Enhancement by calcitonin gene-related peptide of nicotinic receptor-operated noncontractile Ca^{2+} mobilization at the mouse neuromuscular junction

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1 The involvement of calcitonin gene-related peptide (CGRP) in the mechanism of nicotinic acetylcholine receptor-operated noncontractile Ca^{2+} mobilization (not accompanied by twitch tension) was investigated by measuring Ca^{2+} -aequorin luminescence at the neuromuscular junction of mouse diaphragm muscle treated with neostigmine.

2 Noncontractile Ca^{2+} transients were enhanced by 4-aminopyridine (100 μM), a K^+ channel blocker, and inhibited by botulinum toxin (1–100 μg , i.p.) and hexamethonium (10–100 μM), a neuronal nicotinic receptor antagonist.

3 Noncontractile Ca^{2+} transients were diminished by CGRP_{8-37} (10–20 μM), a CGRP antagonist. CGRP (0.3–10 nM) prolonged the duration of noncontractile Ca^{2+} transients. The effect of CGRP was suppressed by CGRP_{8-37} (0.1 μM).

4 Noncontractile Ca^{2+} transients were inhibited by H-89 (0.1–1 μM), a protein kinase-A inhibitor. The catalytic subunit of protein kinase-A and AA373 (300 μM), a protein kinase-A activator, prolonged the duration of noncontractile transients. The prolongations either by CGRP or by AA373 were not observed in the presence of H-89 (0.1 μM).

5 Contractile (accompanied by twitch tension) but not noncontractile Ca^{2+} transients were decreased by 12-O-tetradecanoyl phorbol 13-acetate (TPA, 0.3–1 μM), a protein kinase-C activator. Phospholipase A_2 increased only contractile Ca^{2+} transients. Calmodulin-related agents affected neither type of Ca^{2+} transients.

6 These results provide the first evidence that nicotinic acetylcholine receptor-operated noncontractile Ca^{2+} mobilization is promoted by nerve-released CGRP activating protein kinase-A, and is dependent on the accumulated amounts of acetylcholine at the neuromuscular junction where desensitization might readily develop.

Keywords: Calcitonin gene-related peptide; protein kinase-A; nicotinic acetylcholine receptor; intracellular calcium; protein kinase-C; phospholipase A_2

Introduction

Noncontractile and contractile Ca^{2+} transients are simultaneously generated at the endplate region by nerve stimulation in the presence of a cholinesterase inhibitor in mouse diaphragm muscles (Kimura *et al.*, 1991a). The noncontractile transients are triggered by the prolonged action of acetylcholine (ACh) on the postsynaptic nicotinic ACh receptor (AChR). Competitive nicotinic AChR antagonists are 10 fold more potent on noncontractile Ca^{2+} transients than on contractile Ca^{2+} transients (Kimura *et al.*, 1989).

Noncontractile Ca^{2+} mobilization occurs neither by Ca^{2+} release from sarcoplasmic reticulum, nor by simple Ca^{2+} influx through the nicotinic AChR-channel as a component of endplate current (Kimura *et al.*, 1991a,b). Contractile Ca^{2+} transients are related partly to Ca^{2+} release from the sarcoplasmic reticulum (Kimura *et al.*, 1990). The duration of noncontractile Ca^{2+} transients was much longer than that of contractile transients. We considered the possibility that the long-lasting, noncontractile Ca^{2+} may be mobilized via some pathway of signal transduction.

The neuropeptide calcitonin gene-related peptide (CGRP) coexists with ACh in the nerve terminals of the rodent neuromuscular junction (Takami *et al.*, 1985). CGRP enhances the nicotinic AChR desensitization through the phosphorylation of the nicotinic AChR by protein kinase-A (Mulle *et al.*, 1988). Hence, the properties of noncontractile Ca^{2+} mobilization which is triggered by the accumulated ACh in the synaptic cleft were studied to test whether they

are also affected by CGRP and protein kinase A-related agents.

