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Calcitonin gene-related peptide potentiates nicotinic acetylcholine receptor-operated slow Ca^{2+} mobilization at mouse muscle endplates

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1 The involvement of calcitonin gene-related peptide (CGRP) in the non-contractile slow Ca^{2+} mobilization induced by prolonged nicotinic stimulation was investigated by measurement of $[Ca^{2+}]_i$ levels in mouse single muscle cells (flexor digitorum brevis; FDB) loaded with a Ca^{2+} indicator fluo-3 using confocal laser scanning microscopy.

2 CGRP (3–30 nM) potentiated acetylcholine (ACh, 1 μM)-elicited slow Ca^{2+} mobilization in a concentration-dependent manner.

3 The potentiation by CGRP of the slow Ca^{2+} component was greatly depressed by a competitive nicotinic antagonist (+)-tubocurarine (5 μM). The Ca^{2+} channel blocker nitrendipine (1 μM) affected neither ACh responses nor the CGRP potentiation.

4 The slow Ca^{2+} component was completely abolished by reducing $[Ca^{2+}]_0$ from 2.5 to 0.25 mM whereas the fast component was not affected. The CGRP-induced potentiation of slow Ca^{2+} signal was also depressed by decreasing $[Ca^{2+}]_0$.

5 Isoproterenol (30 μM) and 8-bromo-adenosine 3′,5′-cyclic monophosphate (1 mM) potentiated the ACh-elicited slow Ca^{2+} response. The potentiation by CGRP of the slow Ca^{2+} component was completely abolished by a protein kinase-A inhibitor H-89 (1 μM).

6 These findings indicate that CGRP potentiates the nicotinic ACh receptor-operated slow Ca^{2+} signal via the activation of protein kinase-A system at the skeletal muscle endplates.

Keywords: nicotinic acetylcholine receptor; neuromuscular junction; calcitonin gene-related peptide; protein kinase-A; confocal microscope; fluo-3; non-contractile slow Ca^{2+} mobilization

Introduction

The neuropeptide calcitonin gene-related peptide (CGRP) coexists with acetylcholine (ACh) in the motor nerve endings of rodent neuromuscular junctions (Takami *et al*., 1985a; Matteoli *et al*., 1988). The CGRP binds to receptors located at the postsynaptic membranes (Jennings & Mudge, 1989; Poper & Micevych, 1989; Roa & Changeux, 1991) and induces a localized increase in the concentration of intracellular adenosine 3′,5′-cyclic monophosphate (cyclic AMP) 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 regulates the function of nicotinic ACh receptor (AChR) at the neuromuscular junction (Mulle *et al*., 1988; Kimura *et al*., 1993; Lu *et al*., 1993; Dezaki *et al*., 1996; Kimura *et al*., 1997).

Abnormal accumulation of ACh causes a long-lasting non-contractile Ca^{2+} mobilization measured by Ca^{2+}-aequorin luminescence, in addition to the transient increase of usual contractile Ca^{2+} mobilization in nerve-stimulated mouse diaphragm muscles with the cholinesterase inhibitor, neostigmine (Kimura *et al*., 1990). This non-contractile Ca^{2+} mobilization is independent of the contractile Ca^{2+} released from sarcoplasmic reticulum via nicotinic AChR activation (Kimura *et al*., 1991a,b). The non-contractile Ca^{2+} mobilization physiologically desensitizes nicotinic AChR (Kimura *et al*., 1995). Biphasic elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) is elicited by prolonged nicotinic action at mouse single muscle endplates and the pharmacological properties of the slow Ca^{2+} component are sufficiently similar to those of nerve-evoked non-contractile Ca^{2+} mobilization in the diaphragm muscles (Tsuneki *et al*., 1997). The slow Ca^{2+} component is generated much more slowly in isolated single muscle cells than in intact nerve-stimulated muscles. We therefore suppose that nerve-released CGRP may produce a quicker response due to the proximity of the release site to the postsynaptic receptors. In the present study, to confirm whether CGRP and cyclic AMP-related agents enhance the nicotinic AChR-operated slow Ca^{2+} signal, $[Ca^{2+}]_i$ levels in the endplate of mouse single muscle cells were measured with a real-time confocal laser scanning microscope system.

