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Acetylcholine sensitivity of biphasic Ca^{2+} mobilization induced by nicotinic receptor activation at the mouse skeletal muscle endplate

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1 Acetylcholine (ACh) was locally applied onto the endplate region in a mouse phrenic nerve-diaphragm muscle preparation to measure intracellular free calcium ($[Ca^{2+}]_i$) entry through nicotinic ACh receptors (AChRs) by use of Ca^{2+}-aequorin luminescence.

2 ACh (0.1–3 mM, 20 μl) elicited biphasic elevation of $[Ca^{2+}]_i$ (fast and slow Ca^{2+} mobilization) in muscle cells. The peak amplitude of the slow Ca^{2+} mobilization (not accompanied by twitch tension) was concentration-dependently increased by ACh, whereas that of the fast component (accompanied by twitch tension) reached a maximum response at a lower concentration (0.1 mM) of applied ACh.

3 A pulse of nicotinic agonists, (−)-nicotine (10 mM) and 1,1-dimethyl-4-phenyl-piperazinium (10 mM), but not a muscarinic agonist pilocarpine (10 mM), also elicited a biphasic Ca^{2+} signal.

4 Even though ACh release from motor nerve endings was blocked by botulinum toxin (5 μg, bolus i.p. before isolation of the tissue), the generation of both a fast and slow Ca^{2+} component caused by ACh application was observed.

5 These results strongly suggest that ACh locally applied onto the endplate region of skeletal muscle induces a slow Ca^{2+} signal reflecting Ca^{2+} entry through a postsynaptic nicotinic AChR, which has a low sensitivity to transmitter ACh.

Keywords: Nicotinic acetylcholine receptor; acetylcholine; skeletal muscle endplate; calcium mobilization; botulinum toxin; neuromuscular postsynapse

Introduction

We have shown that non-contractile Ca^{2+} mobilization is generated in mouse diaphragm muscles by nerve stimulation in the presence of anti-cholinesterase agents (Kimura *et al*., 1990b), although a rise in the concentration of cytosolic Ca^{2+} is well known to generate skeletal muscle contraction. Non-contractile Ca^{2+} is mobilized under desensitizing conditions, since desensitization to nerve stimulation readily occurs after inhibition of cholinesterase (Magleby & Pallotta, 1981). This Ca^{2+} mobilization depresses the contractile Ca^{2+} mobilization via protein kinase-C activation, suggesting that non-contractile Ca^{2+} may prevent the nicotinic acetylcholine receptor (AChR) from responding to excessive stimulation by accumulated acetylcholine (ACh) (Kimura *et al*., 1995; Dezaki *et al*., 1996). Non-contractile Ca^{2+} may be mobilized independently of contractile Ca^{2+} mobilization, because (1) the non-contractile Ca^{2+} mobilization is generated in the presence of anti-cholinesterase agents and a higher concentration (5 mM) of external Ca^{2+} increases non-contractile Ca^{2+} mobilization, but decreases contractile Ca^{2+} transients somewhat (Kimura *et al*., 1990b), (2) non-contractile Ca^{2+} mobilization is not due to Ca^{2+} release from sarcoplasmic reticulum (Kimura *et al*., 1991a, b), but due to signal reflecting Ca^{2+} entry through activated nicotinic AChR-channels in contrast to the low Ca^{2+} permeability of muscle nicotinic AChRs and (3) activation of protein kinase-A within skeletal muscle cells enhances non-contractile Ca^{2+} response (Kimura *et al*., 1993). We therefore speculate that the prolonged action of acetylcholine (ACh) or a high concentration of ACh in the synaptic cleft may evoke the generation of a non-contractile Ca^{2+} signal, though the possibility that anti-cholinesterase agents have a direct effect on nicotinic AChRs without hydrolysis of ACh cannot be completely ruled out. However, all previous data for non-contractile Ca^{2+} mobilization were determined in the presence of anti-cholinesterase agents.

In this study, we applied ACh directly onto skeletal muscles in the absence of anti-cholinesterase agents, to induce non-contractile Ca^{2+} mobilization under physiological conditions and to clarify the sensitivity of contractile and non-contractile Ca^{2+} response to ACh.

Methods

Muscle preparations

Male ddY mice (7–9 weeks old, 28–42 g) were lightly anaesthetized with diethyl ether and then killed by decapitation. The right phrenic nerve-diaphragm muscle with 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 rapidly perfused through the chamber (1 ml bath volume) at a rate of 6 ml min^{-1}. The tendon was tied with a silk thread and connected to an isometric transducer (Nihon Kohden, Tokyo, Japan), and the resting tension was adjusted to 2 mN. 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), and equilibrated with 95% O_2 and 5% CO_2.