The present study was designed to elucidate which pathway of signal transduction is involved in postsynaptic nicotinic AChR-operated noncontractile Ca^{2+} mobilization at the neuromuscular junction. Two characteristic properties are described here concerning noncontractile Ca^{2+} transients: These are: (1) their dependence on the amount of ACh released from nerve terminal, and (2) whether they are promoted by released CGRP.

Methods

Muscle preparations

Male ddY mice (7–9 weeks old, 28–42 g) were killed by decapitation. A segment of the phrenic nerve-diaphragm muscle was isolated and fixed 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 equilibrated with 95% O_2 and 5% CO_2 , maintained at 36°C and perfused through the chamber.

Measurement of Ca^{2+} transients

We adopted the procedures used in a previous study for measuring Ca^{2+} -aequorin luminescence (Ca^{2+} transients) and twitch tension (Kimura *et al.*, 1990). In brief, the aequorin

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solution (1 mg ml⁻¹) was pressure-injected into 40–50 fibres near the ends of the fine branches where miniature endplate potentials were obtained. The phrenic nerve was then stimulated at 0.1 Hz with supramaximal square pulses (0.4–0.8 V) of 0.1 ms duration. Aequorin luminescence was led to a photomultiplier tube (Hamamatsu Photonics, Shizuoka, Japan) and measured by a photon counter (Hamamatsu Photonics). The signals were averaged 6 or 30 times by a signal processor (San-ei, Tokyo, Japan).

Drugs and solutions

Type A botulinum toxin (Wako Pure Chemical, Osaka, Japan) was injected into mice by a bolus i.p. injection 40 min prior to dissection.

Adenosine 3':5'-cyclic monophosphate (cyclic AMP; Sigma Chemical, MO, U.S.A.), calmodulin (Calbiochem, Tokyo), catalytic subunit of protein kinase-A (Sigma) and phospholipase A₂ (Sigma) were dissolved in 5 µM EDTA, and then passed through a column of Chelex 100 (50–100 mesh; Bio-Rad Laboratories, CA, U.S.A.). A similar amount of the above solutions was injected by nitrogen gas at 5 to 6 atm for 2 s through a micropipette into the muscle fibres with aequorin (Wako). Injection pipettes were filled with the above drugs at 100 fold higher concentrations than those reported as effective concentrations in other tissues, because the volume of muscle fibre within which aequorin is thought to diffuse was estimated as about 100 fold larger than that of aequorin droplets expelled by pressure at 5 atm for 2 s in the air.

AA373 (3-(2'-hydroxy-4',5'-diethoxybenzoyl)propionic acid) (Takeda Chemical, Osaka), 4 α -phorbol 12, 13-didecanoate (0.1% *N,N*-dimethylformamide solution; Funakoshi, Tokyo), 4-aminopyridine (Nacalai Tesque, Kyoto, Japan), 8-bromo cyclic AMP (Sigma), α -CGRP (human; Asahi-Kasei Industrial, Shizuoka), CGRP₈₋₃₇ (human; Peninsula Laboratories, CA), H-7 (1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; MBL, Tokyo), H-85 (*N*-[2-(*N*-formyl-*p*-chlorocinnamylamino)ethyl]-5-isoquinolinesulphonamide), H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide) and KN-62 (1-[*N,O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) (0.001–0.05% dimethyl sulphoxide solution; provided by Prof. H. Hidaka, Department of Pharmacology, Nagoya University School of Medicine), indomethacin (0.1% *N,N*-dimethylformamide solution; Sigma), isoprenaline (Kaken Pharmaceutical, Tokyo), neostigmine methylsulphate (Sigma), 12-O-tetradecanoyl phorbol 13-acetate (TPA, 0.03–0.1% *N,N*-dimethylformamide solution; Sigma), staurosporine (0.01% *N,N*-dimethylformamide solution; Kyowa Medix, Tokyo), theophylline (Nacalai), W-5 (*N*-(6-aminohexyl)-1-naphthalenesulphonamide hydrochloride; MBL) and W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride; Nacalai) were bath-applied.