Methods

Muscle preparation

Male ddY mice (7–9 weeks old, 30–42 g) were killed by decapitation under slight anesthesia with ether. The flexor digitorum brevis (FDB) muscles were isolated from the hind feet of the mice, incubated with 0.2% collagenase at 36°C for 75 min and then with 0.5% trypsin for 15 min in the 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). The solution was saturated with 95% O_2 and 5% CO_2. The preparation was subsequently rinsed in the nutrient solution and gently triturated to obtain single cells. After washing for 1.5 h, the dispersed muscle cells were incubated in 10 μM fluo-3/AM with the addition of 0.1% pluronic F127 for 45 min at room temperature and washed for 1 h.

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Measurement of intracellular Ca^{2+}

The $[Ca^{2+}]_i$ levels of muscle cells loaded with fluo-3 were imaged using a confocal microscope system (Insight Biomedical, MI, U.S.A.). Fluo-3/AM is easily loaded into cells as a cell permeant ester that is quickly cleaved by intracellular esterases to an impermeant acid. Fluo-3 fluorescence was excited by using 488 nm argon laser line (23 mW), and the emission, passed through the 530 ± 15 nm filter, was detected using a CCD camera with an intensifier. ACh was applied through a micropipette to the cell suspension put on glass bottom microwells (Mat Tek, MA, U.S.A.). The cells were pretreated with (+)-tubocurarine, nitrendipine and H-89 for 10–30 min before ACh application. CGRP solution was applied 2 min after ACh application. Similar extents of $[Ca^{2+}]_i$ changes were elicited in response to the same concentration (1 μM) of ACh applied after the first scanning. Moreover, the similar time-dependent changes of the Ca^{2+}-concentration to fluo-3 signals were observed, when muscle cells were co-loaded with the two Ca^{2+}-indicators, fluo-3 and fura-red, and confocal ratio imaging was used to monitor the $[Ca^{2+}]_i$ changes. Therefore, fluo-3 signal may be stable during the scannings in the present study. During scanning, cellular events and fluorescent images (of XY planes) were recorded and directly visualized through the oculars captured by a CCD camera onto the monitor. Individual images were then transferred to the computer through a frame grabber which can be subsequently analysed by INSIGHT-IQ analysis software. A typical image of fluo-3 fluorescence exhibited different responses in mouse FDB muscle cells after bath application of different drugs and solutions in which the pseudocolor pallet shows the color correspondence within the pixel intensity range of 0 (violet) to 4095 (white). The % difference of the intensity of fluo-3 signals at the endplate region was estimated, and was normalized against that in the resting state. The % difference $= (F_i - F_0) \times 100$; where F_i is the ratio of increase in fluorescence intensity inside the endplate region, and F_0 is that outside the endplate region of muscle cell.

Drugs and solutions

Collagenase (Wako Pure Chemical, Osaka, Japan) and Trypsin Type III (Sigma Chemical, St. Louis, MO, U.S.A.) were used for incubation and single cell isolation. Fluo-3/AM (Dojindo, Kumamoto, Japan) with addition of Pluronic F127 (Molecular Probes, Oregon, U.S.A.) was used as the Ca^{2+} indicator. CGRP (rat α; Peptide Institute, Osaka, Japan), H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide; Biomol Research Laboratories, U.S.A.), nitrendipine (Yoshitomi Pharmaceutical Industries, Osaka, Japan), (+)-tubocurarine chloride (Nacalai Tesque, Kyoto, Japan), dl-isoproterenol HCl (Wako) and 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cyclic AMP; Biomol) were bath applied.

Statistical analysis

Data were expressed as mean ± s.e.mean. The significant difference was expressed with one-way analysis of variance (ANOVA) followed by Scheffé's test at $P = 0.05$ or 0.01.

Results

CGRP potentiates ACh-induced slow Ca^{2+} mobilization

Bath application of ACh generates a biphasic elevation (fast and slow components) in $[Ca^{2+}]_i$ at the single muscle cell endplate of mice as previously reported (Tsuneki *et al*., 1997). Within the first 5–30 s after bath-application of ACh, a localized rise in $[Ca^{2+}]_i$ was observed at the endplate, and thereafter, it slowly declined with time and disappeared about 2 min after ACh application. However, in the continued presence of ACh, the $[Ca^{2+}]_i$ was subsequently increased again to a larger extent within 8–10 min. To elucidate the effect of CGRP on the slow Ca^{2+} component, CGRP was applied into the bath 2 min after ACh application, and the Ca^{2+}