Measurement of intracellular Ca^{2+} transients

We adopted the procedures for measuring Ca^{2+}-aequorin luminescence (Ca^{2+} transients) (Figure 1) described previously

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(Kimura *et al*., 1990a; 1991b). Aequorin is a fast-responding intracellular Ca^{2+} indicator. The aequorin solution (1 mg ml^{-1}) was intracellularly expelled from the micropipette into the myoplasm at an endplate region of 1–1.5 mm diameter, where miniature endplate potentials (m.e.p.ps) were generated. Usually, a total of 40 to 50 fibres in a diaphragm muscle segment was loaded with aequorin from the intracellular microelectrode used to measure m.e.p.ps by 2 s-nitrogen gas pressure pulses of 5–6 kg cm^{-2} delivered by a pressure system with 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 3 mm in diameter (Mitsubishi Rayon, Toyama, Japan) used as a light guide was placed near the aequorin-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 platinum electrodes placed 1 mm apart, Ca^{2+} transients and twitch tension were recorded simultaneously on a polygraph (San-ei, Tokyo, Japan) and a signal processor (7T07A, San-ei). Without aequorin loading, nerve stimulation alone did not produce light. The intracellular Ca^{2+} concentration was represented as the intensity of Ca^{2+}-aequorin luminescence (kcps; k counts s^{-1}).

Local application of ACh or other agonists onto the skeletal muscle endplate

ACh solution (20 μl) was injected into a puffer microtube and one end of the microtube was immersed in Krebs solution and placed near the endplate region, 20 μm away from the surface of the muscle. The other end of the microtube was connected to a pressure system, which was the same as that used for aequorin injection. We set a small bubble of air at the nozzle of microtube and the ACh solution was kept without leakage into Krebs' solution in the chamber to assure no leakage of ACh. ACh was acutely applied onto the endplate region of the muscle preparation by nitrogen gas pressure pulses of 0.3–0.5 kg cm^{-2}. In this condition, the puffed solution (20 μl coloured with blue ink) was quickly diffused and removed from the muscle endplate by perfusing the solution within 1 s, although the duration of ACh application was not controlled accurately. The similar extents of $[Ca^{2+}]_i$ changes could be consecutively elicited in response to the same concentration of ACh applied at 10 min intervals. Therefore, the desensitization may not occur during a pulse of ACh in this study. Solutions (20 μl) of nicotinic agonists, (−)-nicotine and 1,1-dimethyl-4-phenyl-piperazinium, or a muscarinic agonist pilocarpine, similarly, were locally applied onto the endplate region by use of this system.

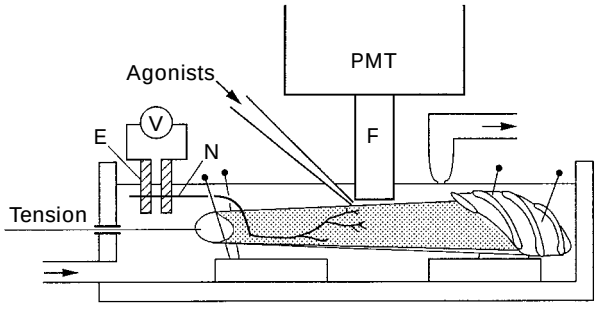

Figure 1 Apparatus used to measure aequorin signals and tension in the mouse phrenic nerve-diaphragm muscle preparation. The modified Krebs solution perfused the preparation at a rate of 6 ml min^{-1}. The tendon was tied with a silk thread and connected to an isometric transducer. The phrenic nerve (N) was stimulated via a pair of platinum wire electrodes 1 mm apart (E). Aequorin luminescence emitted from the aequorin-injected area was directed through the optical fibre (F) onto a photomultiplier tube (PMT) of which the output was led into a photon counter.

Drugs and solutions

Aequorin (Wako Pure Chemical, Osaka, Japan) was dissolved in Ca^{2+}-free distilled water containing 5 μM EDTA. Acetylcholine chloride (Daiichi, Tokyo, Japan), 1,1-dimethyl-4-phenyl-piperazinium iodide (Sigma Chemical, St. Louis, MO, U.S.A.), neostigmine methylsulphate (Sigma), (−)-nicotine (Wako), pilocarpine hydrochloride (Wako) were dissolved in distilled water and diluted with modified Krebs solution. Type A botulinum toxin (Wako) was administered to mice as a bolus i.p. injection under light anaesthesia with diethyl ether. The mice were paralyzed and died 40–60 min after the injection of botulinum toxin, and the phrenic nerve-diaphragm muscle was then isolated.

Statistical analysis

Data are expressed as means ± s.e.mean. One-way analysis of variance with the Scheffé's multiple-comparison test was used to determine statistical significances. The 50% effective concentrations (EC_{50} with 95% confidence limit) of ACh on the peak amplitude of Ca^{2+} transients were calculated by analysing log concentration-response curves.

Results

Slow Ca^{2+} mobilization elicited by local application of ACh onto the endplate region of skeletal muscle

After an increase in the peak amplitude of contractile Ca^{2+} transients (accompanied by twitch tension), generation of non-contractile Ca^{2+} transients (not accompanied by muscle contraction) with a long duration was observed in response to nerve stimulation in the presence of neostigmine (Figure 2 left), as previously shown (Kimura *et al*., 1990b). Using the same preparation (90 min after washout of neostigmine), ACh (0.3 mM) was locally applied onto the muscle endplate region in the absence of neostigmine. ACh elicited biphasic elevation of $[Ca^{2+}]_i$, fast contractile Ca^{2+} transients and slow non-contractile Ca^{2+} transients in muscle cells (Figure 2 right), demonstrating that ACh in the absence of neostigmine produces a similar qualitative response.