Experimental protocol

After intracellular injection by pressure or preincubation with the above drugs, neostigmine was added for 15 min. The records for 30 signals of Ca²⁺ transients obtained during the last 5 min were analyzed. The duration of noncontractile Ca²⁺ transients consists of time to peak (T_p) and decay time (T_d). Decay time was expressed as the period from peak to e⁻¹ amplitude of the signal.

Statistical analysis

Data are expressed as mean $\pm$ s.e. One-way analysis of variance (ANOVA), multiple-range test and Student's *t* test were used to evaluate statistical differences between the means. The levels of significance were taken as *P* = 0.05 or 0.01. The 50% inhibitory concentrations (IC₅₀) on peak amplitude of noncontractile Ca²⁺ transients were calculated by direct assay on log concentration-response curves, where

each response was presented as a percentage of the noncontractile response in the presence of neostigmine alone.

Results

Involvement of ACh in noncontractile Ca²⁺ mobilization

4-Aminopyridine (100 µM), a K⁺ channel blocker that enhances the release of ACh (Somogyi *et al.*, 1987), increased the peak amplitude of both noncontractile and contractile Ca²⁺ transients, but did not affect the duration of noncontractile transients (data not shown). Botulinum toxin produces a highly effective blockade of quantal ACh release (Stanley & Drachman, 1983). Type A botulinum toxin (1 to 100 µg, bolus i.p.) completely suppressed the generation of both types of Ca²⁺ transient but did not affect mechanical or luminescence responses following direct stimulation of muscle segments (data not shown). Hexamethonium (10 to 100 µM), an antagonist of presynaptic nicotinic AChRs mediating a positive feed-back mechanism (Wessler *et al.*, 1986), inhibited the peak amplitude of noncontractile Ca²⁺ transients but not their duration (Figure 1). The IC₅₀ for hexamethonium was

Figure 1 Involvement of a presynaptic nicotinic AChR in the mobilization of noncontractile Ca²⁺ in diaphragm muscles treated with 0.3 µM neostigmine. (a) Typical traces of Ca²⁺ transients in the presence of hexamethonium at the concentrations indicated (µM). The large, rapid increase in Ca²⁺ represents the contractile transients, and the slower prolonged increase represents the noncontractile transients. Ordinate calibration bar represents 50% amplitude. (b) Log concentration-response curves of hexamethonium on the peak amplitude and the duration of noncontractile Ca²⁺ transients, respectively. Increasing concentrations of hexamethonium were added to the neostigmine solution every 5 min after a 15 min application of neostigmine, and the records for 6 signals during the last 1 min were analysed. Peak amplitude of either type of Ca²⁺ transients was expressed as a percentage of contractile Ca²⁺ transients obtained before the application of neostigmine. *n* = 4 to 8. **P* < 0.05 and ***P* < 0.01; significantly different from the control response to neostigmine alone, by multiple-range test.

30 μM (23.3–38.7, 95% confidence limits). Contractile Ca^{2+} transients were not affected by hexamethonium (data not shown).

Enhancing effect of CGRP on noncontractile Ca^{2+} transients

CGRP_{8–37} (10 to 20 μM), a competitive CGRP antagonist (Poyner *et al.*, 1992), shortened the duration of noncontractile Ca^{2+} transients, whereas it did not affect the peak amplitude (Figure 2a). CGRP (0.3 to 10 nM) prolonged the duration of noncontractile Ca^{2+} transients without affecting their peak amplitude (Figure 2b). When the duration was divided into two time phases, T_1 and T_2 (see methods), the lengthening of the duration by CGRP could be attributed to an increase in T_2 (Table 1), suggesting a delay of the inactivation process. A low concentration (0.1 μM) of CGRP_{8–37}, which had no effect on noncontractile Ca^{2+} transients, suppressed the enhancing effect of 10 nM CGRP on the duration of