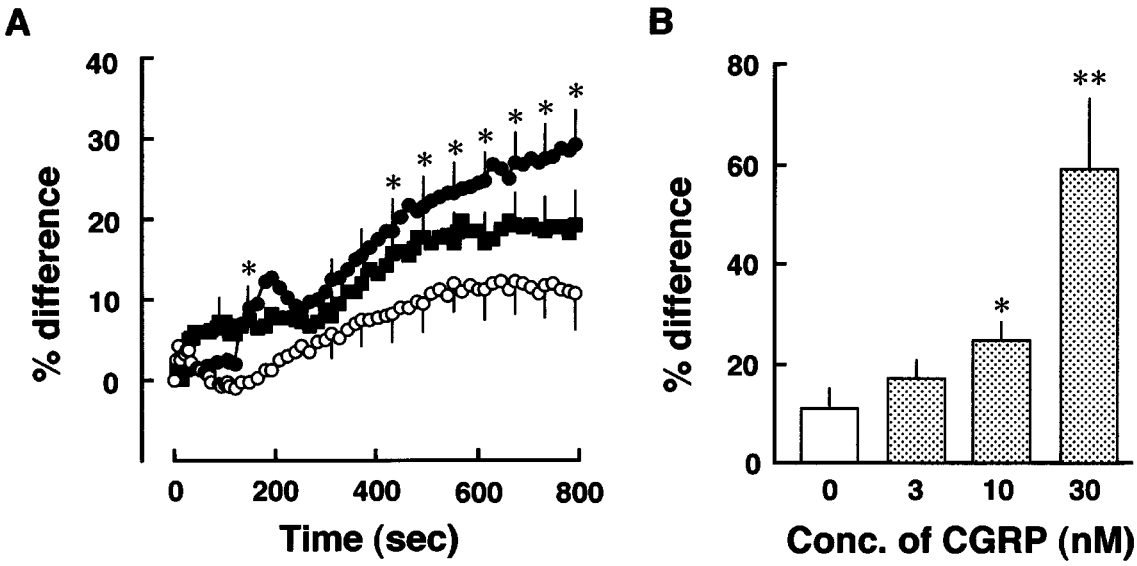

Figure 1 Potentiating effect of CGRP on the ACh (1 μM)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. (A) Time course of changes in the fluorescence intensity of fluo-3 at the endplate. The cells were exposed to CGRP (○: 0 nM, ■: 3 nM and ●: 10 nM) 2 min after ACh application. The % difference $= (F_i - F_0) \times 100$; where F_i is the ratio of increase in fluorescence intensity inside the endplate region, and F_0 is that outside the endplate region of muscle cell. (B) CGRP concentration-dependency of the ACh-elicited slow Ca^{2+} response at the 600 s after the ACh application. Data are expressed as mean ± s.e.mean ($n = 27–30$). $^*P < 0.05$, $^*P < 0.01$: significantly different from control response with ACh alone (0 nM CGRP) based on one-way ANOVA followed by Scheffé's test.

fluorescence was observed by confocal imaging. CGRP (10 nM) significantly potentiated ACh (1 μM)-induced slow Ca^{2+} mobilization (Figure 1A). The peak amplitude of the slow component of the Ca^{2+} signal depended on the CGRP concentrations (3–30 nM) (Figure 1B). A transient rise in $[Ca^{2+}]_i$ was observed at the endplate region within a few seconds after CGRP application which quickly declined. Pretreatment of the CGRP (10 nM) with the cells for 2–20 min before ACh-application did not affect the ACh-induced fast or the slow Ca^{2+} response (data not shown).

To examine the effect of CGRP alone on the basal $[Ca^{2+}]_i$ level, the mouse FDB muscle cells were exposed to CGRP (10 nM) with successive scanning by the confocal microscope. The fluorescence intensity inside and outside the endplate region indicated that CGRP alone slightly but significantly increased the basal $[Ca^{2+}]_i$ levels at the endplate regions. The increase of fluo-3 signal elicited by ACh with CGRP was larger than the addition of those elicited each by ACh or by CGRP. The % difference of fluorescence intensity (increase in the basal $[Ca^{2+}]_i$ level) elicited by ACh (1 μM) with CGRP (10 nM), ACh (1 μM) alone and CGRP (10 nM) alone at the endplate region was $24.9 \pm 3.4\%$, $11.2 \pm 3.8\%$ and $2.1 \pm 0.6\%$, respectively. Thus, the enhancing effect of CGRP on the ACh-elicited slow Ca^{2+} signal may be synergistic and not just additive.