On comparison of the typical data of ACh application with that of nerve stimulation in the presence of neostigmine, the former tension apparently decreased (although prolonged) despite an increase in the initial calcium transients. Nerve stimulation elicits efficiently activation of all the endplate and induces contraction in the whole muscle preparation, but local application of ACh induces muscle contraction only at some fibres exposed with ACh. Thus, nerve stimulation-evoked twitch tension is efficiently larger than local application of ACh is, although the nerve-stimulated calcium signals in aequorin-injected fibres are almost in the similar (or somewhat smaller) extent to those elicited by the ACh application.

Figure 3 shows changes in $[Ca^{2+}]_i$ measured in response to five successive applications of ACh at 10 min intervals on the same muscle preparation. The results indicate that similar $[Ca^{2+}]_i$ changes could be consecutively elicited in response to the same concentration of applied ACh under these conditions. However, at pulse intervals of 3 min the response to successive application of ACh (0.3 mM) gradually diminished (data not shown), suggesting desensitization of the nicotinic AChRs. Thus, ACh or other agonists in the following experiments were applied at 10 min intervals to avoid the potential desensitization.

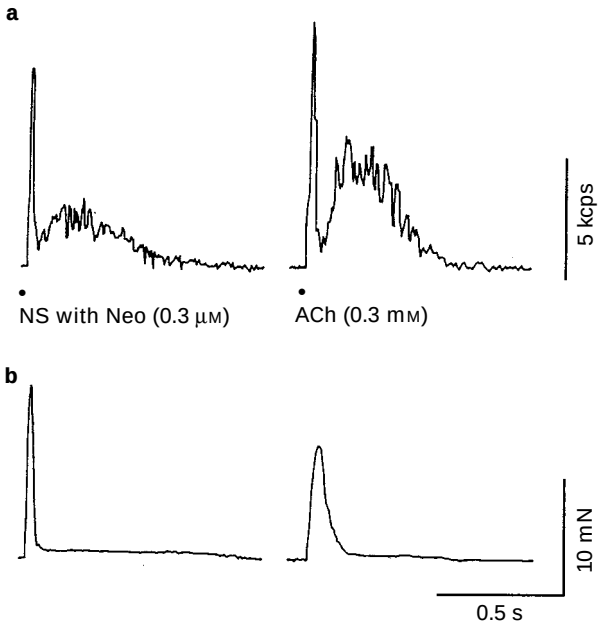

Figure 2 Biphasic elevation of $[Ca^{2+}]_i$ in muscle cells at the mouse neuromuscular junction. The trace shows Ca^{2+}-aequorin luminescence (Ca^{2+} transients, a) and twitch tension (b) elicted by nerve stimulation (NS) with 0.3 μM neostigmine (Neo; left) or by ACh (0.3 mM) locally applied onto the endplate region (right) at the mouse phrenic nerve-diaphragm muscle. The large, rapid increase in Ca^{2+} represents the contractile (accompanied by twitch tension) fast transients, and the prolonged increase represents the non-contractile (not accompanied by muscle contraction) slow transients.

Figure 4 shows the ACh concentration-dependency of Ca^{2+} transients. Slow Ca^{2+} transients elicited by ACh applied directly were initiated at ACh concentrations exceeding 0.1 mM, and the peak amplitude of slow Ca^{2+} transients was concentration-dependently increased by ACh (0.1–3 mM). At the ACh concentrations (0.1–1 mM), the large slow Ca^{2+} component was not accompanied by local contracture except at a higher concentration (3 mM) (data not shown). Fast Ca^{2+} transients were generated at a lower concentration (10 μM) of ACh, and the peak amplitude reached a maximum response at approximately 30 μM. This augmenting effect of ACh on the peak amplitude of the fast component tended to decrease at higher concentrations (>1 mM). The maximum peak response of slow Ca^{2+} transients elicited by 3 mM ACh was $266 \pm 38\%$ (10 separate experiments) of the peak amplitude of fast transients at 0.1 mM ACh. The relative response of the fast Ca^{2+} signal to the slow signal was not changed ($254 \pm 51\%$ for 5 separate experiments) even when the volume of aequorin injected into the muscle cell was increased, by prolonging the duration of intracellular pressure-injection. Therefore, the aequorin signal is unsaturated during the fast response, and so an accurate measurement of the Ca^{2+} signals is obtained. The EC_{50} value of ACh was 17.0 μM (15.5–18.7) for the fast Ca^{2+} transients and 0.42 mM (0.37–0.48) for the slow Ca^{2+} signal.

The slow Ca^{2+} response was not generated by the ACh (0.3 mM)-puff in which aequorin was injected into the extrajunctional area (near the rib), whereas fast contractile Ca^{2+} transients were generated at both junctional and extrajunctional areas (Figure 5). The slow Ca^{2+} response was not observed even when the higher concentration (3 mM) of ACh was applied at extrajunctional area (data not shown), indicating that the slow Ca^{2+} signal is restricted to the endplate region.