Table 1 Influence of protein kinase-A system on duration of noncontractile Ca^{2+} transients

Drug	T_1 (ms)	T_2 (ms)	Total duration ^a (ms)	n
Control ^b	154 $\pm$ 8	429 $\pm$ 40	583 $\pm$ 40	9
CGRP (10 nM)	193 $\pm$ 23	550 $\pm$ 29*	743 $\pm$ 27**	8
PKA (10 μM) ^c	162 $\pm$ 13	519 $\pm$ 28	681 $\pm$ 27†	7
AA373 (300 μM)	161 $\pm$ 8	559 $\pm$ 20*	719 $\pm$ 18*	7
H-89 (0.3 μM)	171 $\pm$ 12	343 $\pm$ 17‡	514 $\pm$ 23‡‡	7
H-85 (0.3 μM)	199 $\pm$ 17	434 $\pm$ 32	634 $\pm$ 29	7

^aTotal duration consists of time to peak (T_1) and decay time (T_2). ^bDuration of noncontractile Ca^{2+} transients in the presence of 0.3 μM neostigmine alone. ^cCatalytic subunit of protein kinase-A (PKA) was intracellularly injected with aequorin.

Statistical differences were evaluated by Student's *t* test.

* $P < 0.05$ and ** $P < 0.01$; significantly different from control responses to neostigmine alone, determined by two-tail test.

† $P < 0.05$ represents one-tail significance.

‡ $P < 0.05$ and ‡‡ $P < 0.01$; significantly different from the effect of H-85, determined by two-tail test.

Figure 2 Involvement of calcitonin gene-related peptide (CGRP) in the mobilization of noncontractile Ca^{2+} in diaphragm muscles treated with 0.3 μM neostigmine. CGRP_{8–37} was preincubated for 90 min before neostigmine was added. Responses to CGRP_{8–37} and their control responses to neostigmine alone were examined in nonperfused solution to minimize losses. CGRP was preincubated for 15 min before neostigmine was added. CGRP_{8–37} (0.1 μM) or H-89 were added 10 min before addition of CGRP. (a) Upper: typical traces of Ca^{2+} transients in the presence of CGRP_{8–37} at the concentrations indicated (μM). Ordinate calibration bars represent 50% amplitude. Middle and lower: log concentration-response curves of CGRP_{8–37} (●) on the peak amplitude and the duration of noncontractile Ca^{2+} transients, respectively. (b) Upper: typical traces in the presence of CGRP (0.3–10 nM). Middle and lower: log concentration-response curves of CGRP without any blockers (■), with 0.1 μM CGRP_{8–37} (Δ) and with 0.1 μM H-89 (□) on the peak amplitude and the duration, of noncontractile transients, respectively. Peak amplitude of either type of Ca^{2+} transients was expressed as a percentage of contractile Ca^{2+} transients obtained before the application of neostigmine. $n = 5$ to 10. ** $P < 0.01$; significantly different from the control response to neostigmine alone, by one-way ANOVA. † $P < 0.05$ and ‡† $P < 0.01$; significantly different from the effects of CGRP without CGRP_{8–37} and H-89, by Student's *t* test.

noncontractile transients (Figure 2b). H-89 (0.1 μM), a selective inhibitor of protein kinase-A (Chijiwa *et al.*, 1990), also suppressed the lengthening effect of 10 nM CGRP. Contractile Ca^{2+} transients were affected neither by CGRP_{8–37} nor by CGRP (data not shown).