(+)-Tubocurarine depresses the CGRP-induced potentiation of slow Ca^{2+} response

To clarify the mechanism on the CGRP-induced potentiation of the slow Ca^{2+} response, the effect of a competitive nicotinic antagonist (+)-tubocurarine was examined. The FDB muscle cells were pretreated with (+)-tubocurarine (5 μM) 10–20 min before the application of ACh, then CGRP was added 2 min after ACh application. ACh (1 μM) responses were completely depressed in both Ca^{2+} components, and the potentiation of slow Ca^{2+} component by CGRP (10 nM) was also depressed by 5 μM (+)-tubocurarine (Figure 2). These results indicate that the CGRP enhances nicotinic AChR-operated slow Ca^{2+} signal. (+)-Tubocurarine did not block the increase in $[Ca^{2+}]_i$ to CGRP alone (data not shown).

CGRP-induced potentiation of slow Ca^{2+} signal is dependent on the external Ca^{2+} concentrations

To examine the external Ca^{2+} ($[Ca^{2+}]_0$)-dependency of the potentiating effect of CGRP on the slow Ca^{2+} component elicited by ACh, $[Ca^{2+}]_0$ was decreased from 2.5 to 0.25 mM. The slow Ca^{2+} signal elicited by ACh (1 μM) was depressed by 0.25 mM $[Ca^{2+}]_0$, whereas, the fast component was not affected as shown previously (Tsuneki *et al*., 1997). The CGRP (10 nM)-induced potentiation of the slow Ca^{2+} component was also significantly depressed by decreasing $[Ca^{2+}]_0$ (Figure 3), indicating that CGRP-induced potentiation of slow Ca^{2+} signal is dependent on the external Ca^{2+} concentrations.

No effect of nitrendipine on the CGRP-induced potentiation of slow Ca^{2+} mobilization

To confirm whether Ca^{2+} influx through voltage-dependent Ca^{2+} channels is involved in the mechanism of CGRP-induced potentiation of the slow Ca^{2+} signal, the effect of the Ca^{2+} channel blocker, nitrendipine, was examined. The FDB muscle cells were pretreated with nitrendipine (1 μM) for 10–20 min before the application of ACh, then CGRP was added 2 min after ACh application and compared with the control response of ACh alone after the same concentration of nitrendipine. Nitrendipine did not affect either the ACh responses or the potentiating effect of CGRP (Figure 4), suggesting that

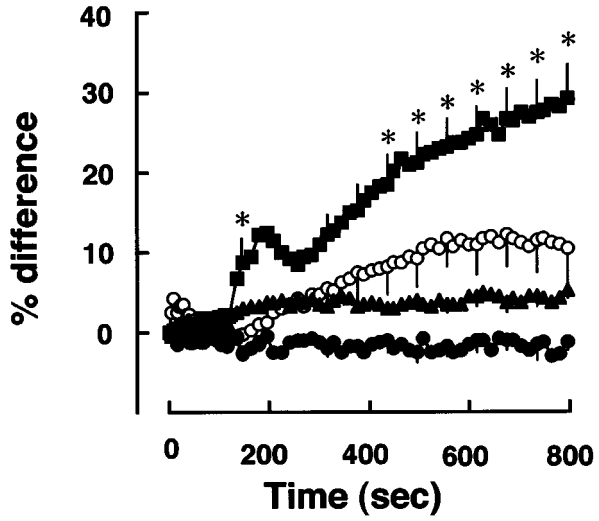

Figure 2 Depression by (+)-tubocurarine of the CGRP-induced potentiation of ACh (1 μM)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were pretreated with (+)-tubocurarine (5 μM) for 10–20 min before the application of ACh, then CGRP was added 2 min after ACh application. The effect of CGRP (10 nM) was determined in the absence (■) and presence of (+)-tubocurarine (▲) and compared to ACh response in the presence of (+)-tubocurarine (●) without CGRP. The % difference = $(F_i - F_0) \times 100$; where F_i is the ratio of increase in fluorescence intensity inside the endplate region, and F_0 is that outside the endplate region of muscle cell. Data are expressed as mean ± s.e.mean ($n = 23$–30). *$P < 0.05$: significantly different from control response with ACh alone (○) based on one-way ANOVA followed by Scheffé's test.