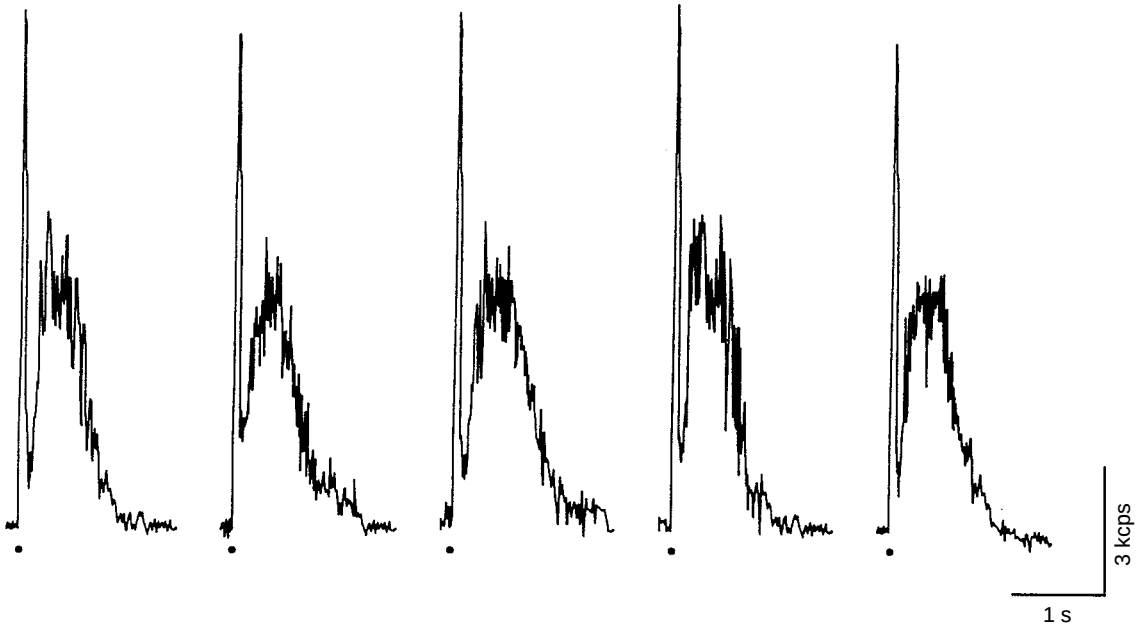

Figure 3 Stability of aequorin signals in response to successive applications of ACh (0.3 mM) onto the endplate region of a skeletal muscle preparation. The figure shows five consecutive $[Ca^{2+}]_i$ responses (from left to right) elicited in the same muscle preparation by ACh-puff at 10 min intervals, indicating that the desensitization does not occur during ACh pulses. Similar results were obtained in 3 muscle preparations.

Skeletal muscle fibres are classified as fast-twitch or slow-twitch, based on histochemical and contractile properties (Barnard *et al*., 1971). To determine whether the difference between fast and slow Ca^{2+} component is attributable to muscle fibre types, ACh-induced Ca^{2+} response was measured in the flexor digitorum brevis (FDB), a fast-twitch muscle (Carlsen *et al*., 1985), and the soleus, a slow-twitch muscle (Ariano *et al*., 1973). A slow Ca^{2+} signal elicited by ACh application was observed in both FDB and soleus muscle preparations of mice (data not shown), indicating that this Ca^{2+} response occurs in both kinds of skeletal muscle cells.

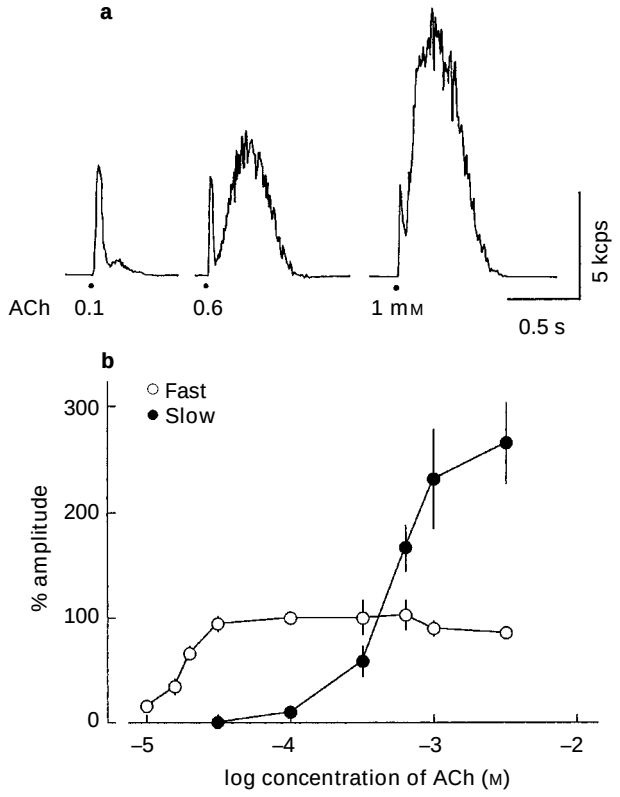

Figure 4 Concentration-dependence of fast and slow Ca^{2+} transient signals elicited by ACh locally applied onto muscle endplate region. (a) Typical traces of Ca^{2+} transients elicited by local application of ACh at 10 min intervals at a concentration of 0.1 mM, 0.6 mM or 1 mM. (b) Log concentration-response curves of ACh for the peak amplitudes of fast and slow Ca^{2+} transients. Data (means with vertical lines showing s.e.mean for 6 to 10 separate experiments) are expressed as percentages of ACh (0.1 mM)-induced fast Ca^{2+} transients.