Promoting effect of protein kinase-A activator and suppressing effect of a kinase inhibitor on noncontractile Ca^{2+} transients

H-89 (0.1 to 1 μM) decreased both the peak amplitude and the duration of noncontractile Ca^{2+} transients, whereas H-85 (0.1 to 1 μM), a noneffective analogue of H-89, had no effect (Figure 3a). The IC_{50} for H-89 on the peak amplitude was 0.14 μM (0.07–0.50). The shortening of the duration by H-89 (0.3 μM) was due to a decrease in T_2 (Table 1). These results demonstrate that H-89 decreased noncontractile transients both by suppressing the activation process and by promoting the inactivation process for Ca^{2+} mobilization. AA373 (300 μM), a protein kinase-A activator (Kimura *et al.*, 1977), prolonged the duration of noncontractile transients 1.2 fold, especially T_2 , without affecting the peak amplitude of both noncontractile and contractile Ca^{2+} transients (Figure 3b, Table 1). AA373 failed to prolong the duration in the presence of H-89 (0.1 μM). The catalytic subunit of protein kinase-A (10 μM in an injection pipette) also prolonged the duration 1.2 fold (Table 1). These results indicate that the influence of protein kinase-A activation is similar to that of the CGRP application.

Enhancing effect of cyclic AMP on contractile Ca^{2+} transients

The increase in contractile Ca^{2+} transients after cholinesterase inhibition was enhanced by 10 μM isoprenaline (30 min treatment) (Table 2). Cyclic AMP (0.3 mM in the pipette) and 100 μM 8-bromo cyclic AMP (30 min), a cyclic AMP analogue able to enter the cell membrane, also enhanced the contractile responses 2.3 and 1.4 fold, respectively. The duration of noncontractile transients, however, was not prolonged under such conditions. The prolonging effects of cyclic AMP and isoprenaline were observed only at 3–30 fold higher concentrations. Theophylline (100 μM for 30 min) significantly increased only the duration of noncontractile transients.

Figure 3 Involvement of protein kinase A (PKA)-activation in the mobilization of noncontractile Ca^{2+} in diaphragm muscles treated with $0.3 \mu\text{M}$ neostigmine. H-89, H-85 and AA373 were present for 30 min before neostigmine was added. H-89 ($0.1 \mu\text{M}$) was added 10 min before addition of AA373. (a) Upper: typical traces of Ca^{2+} transients in the presence of H-89 at the concentrations indicated (μM). Ordinate calibration bar represents 50%. Middle and lower: log concentration-response curves of H-89 (●) and H-85 (▲) on the peak amplitude and the duration, of noncontractile Ca^{2+} transients, respectively. (b) Upper: typical traces in the presence of $300 \mu\text{M}$ AA373. Middle and lower: log concentration-response curves of AA373 without (■) and with $0.1 \mu\text{M}$ H-89 (□) on the peak amplitude and the duration, of noncontractile transients, respectively. Peak amplitude of either type of Ca^{2+} transients was expressed as a percentage of contractile Ca^{2+} transients obtained before the application of neostigmine. $n = 4$ to 9 . * $P < 0.05$ and ** $P < 0.01$; significantly different from the control response to neostigmine alone, by one-way ANOVA. †† $P < 0.01$, significantly different from the effects of AA373 without H-89, by Student's t test.

Suppressing effect of a protein kinase-C activator on contractile Ca^{2+} transients

TPA (0.3 to $1 \mu\text{M}$, 30 min treatment), a specific activator of protein kinase-C (Nishizuka, 1984), reduced the increase in contractile Ca^{2+} transients to 0.5 fold but not the noncontractile component, whereas the noneffective analogue 4α -phorbol 12, 13-didecanoate ($1 \mu\text{M}$, 30 min) affected neither type of Ca^{2+} transient (Table 3). Staurosporine (10 nM , 30 min) and H-7 ($10 \mu\text{M}$, 30 min), nonselective protein kinase-C inhibitors (Rüegg & Burgess, 1989), did not alter either component of the Ca^{2+} signal.