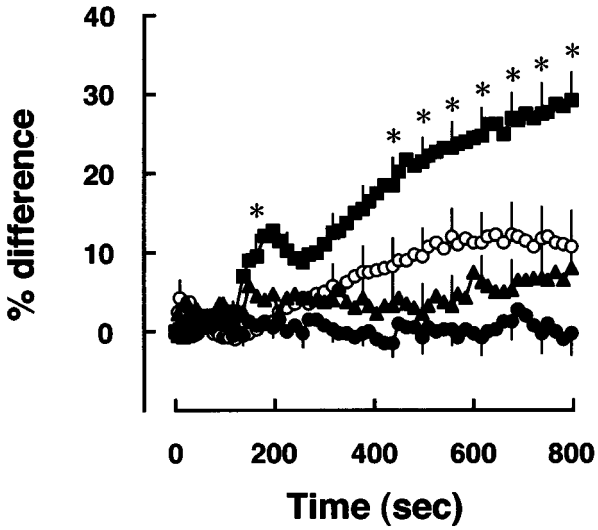

Figure 3 Depressing effect of low external Ca^{2+} concentration on the CGRP-induced potentiation of ACh (1 μM)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were pretreated with Krebs' solution containing low Ca^{2+} concentration for 5–10 min before the application of ACh, then CGRP was added 2 min after ACh application. The effect of CGRP (10 nM) was determined in the presence of low Ca^{2+} concentration (▲: 0.25 mM) and Krebs' solution containing normal Ca^{2+} concentration (■: 2.5 mM) and compared to ACh response in the presence of low Ca^{2+} concentration (●: 0.25 mM). Data are expressed as mean ± s.e.mean ($n = 23$–30). *$P < 0.05$: significantly different from control response with ACh alone (○) based on one-way ANOVA followed by Scheffé's test.

voltage-dependent Ca^{2+} channels are not involved in the effect of CGRP.

Isoproterenol and 8-Br-cyclic AMP potentiate the ACh-elicited slow Ca^{2+} mobilization

We further examined the effects of cyclic AMP-related agents, isoproterenol and 8-Br-cyclic AMP. The cells were exposed to isoproterenol (30 μM) 2 min after ACh (1 μM) application. A localized, brief rise in $[Ca^{2+}]_i$ was observed just after isoproterenol application, which was gradually and significantly increased again by time (Figure 5). Pretreatment of the FDB cells with isoproterenol (3–30 μM) for 20–30 min before ACh application produced a concentration-dependent potentiation of both fast and slow Ca^{2+} mobilization not only inside the endplate region but also outside the region. In fact, many cells were contracted after ACh application in the presence of isoproterenol (data not shown). Therefore, we examined the effect of isoproterenol alone (1, 10 and 30 μM) on the resting $[Ca^{2+}]_i$ in mouse FDB muscle cells. The cells were exposed to isoproterenol with successive scanning with the confocal microscope. The fluorescence intensity of fluo-3 was increased by isoproterenol not only in the endplate region but also all over the whole muscle cell (data not shown). Thus, no difference of the fluorescence intensity between inside and outside the endplate region was observed. The increase depended on time and isoproterenol concentration.

8-Br-cyclic AMP (1 mM), a membrane-permeable cyclic AMP analogue applied 2 min after ACh application, also potentiated the slow Ca^{2+} signal. A significant prolonged rise in the $[Ca^{2+}]_i$ was observed just after 8-Br-cyclic AMP application until 400 s, after which the response slightly declined and was then maintained at a constant level (Figure 6). 8-Br-cyclic AMP (1 mM) alone alike isoproterenol increased the resting $[Ca^{2+}]_i$ level not only in the endplate region but also all over the whole muscle cell (data not shown).

