.*, 1986; Huganir *et al.*, 1986; Middleton *et al.*, 1988). CGRP induces the phosphorylation of nicotinic AChR (Miles *et al.*, 1989) and enhances the desensitization, through protein kinase-A activation (Mulle *et al.*, 1988). However, activation of protein kinase-A has been reported to have no effect on the desensitization (Wagoner & Pallotta, 1988), or to cause an increase rather than a decrease in nicotinic AChR activity (Ferrer-Montiel *et al.*, 1991). In this study, we investigated the enhancing effect of CGRP and protein kinase-A-related agents on the non-contractile Ca^{2+}-induced depression of contractile Ca^{2+} mobilization, to elucidate the involvement of CGRP in the process of nicotinic AChR desensitization. When a protein kinase-A inhibitor, H-89, blocked non-contractile Ca^{2+} mobilization, the depression of contractile Ca^{2+} transients elicited by the second pulse was diminished. In contrast, the depression was enhanced by CGRP and protein kinase-A activator AA373, which prolonged the duration of non-contractile Ca^{2+} mobilization. The

enhancement of depression by CGRP was completely inhibited by H-89. Both CGRP and AA373, however, failed to enhance the depression of contractile Ca^{2+} transients elicited by the second pulse at low external Ca^{2+} concentration (1.3 mM), where the non-contractile Ca^{2+} mobilization does not occur. Using the patch clamp technique in adult mouse muscle cells, we have demonstrated that activation of protein kinase-A cannot accelerate the nicotinic AChR desensitization in external Ca^{2+}-free solution (Nojima *et al.*, 1994), where non-contractile Ca^{2+} mobilization also does not occur (Kimura *et al.*, 1989). These results suggest that the protein kinase-A-induced phosphorylation of nicotinic AChR does not cause the enhancement of desensitization, because the nicotinic AChR subunits are phosphorylated without Ca^{2+} (Huganir & Greengard, 1983). Thus, the CGRP-induced activation of protein kinase-A may promote the nicotinic AChR desensitization via the mobilization of non-contractile Ca^{2+}, even though the phosphorylation of the isolated nicotinic AChR by protein kinase-A can partly enhance the desensitization directly (Huganir *et al.*, 1986).

Although the nicotinic AChR desensitization in skeletal muscle is enhanced by intracellular Ca^{2+}, protein kinase-A and protein kinase-C (Miledi, 1980; Huganir *et al.*, 1986; Eusebi *et al.*, 1987), their mutual relationships in the process of desensitization has not yet been established. In the present study, since the CGRP-enhanced depression of contractile Ca^{2+} transients was inhibited by low concentrations of staurosporine without affecting either contractile or non-contractile Ca^{2+} mobilization alone, we have established the chain reactions between intracellular Ca^{2+}, protein kinase-A and protein kinase-C in the process of nicotinic AChR desensitization. The activation of protein kinase-A may mobilize non-contractile Ca^{2+}, which in turn may activate protein kinase-C, then stabilize the postsynaptic nicotinic AChR in refractory states and protect it against over-excitation. Hence, nicotinic AChR desensitization may be developed by the 'cross-talk' between the protein kinase-A system and the protein kinase-C system through non-contractile Ca^{2+} mobilization.

In conclusion, our present study suggests that the CGRP activating protein kinase-A mobilizes non-contractile Ca^{2+}, in turn activates protein kinase-C, then may enhance the desensitization of postsynaptic nicotinic AChRs, depressing the contractile Ca^{2+} mobilization at the neuromuscular junction.

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