No involvement of calmodulin in Ca^{2+} mobilization

Intracellular injection of calmodulin ($10000 \text{ unit ml}^{-1}$ in the pipette) changed neither contractile nor noncontractile Ca^{2+} transients (Table 3). Both W-7 ($10 \mu\text{M}$, 30 min treatment), a calmodulin inhibitor (Hidaka *et al.*, 1980), and W-5 ($10 \mu\text{M}$, 30 min), a noneffective analogue of W-7, suppressed contrac-

Table 2 Influence of cyclic AMP on contractile and noncontractile Ca^{2+} transients

Drug	Contractile amplitude (%)	Noncontractile amplitude (%)	duration (ms)	n
Control ^a	228 ± 25	42 ± 8	560 ± 43	9
8-Br cyclic AMP ($100 \mu\text{M}$)	317 ± 36†	44 ± 8	609 ± 16	8
Isoprenaline ($10 \mu\text{M}$)	417 ± 35**	48 ± 8	583 ± 36	8
($30 \mu\text{M}$)	308 ± 33†	46 ± 8	720 ± 19**	8
Theophylline ($100 \mu\text{M}$)	279 ± 42	41 ± 5	691 ± 22**	16
	(kcps) ^b	(kcps)	(ms)	
Cyclic AMP (0 mM) ^c	12.0 ± 2.4	2.2 ± 0.3	574 ± 25	9
(0.3 mM) ^c	27.0 ± 6.0*	3.7 ± 0.7	588 ± 25	10
(10 mM) ^c	32.5 ± 4.8**	3.8 ± 0.7	719 ± 29**	8

^aContractile Ca^{2+} transients before pretreatment with cyclic AMP-related agents were regarded as 100% amplitude only in this table, since contractile transients were increased by these agents alone. ^bKilocounts per second. Ca^{2+} signals before injection of cyclic AMP could not be obtained. Then data are expressed as absolute luminescence values. ^cDrug concentrations in an injection pipette.

Statistical differences were evaluated by Student's t test.

* $P < 0.05$ and ** $P < 0.01$; determined by two-tail test.

† $P < 0.05$ represents one-tail significance.

Table 3 Involvement of signal transduction systems in contractile and noncontractile Ca^{2+} mobilization

Drug	Contractile amplitude (%)	Noncontractile amplitude (%)	duration (ms)	n
Control ^a	250 ± 13	50 ± 10	583 ± 40	9
Protein kinase-C				
TPA ($0.3 \mu\text{M}$)	148 ± 16**	50 ± 28	550 ± 27	4
($1 \mu\text{M}$)	126 ± 15**	49 ± 9	541 ± 18	9
4α -PDD ^b ($1 \mu\text{M}$)	227 ± 14	46 ± 3	626 ± 36	7
Staurosporine (10 nM)	238 ± 21	45 ± 8	573 ± 27	7
H-7 ($10 \mu\text{M}$)	244 ± 17	47 ± 6	597 ± 39	8
Calmodulin				
Calmodulin (10000 u ml^{-1}) ^d	233 ± 16	50 ± 11	629 ± 29	9
W-7 ($10 \mu\text{M}$)	179 ± 18**	16 ± 3*	154 ± 25**	7
W-5 ($10 \mu\text{M}$)	187 ± 20*	15 ± 2*	387 ± 61*	7
KN-62 ($5 \mu\text{M}$)	231 ± 43	50 ± 9	546 ± 29	8
Phospholipase A_2				
PLA ₂ ^c (1000 u ml^{-1}) ^d	330 ± 31	51 ± 6	605 ± 16	9
(10000 u ml^{-1}) ^d	457 ± 76*	52 ± 10	580 ± 21	9
Indomethacin ($10 \mu\text{M}$)	240 ± 22	51 ± 8	642 ± 37	9

^aResponses of each type of Ca^{2+} transients to $0.3 \mu\text{M}$ neostigmine alone. ^b 4α -Phorbol 12, 13-didecanoate. ^cPhospholipase A_2 ($1000 \text{ unit ml}^{-1}$). ^dDrug concentrations in an injection pipette.