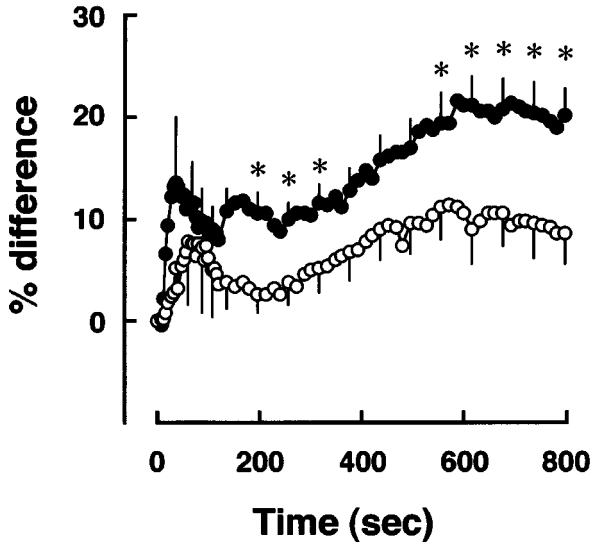

Figure 4 No effect of a Ca^{2+} channel blocker nitrendipine on the CGRP-induced potentiation of ACh (1 μM)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were pretreated with nitrendipine (1 μM) for 10–20 min before the application of ACh, then CGRP was added 2 min after ACh application. The effect of CGRP (10 nM) was determined in the presence of nitrendipine (●) and compared to ACh response in the presence of nitrendipine without CGRP (○). Data are expressed as mean±s.e.mean ($n=18-22$). *$P<0.05$: significantly different from control response with ACh alone (○) based on one-way ANOVA followed by Scheffé's test.

CGRP-induced potentiation of slow Ca^{2+} mobilization is depressed by protein kinase-A inhibition

To investigate the involvement of protein kinase-A in the potentiating effect of CGRP on the slow Ca^{2+} mobilization, the FDB muscle cells were pretreated with a protein kinase-A inhibitor H-89 (1 μM) for 20–30 min. The CGRP (10 nM)-induced potentiation of the slow Ca^{2+} component was completely blocked by H-89 (Figure 7), demonstrating that CGRP potentiates the slow Ca^{2+} mobilization via activation of protein kinase-A within muscle cells.

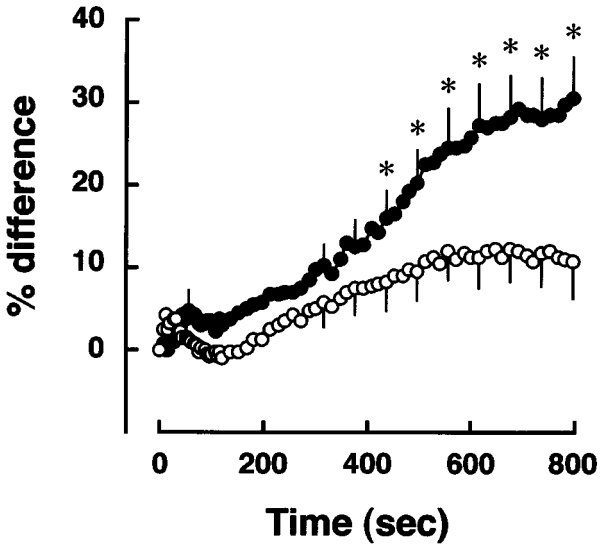

Figure 5 Potentiating effect of isoproterenol on the ACh-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were exposed to isoproterenol (●: 30 μM) 2 min after ACh (1 μM) application. Data are expressed as mean±s.e.mean ($n=24-27$). *$P<0.05$: significantly different from control response with ACh alone (○) based on one-way ANOVA followed by Scheffé's test.

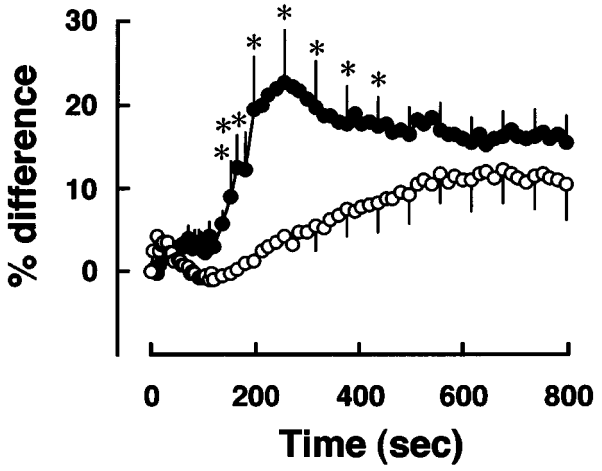

Figure 6 Potentiating effect of 8-Br-cyclic AMP on the ACh (1 μM)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were exposed to 8-Br-cyclic AMP (●: 1 mM) 2 min after ACh application. Data are expressed as mean±s.e.mean ($n=27-30$). *$P<0.05$: significantly different from control response with ACh alone (○) based on one-way ANOVA followed by Scheffé's test.