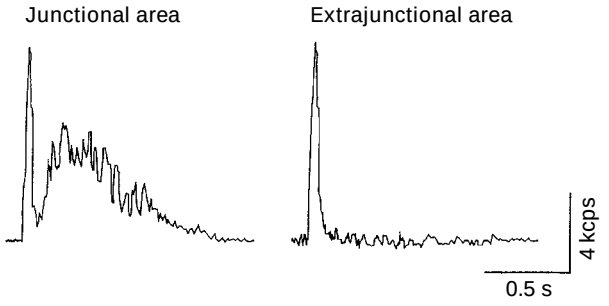

Figure 5 ACh application-induced slow Ca^{2+} transients observed only at the muscle endplate region. The trace shows Ca^{2+} transient signals elicited by ACh (0.3 mM) applied onto the junctional (endplate) (left) and extra-junctional areas (right). Similar results were observed in 3 separate experiments.

The generation of slow Ca^{2+} mobilization by nicotinic but not muscarinic agonists

The typical responses to nicotinic and muscarinic agonists, rather than ACh, are shown in Figure 6. (−)-Nicotine and 1,1-dimethyl-4-phenyl-piperazinium (DMPP), nicotinic agonists, were acutely applied onto the endplate region of muscle. Both (−)-nicotine (10 mM) and DMPP (10 mM) elicited biphasic Ca^{2+} mobilization, wheres the muscarinic agonist pilocarpine (10 mM) elicited neither type of Ca^{2+} mobilization, suggesting that these Ca^{2+} signals are generated via activation of the nicotinic AChR. It appears that the sensitivities of these nicotinic agonists were low compared with that of ACh to the slow Ca^{2+} response and an equal concentration of DMPP was more effective than (−)-nicotine at producing the slow response, but less effective at generating the fast response.

Presynaptic nicotinic autoreceptor is not involved in ACh-induced slow Ca^{2+} signal

Transmitter release from motor nerve endings is modulated by presynaptic nicotinic autoreceptors (Bowman *et al*., 1988). To investigate the involvement of ACh release caused by ACh-induced activation of the nicotinic autoreceptor on motor nerve endings in slow Ca^{2+} mobilization, ACh release was blocked by botulinum toxin. The toxin produces highly effective blockade of quantal ACh release (Stanley & Drachman, 1983). Type A botulinum toxin (5 μg, bolus i.p. injection)

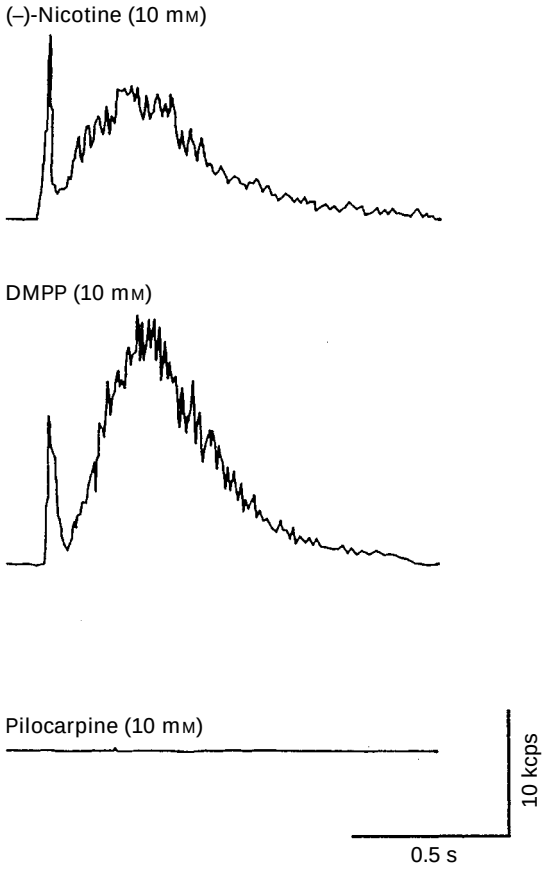

Figure 6 The generation of Ca^{2+} transients caused by local application of nicotinic but not muscarinic agonists. (−)-Nicotine (10 mM), DMPP (10 mM) or pilocarpine (10 mM) was locally applied onto the endplate region. Each trace shows the changes in $[Ca^{2+}]_i$ in response to (−)-nicotine (upper), DMPP (middle) and pilocarpine (lower). Similar results were obtained in 3 separate experiments.

completely suppressed the generation of contractile Ca^{2+} transients elicited by nerve stimulation (Figure 7b left). No twitch tension was produced by nerve stimulation under these conditions (data not shown). Despite neostigmine (0.3 μM) being applied to the muscle preparation, no elevation of intracellular Ca^{2+} was observed in response to nerve stimulation (Figure 7b right). Nevertheless, local application of ACh (0.3 mM) onto the endplate region generated both fast and slow Ca^{2+} transients in this preparation (Figure 7b middle). In the vehicle without botulinum toxin, fast Ca^{2+} transients were generated by nerve stimulation, and the large fast and slow Ca^{2+} signals were also elicited either by locally applied ACh (0.3 mM) or by stimulating the nerve with neostigmine (0.3 μM) (Figure 7a). These results suggest that the modulation of ACh release via activation of a presynaptic autoreceptor does not cause ACh-induced slow Ca^{2+} signal.