* $P < 0.05$ and ** $P < 0.01$; determined by one-way ANOVA.

tile and noncontractile Ca^{2+} transients by the same extent, demonstrating that the calmodulin inhibitor affected Ca^{2+} transients by a mechanism unrelated to the inhibition of calmodulin; the effect may be due to nicotinic AChR-channel blockade. KN-62 ($5 \mu\text{M}$, 30 min), a calmodulin kinase II inhibitor (Tokumitsu *et al.*, 1990), affected neither type of Ca^{2+} transient.

Enhancing effect of phospholipase A_2 on contractile Ca^{2+} transients

Phospholipase A_2 (1000 to $10000 \text{ unit ml}^{-1}$ in the injection pipette) promoted only the increase in contractile transients, but not noncontractile transients (Table 3). Indomethacin ($10 \mu\text{M}$, 30 min treatment), a cyclo-oxygenase inhibitor, had no effect on either type of Ca^{2+} mobilization.

Discussion

Noncontractile Ca^{2+} mobilization is induced by the accumulation of ACh after cholinesterase inhibition, at the neuromuscular junction. However, the mechanism of noncontractile Ca^{2+} mobilization has not been clarified. The duration of endplate potentials is prolonged by the continued action of ACh in anticholinesterase-treated muscle (Katz & Miledi, 1975; Burd & Ferry, 1987), then desensitization of the nicotinic AChR can readily occur to nerve stimulation (Magleby & Pallotta, 1981). Noncontractile Ca^{2+} which is mobilized under such a desensitizable condition may prevent the nicotinic AChR from responding to excessive stimuli.

The peak amplitude of noncontractile Ca^{2+} transients was enhanced by 4-aminopyridine and inhibited either by botulinum toxin or by hexamethonium. These results demonstrate that the peak amplitude of noncontractile transients depends on evoked ACh release from presynapse. In particular, the inhibitory effect of hexamethonium suggests that the autofacilitation of ACh release is an essential process in noncontractile Ca^{2+} mobilization, since hexamethonium reduces the ACh release by blocking a presynaptic nicotinic AChR which mediates the positive feed-back mechanism (Wessler *et al.*, 1986). Hexamethonium has little effect on the postsynaptic nicotinic AChR at the low concentrations used in this study (Milne & Byrne, 1981). We cannot, however, rule out the possibility that another type of nicotinic AChR, such as a neuronal nicotinic AChR, might exist on the postsynaptic side to mobilize noncontractile Ca^{2+} . The direct activation of the postsynaptic nicotinic AChR by neostigmine can be excluded from the mechanism of Ca^{2+} mobilization, because it did not generate noncontractile Ca^{2+} transients after the blockade of quantal ACh release by botulinum toxin.

Noncontractile Ca^{2+} transients were shortened by the CGRP antagonist CGRP₈₋₃₇, and prolonged by CGRP. It has been reported that the CGRP is released in part from the motor nerve by prolonged terminal depolarization, although the major source of CGRP release is from sensory terminals of skeletal muscle (Sakaguchi *et al.*, 1991). The CGRP binds to CGRP receptors located at the neuromuscular junction (Poyner *et al.*, 1992; Popper & Micevych, 1989). Our results suggest that the mobilization of noncontractile Ca^{2+} may be promoted by endogenous CGRP of the neuromuscular synapse. The release of CGRP may be initiated through the positive feed-back mechanism of ACh release during the accumulation of ACh.