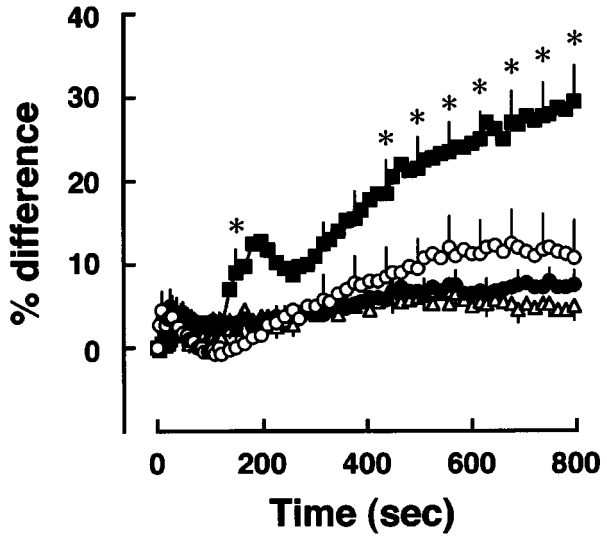

Figure 7 Depressing effect of a protein kinase-A inhibitor H-89 on the CGRP-induced potentiation of slow Ca^{2+} component. The cells were pretreated with H-89 (1 μM) for 20–30 min before the application of ACh, then CGRP was added 2 min after ACh application. The effect of CGRP (10 nM) was determined in the absence (■) and presence of H-89 (△) and compared to ACh response in the presence of H-89 (●). Data are expressed as mean ± s.e.mean ($n = 20-30$). *$P < 0.05$: significantly different from control response with ACh alone (○) based on one-way ANOVA followed by Scheffé's test.

Discussion

CGRP, a major neuropeptide coexists with ACh at the motor nerve endings (Takami *et al*., 1985a,b; Matteoli *et al*., 1988), is released by electrical nerve stimulation or by cholinesterase inhibition-induced ACh accumulation activating presynaptic nicotinic AChR in intact skeletal muscle, and may negatively modulate nerve stimulation-evoked acetylcholine release (Kimura *et al*., 1997). CGRP binds to its receptors located at the neuromuscular junction (Jennings & Mudge, 1989; Poper & Micevych, 1989; Roa & Changeux, 1991). This peptide prolongs the open time of nicotinic AChR channel currents in cultured myotubes (Lu *et al*., 1993; Owens & Kullberg, 1993) and regulates neuromuscular transmission by modulation of nicotinic AChR phosphorylation and desensitization (Dezaki *et al*., 1996; Miles *et al*., 1989). CGRP activates adenylate cyclase and induces a localized increase in the concentration of intracellular cyclic AMP leading to the activation of protein kinase-A, in the endplate rich regions of skeletal muscle (Kobayashi *et al*., 1987; Matsumoto *et al*., 1992). We have previously reported that CGRP prolongs the duration of nerve-stimulated non-contractile slow Ca^{2+} mobilization and $CGRP_{8-37}$, a CGRP antagonist, shortens its duration (Kimura *et al*., 1993), suggesting that endogenous CGRP may modulate the slow Ca^{2+} response.