Discussion

The present results indicate that non-contractile slow Ca^{2+} mobilization is elicited by the local application of ACh onto the endplate region of skeletal muscle. This Ca^{2+} signal is weakly sensitive to ACh, because it was about 25 fold less potent than in the activation of fast Ca^{2+} transients. The slow Ca^{2+} response observed with ACh was evoked via nicotinic AChRs, as it was generated by local application of (−)-nicotine or DMPP, nicotinic agonists, but not by a muscarinic agonist pilocarpine. These results suggest that the slow Ca^{2+} mobilization elicited by local application of ACh may be operated by nicotinic AChR which is weakly sensitive to ACh. The slow Ca^{2+} mobilization rarely generates muscle contraction. Only a very high concentration of ACh, which produced a large amount of the slow Ca^{2+} signal, barely generated a delayed tonic contracture following the phasic twitch tension which was produced by the fast Ca^{2+} mobilization. The slow Ca^{2+} response, therefore, may occur via a mechanism located just beneath the plasma membrane of muscle cells, making it difficult to produce muscle contraction. The ACh concentration estimated is about 300 μM in the synaptic cleft (Kuffler & Yoshikami, 1975), which is sufficient for the generation of slow Ca^{2+} signal. Under physiological conditions, this response may be present, but not be observed by nerve stimulation without anti-cholinesterase agents with the present technique.

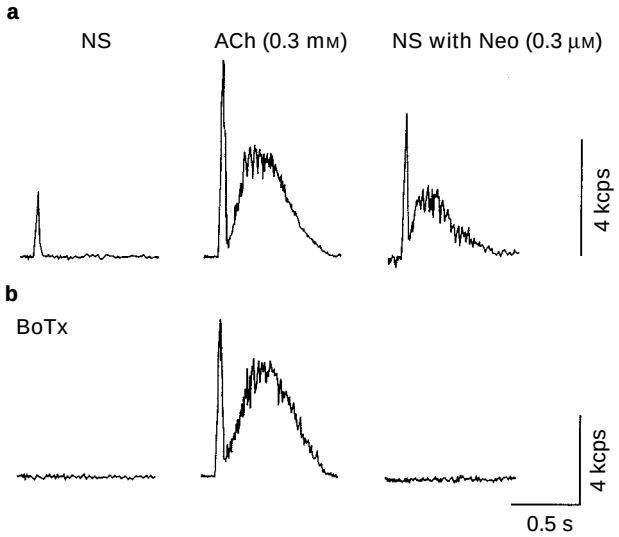

Figure 7 Intracellular Ca^{2+} responses in phrenic nerve-diaphragm muscle preparation from a botulinum toxin-injected mouse. Type A botulinum toxin (BoTx; 5 μg) was administered into one mouse as a bolus i.p. injection 60 min before dissection (b). For the control, saline was injected into another mouse (a). Each trace shows the changes in $[Ca^{2+}]_i$ in response to nerve stimulation (NS), local application of ACh (0.3 mM) onto the endplate region and nerve stimulation with 0.3 μM neostigmine (Neo) of the same preparation. Similar results were obtained in 4 separate experiments.

Diaphragm muscles were used as the skeletal muscle preparation for measurement of intracellular Ca^{2+} level in our previous and present study. The diaphragm muscles are mixed-types of fast- and slow-twitch muscle fibres (Keens *et al*., 1978). Differences in muscle fibres do not contribute to the two types (fast and slow) of Ca^{2+} response because ACh-induced slow Ca^{2+} response was generated in both FDB muscle (fast-twitch muscle, Carlsen *et al*., 1985) and soleus muscle (slow-twitch muscle, Ariano *et al*., 1973). Therefore, the slow Ca^{2+} signal appears to occur in both kinds of skeletal muscle cells. The kinetics of nicotinic AChR activation, inactivation and physiological characteristics of these muscle fibre types are potentially important and should be elucidated in the near future.

Although botulinum toxin (5 μg, bolus i.p. injection), which produces highly effective blockade of ACh release (Stanley & Drachman, 1983), completely depressed the generation of contractile Ca^{2+} transients elicited by nerve stimulation, local application of ACh (0.3 mM) onto the endplate region resulted in the generation of both fast and slow Ca^{2+} response. Local application of ACh also elicited slow Ca^{2+} signal on a denervated mouse diaphragm muscles (on the 9–11th day after denervation) (unpublished observation). These results indicate that the modulation of ACh release via activation of presynaptic nicotinic autoreceptors (Bowman *et al*., 1988) does not lead to ACh-induced slow Ca^{2+} signal, though non-contractile Ca^{2+} mobilization elicited by nerve stimulation with anti-cholinesterase is modulated by a neuropeptide calcitonin gene-related peptide (Kimura *et al*., 1993), which co-exists with ACh in motor nerve endings (Takami *et al*., 1985; Fontaine *et al*., 1986; New & Mudge, 1986; Matteoli *et al*., 1988). Therefore, ACh application may elicit a slow Ca^{2+} response within muscle cells through postsynaptic nicotinic AChR-channels. In addition, the slow Ca^{2+} signal was not generated by the puffed ACh in the extrajunctional area (near the rib), whereas the fast contractile Ca^{2+} transients were generated in both junctional and extrajunctional areas. Using confocal imaging with a fluorescent Ca^{2+}-indicator fluo-3, we observed the local slow increase of $[Ca^{2+}]_i$ elicited by bath-application of ACh at the endplate region of single mouse FDB cells (Tsuneki *et al*., 1997). Thus, the slow Ca^{2+} signal may be observed only at the endplate region of skeletal muscle.