CGRP induces a localized increase in the cyclic AMP level in endplate-rich region, indirectly leading to the activation of protein kinase-A (Matsumoto *et al.*, 1992). The protein kinase-A inhibitor H-89 suppressed noncontractile Ca^{2+} transients. The duration of noncontractile transients was prolonged either by the protein kinase-A activator, AA373 or by the catalytic subunit of the kinase. The prolongations by CGRP were completely suppressed by H-89. These results indicate that CGRP may activate protein kinase-A to mobilize noncontractile Ca^{2+} . The prolonging effects of isoprenaline and cyclic AMP were observed only at higher concentrations than those increasing contractile transients, whereas CGRP prolonged the noncontractile transients without affecting the contractile responses. Although the low sensitivity to added cyclic AMP appears to reflect problems of access of cyclic AMP to the relevant compartment of muscle fibre, cyclic AMP-independent activation of protein kinase-A by CGRP may be one of pathways in noncontractile Ca^{2+} mobilization. A presynaptic role of protein kinase-A (Van der Kloot & Brănisteanu, 1992) contributes less to

the prolongation of noncontractile Ca^{2+} transients, because the prolongation was observed by intracellular injection of the catalytic subunit of protein kinase-A or a high concentration of cyclic AMP into muscle fibres. Nicotinic AChR phosphorylation by protein kinase-A causes agonist-independent activation of the nicotinic AChR in the single-channel recording (Ferrer-Montiel *et al.*, 1991). Hence, CGRP-induced prolongation of noncontractile Ca^{2+} transients, especially T_2 (decay time in the Ca^{2+} signal reflecting an inactivation process), may be related to the receptor-channel opening via phosphorylation by protein kinase-A.

It has been shown that a protein kinase-C activator TPA alters the conductance and the gating behaviour of the nicotinic AChR-channel (Eusebi *et al.*, 1987). The decrease in contractile Ca^{2+} transients by TPA is probably correlated with phosphorylation of the nicotinic AChR by protein kinase-C. The presynaptic effect of TPA may not relate to this decrease, since phorbol ester enhances the ACh release in mouse phrenic nerve-diaphragm preparations (Murphy & Smith, 1987). In contrast to contractile transients, the noncontractile transients were not inhibited by TPA. The protein kinase-C inhibitors, staurosporine and H-7, did not affect noncontractile transients, demonstrating that the mechanism of noncontractile Ca^{2+} mobilization is not related to the enzyme activity of protein kinase-C. On the other hand, phospholipase A_2 increased only contractile transients. This increase may be in part due to arachidonic acid-induced Ca^{2+} release from the sarcoplasmic reticulum (Cheah, 1981). Calmodulin-related agents affected neither type of Ca^{2+} transients. Thus, the postsynaptic signalling pathways examined in this study, other than the protein kinase-A system, were not involved in the processes of noncontractile Ca^{2+} mobilization.

CGRP promotes nicotinic AChR desensitization through phosphorylation by protein kinase-A (Miles *et al.*, 1989). In addition, intracellular Ca^{2+} influx through the nicotinic AChR enhances the desensitization via phosphorylation of the receptor by protein kinase-C (Huganir & Greengard, 1990). However, the interaction between the cyclic AMP (protein kinase-A) system and Ca^{2+} system with respect to desensitization remains unclear. We suggest the possibility that CGRP may mobilize noncontractile Ca^{2+} during desensitization. Noncontractile Ca^{2+} may translocate protein kinase-C to the plasma membrane and promote the kinase activation by co-factors such as diacylglycerol, because protein kinase-C binds to the membrane in a Ca^{2+} -dependent manner (Bazzi & Nelsestuen, 1988). Protein kinase-C phosphorylates the nicotinic AChR, inducing desensitization of the receptor (Eusebi *et al.*, 1987). Thus, the protein kinase-A system may cooperate with the Ca^{2+} system to promote nicotinic AChR desensitization.

In conclusion, noncontractile Ca^{2+} mobilization was promoted by nerve-released CGRP activating protein kinase-A, and was dependent on the accumulated amounts of ACh at the neuromuscular junction, whereas contractile Ca^{2+} mobilization was inhibited by protein kinase-C and enhanced by phospholipase A_2 .

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