In this study, confocal imaging of FDB muscle cells indicated that bath-application of CGRP, after ACh application, potentiated the ACh-elicited slow Ca^{2+} signal. The potentiation of the slow Ca^{2+} signal by cyclic AMP-related agents, isoproterenol and 8-Br-cyclic AMP, occurred even though they have different time courses of potentiation. The much slower generation of the slow Ca^{2+} signal in FDB muscle cells than in nerve-stimulated muscles may be due to the lack of nerve-derived CGRP. CGRP, isoproterenol and 8-Br-cyclic AMP independently slightly increased the intracellular Ca^{2+} level. Nevertheless, cotreatment of muscle cells with ACh and either CGRP or cyclic AMP-related agents led to a synergistic enhancement in the slow Ca^{2+} response compared with those elicited by ACh or these agents alone. The protein kinase-A inhibitor H-89 completely abolished the potentiation by CGRP. These results indicate the activation of protein kinase-A within the muscle cell is essential for the enhancement of the slow Ca^{2+} component by CGRP. These results suggest that these two pathways for ACh and protein kinase-A may be linked as the phosphorylation of the nicotinic AChR by protein kinase-A activation. Protein kinase-A-induced phosphorylation of nicotinic AChR may enhance its receptor activity, then potentiating the nicotinic AChR-operated slow Ca^{2+} signal. The first brief and localized rise in the $[Ca^{2+}]_i$ appeared to be due to the effect of CGRP since the application of the vehicle (Krebs' solution), rather than CGRP, did not produce any observable change in $[Ca^{2+}]_i$. CGRP pretreatment, not but post treatment, had no effect on the ACh-elicited Ca^{2+} signals. This may be explained by the fact that the CGRP (10^{-7} M)-induced increase in cyclic AMP content has a half-time of a few minutes in the rat innervated muscle cells (Eusebi *et al*., 1988).

The nerve-stimulated non-contractile Ca^{2+} mobilization is especially blocked by competitive nicotinic antagonists such as (+)-tubocurarine and pancuronium at concentrations which have no effect on contractile Ca^{2+} transients (Kimura *et al*., 1990). Both components of the ACh responses were completely depressed and the potentiation by CGRP was greatly depressed in the presence of (+)-tubocurarine. Therefore, CGRP may activate its own receptor, then indirectly enhance the nicotinic AChR-operated slow Ca^{2+} signal because CGRP does not activate directly nicotinic AChR at the muscle surface (Takami *et al*., 1985b).

Ca^{2+} influx through skeletal muscle Ca^{2+} channels and the force of concentration are increased in response to β-adrenergic stimulation and high frequency-electrical stimulation (Arreola *et al*., 1987). These effects are considered to be mediated by cyclic AMP-dependent phosphorylation of skeletal muscle Ca^{2+} channels. L-type Ca^{2+} channels in the transverse tubular (T tubule) membranes are activated by depolarization and undergo a conformational change that activates the sarcoplasmic reticulum Ca^{2+} release channels via a physical coupling mechanism resulting in Ca^{2+} release and muscle contraction (Johnson *et al*., 1997; Tanabe *et al*., 1990; Rios *et al*., 1991; Lu *et al*., 1994). The slow Ca^{2+} component was completely abolished by diminishing $[Ca^{2+}]_0$ from 2.5 to 0.25 mM, whereas the fast component was not affected, as shown previously (Tsuneki *et al*., 1997). The CGRP-induced potentiation of the slow Ca^{2+} signal was also significantly depressed by decreasing $[Ca^{2+}]_0$. However, the involvement of the voltage-sensitive L-type Ca^{2+} channels in the CGRP enhancing mechanism may be ruled out because the Ca^{2+} channel blocker, nitrendipine, did not affect either the ACh responses or the CGRP-induced potentiation. The response to CGRP is presumably related to Ca^{2+} influx through activated nicotinic AChR-channel as nicotinic receptor-channels are permeable to Ca^{2+} (Vernino *et al*., 1994). Moreover, a neuronal nicotinic AChR antagonist methyllycaconitine depresses nerve-stimulated and ACh-elicited slow Ca^{2+} responses at concentrations that have no effect on the fast Ca^{2+} component (Tsuneki *et al*., 1997). Neuronal nicotinic AChRs have a higher Ca^{2+} permeability than muscle nicotinic AChR (Vernino *et al*., 1994). Therefore, the slow Ca^{2+} component may be due to signals reflecting Ca^{2+} influx through a neuronal-type nicotinic AChR subtype on the

muscle cells (Kimura *et al*., 1994; Tsuneki *et al*., 1995), different from classical muscle-type receptors that promote the fast Ca^{2+} component from an intracellular Ca^{2+} store.

In conclusion, the present study indicates that CGRP potentiates the nicotinic AChR-operated slow Ca^{2+} signal via protein kinase-A activation in the mouse single muscle cell endplates. Nerve-released CGRP may activate a protein kinase-A system and thus contribute to the regulation of the nicotinic AChR function at the neuromuscular junction.

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