There may be no direct causal relationship between fast and slow Ca^{2+} mobilization as follows: geographutoxin II, a skeletal muscle Na^+ channel blocker, selectively blocks nerve-stimulated contractile Ca^{2+} mobilization without affecting non-contractile Ca^{2+} response in the presence of an anti-cholinesterase agent (Kimura *et al*., 1991b). Also, caffeine (5 mM), which promotes Ca^{2+} release from the sarcoplasmic reticulum, increased only contractile Ca^{2+} signal (Kimura *et al*., 1991a). Moreover, non-contractile slow Ca^{2+} signal depends on external Ca^{2+} concentration (Kimura *et al*., 1990b; Tsuneki *et al*., 1997). It is presumably related to Ca^{2+} influx through activated nicotinic AChR-channels as these receptor-channels are permeable to calcium (Vernino *et al*., 1994; Villarroel & Sakmann, 1996).

Calcium influx via nicotinic AChRs may be physiologically relevant and this Ca^{2+} signal may serve as a safety mechanism, whereby the stimulation of the postsynaptic cell does result in a

local increase in intracellular Ca^{2+} causing calcium-dependent cellular processes. Slow Ca^{2+} is mobilized under desensitizing conditions, since the generation of this Ca^{2+} mobilization is required for high concentrations of ACh in the synaptic cleft. Under the conditions, nicotinic AChR desensitization readily occurs. Our previous results indicate that a non-contractile slow Ca^{2+} signal depresses the contractile fast 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; Dezaki *et al*., 1996). The depression is promoted by increasing the slow Ca^{2+} signal. Moreover, the slow Ca^{2+}-induced depression is diminished by staurosporine, a protein kinase-C inhibitor. The Ca^{2+} influx via nicotinic AChR-channels accelerates the rate of nicotinic AChR desensitization in skeletal muscle (Miledi, 1980). We thus speculate that a slow Ca^{2+} signal may enhance the nicotinic AChR desensitization through activation of protein kinase-C. Using the patch clamp technique in single cells from the adult mouse FDB muscle, we have demonstrated that nicotinic AChR desensitization is accelerated by increasing extracellular Ca^{2+} concentrations (Nojima *et al*., 1992), conditions under which the slow Ca^{2+} response also increases. The slow Ca^{2+} signal, via the highly Ca^{2+}-permeable nicotinic AChR subtype, may stabilize the usual muscle-type AChR in refractory states and protect it from over-excitation at the motor endplate, thereby depressing fast contractile Ca^{2+} mobilization. Thus, there may be a Ca^{2+}-related interaction between the highly Ca^{2+}-permeable nicotinic AChR subtype (a neuronal subtype at least containing $\beta 2$ subunit, Kimura *et al*., 1994; Tsuneki *et al*., 1995) and the usual muscle-type receptor. This interaction affects the neuromuscular transmission and may be analogous to the long-lasting changes in synaptic plasticity in the central nervous system glutamate receptor subtypes which occur via (Bliss & Collingridge, 1993).

In conclusion, the results of our present study strongly suggest that ACh locally applied onto the endplate region of skeletal muscle elicits a slow Ca^{2+} signal, presumably the nerve-stimulated non-contractile Ca^{2+}, via the postsynaptic nicotinic AChR which has a low sensitivity to ACh at the neuromuscular junction.

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References

ARIANO, M.A., ARMSTRONG, R.B. & EDGERTON, V.R. (1973). Hindlimb muscle fiber populations of five mammals. *J. Histochem. Cytochem.,* **21,** 51–55.

BARNARD, R.J., EDGERTON, V.R., FURUKAWA, T. & PETER, J.B. (1971). Histochemical, biochemical, and contractile properties of red, white, and intermediate fibers. *Am. J. Physiol.,* **220,** 410–414.

BLISS, T.V.P. & COLLINGRIDGE, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature,* **361,** 31–39.

BOWMAN, W.C., MARSHALL, I.G., GIBB, A.J. & HARBORNE, A.J. (1988). Feedback control of transmitter release at the neuromuscular junction. *Trends Pharmacol. Sci.,* **9,** 16–20.

CARLSEN, R.C., LARSON, D.B. & WALSH, D.A. (1985). A fast-twitch oxidative-glycolytic muscle with a robust inward calcium current. *Can. J. Physiol. Pharmacol.,* **63,** 958–965.

DEZAKI, K., KIMURA, I., TSUNEKI, H. & KIMURA, M. (1996). Enhancement by calcitonin gene-related peptide on non-contractile Ca^{2+}-induced nicotinic receptor desensitization at the mouse neuromuscular junction. *Br. J. Pharmacol.,* **118,** 1971–1976.

FONTAINE, B., KLARSFELD, A., HOKFELT, T. & CHANGEUX, J.-P. (1986). Calcitonin gene-related peptide, a peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. *Neurosci. Lett.,* **71,** 59–65.

KEENS, T.G., CHEN, V., PATEL, P., O'BRIEN, P., LEVISON, H. & IANUZZO, C.D. (1978). Cellular adaptation of the ventilatory muscles to a chronic increased respiratory load. *J. Appl. Physiol.,* **44,** 905–908.

KIMURA, I., DEZAKI, K., TSUNEKI, H. & KIMURA, M. (1995). Postsynaptic nicotinic receptor desentitized by non-contractile Ca^{2+} mobilization via protein kinase-C activation at the mouse neuromuscular junction. *Br. J. Pharmacol.,* **114,** 461–467.

KIMURA, I., KONDOH, T. & KIMURA, M. (1990a). Changes in intracellular Ca^{2+} produced in the mouse diaphragm by neuromuscular blocking drugs. *J. Pharm. Pharmacol.,* **42,** 626–631.

KIMURA, I., KONDOH, T. & KIMURA, M. (1990b). Postsynaptic nicotinic ACh receptor-operated Ca^{2+} transients with neostigmine in phrenic nerve-diaphragm muscles of mice. *Brain Res.,* **507,** 309–311.

KIMURA, I., KONDOH, T., TSUNEKI, H. & KIMURA, M. (1991a). Reversed effect of caffeine on non-contractile and contractile Ca^{2+} mobilization operated by acetylcholine receptor in mouse diaphragm muscle. *Neurosci. Lett.,* **127,** 28–30.

KIMURA, I., TSUNEKI, H., DEZAKI, K. & KIMURA, M. (1993). Enhancement by calcitonin gene-related peptide of nicotinic receptor-operated noncontractile Ca^{2+} mobilization at the mouse neuromuscular junction. *Br. J. Pharmacol.,* **110,** 639–644.

KIMURA, I., TSUNEKI, H., DEZAKI, K., NOJIMA, H. & KIMURA, M. (1994). Monoclonal antibody to $\beta 2$ subunit of neuronal nicotinic receptor depresses the postjunctional non-contractile Ca^{2+} mobilization in the mouse diaphragm muscle. *Neurosci. Lett.,* **180,** 101–104.

KIMURA, M., KIMURA, I., KONDOH, T. & TSUNEKI, H. (1991b). Noncontractile acetylcholine receptor-operated Ca^{++} mobilization: suppression of activation by open channel blockers and acceleration of desensitization by closed channel blockers in mouse diaphragm muscle. *J. Pharmacol. Exp. Ther.,* **256,** 18–23.

KUFFLER, S.W. & YOSHIKAMI, D. (1975). The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. *J. Physiol.,* **251,** 465–482.

MAGLEBY, K.L. & PALLOTTA, B.S. (1981). A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. *J. Physiol.,* **316,** 225–250.

MATTEOLI, M., HAIMANN, C., TORRI-TARELLI, F., POLAK, J.M., CECCARELLI, B. & DE CAMILLI, P. (1988). Differential effect of α-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 7366–7370.

MILEDI, R. (1980). Intracellular calcium and desensitization of acetylcholine receptors. *Proc. R. Soc. Lond. B.,* **209,** 447–452.

NEW, H.V. & MUDGE, A.W. (1986). Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature,* **323,** 809–811.

NOJIMA, H., MUROI, M., KIMURA, I. & KIMURA, M. (1992). Indirect inhibitory effect of succinylcholine on acetylcholine-activated channel activities and its modulation by external Ca^{2+} in mouse skeletal muscles. *Br. J. Pharmacol.,* **105,** 23–26.

STANLEY, E.F. & DRACHMAN, D.B. (1983). Botulinum toxin blocks quantal but not non-quantal release of ACh at the neuromuscular junction. *Brain Res.,* **261,** 172–175.

TAKAMI, K., KAWAI, Y., UCHIDA, S., TOHYAMA, M., SHIOTANI, Y., YOSHIDA, H., EMSON, P.C., GIRGIS, S., HILLYARD, C.J. & MACINTIRE, I. (1985). Effect of calcitonin gene-related peptide on contraction of striated muscle in the mouse. *Neurosci. Lett.,* **60,** 227–230.

TSUNEKI, H., DEZAKI, K. & KIMURA, I. (1997). Neuronal nicotinic receptor operates slow Ca^{2+} mobilization at mouse muscle endplate. *Neurosci. Lett.,* **225,** 185–188.

TSUNEKI, H., KIMURA, I., DEZAKI, K., KIMURA, M., SALA, C. & FUMAGALLI, G. (1995). Immunohistochemical localization of neuronal nicotinic receptor subtypes at pre- and postjunctional sites in mouse diaphragm muscle. *Neurosci. Lett.,* **196,** 13–16.

VERNINO, S., ROGERS, M., RADCLIFFE, K.A. & DANI, J.A. (1994). Quantitative measurement of calcium flux through muscle and neuronal nicotinic acetylcholine receptors. *J. Neurosci.,* **14,** 5514–5525.

VILLARROEL, A. & SAKMANN, B. (1996). Calcium permeability increase of endplate channels in rat muscle during postnatal development. *J. Physiol.,* **496,** 331–338.

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