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# Indirect inhibitory effect of succinylcholine on acetylcholine-activated channel activities and its modulation by external $Ca^{2+}$ in mouse skeletal muscles

[1]Hiroshi Nojima, Masashi Muroi, Ikuko Kimura & Masayasu Kimura

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

**1** The effect of extracellular calcium on single acetylcholine (ACh)-activated channel activities when desensitizing concentrations of succinylcholine (SuCh) were applied to the surrounding endplate membrane was investigated by the cell-attached patch-clamp technique at endplates of single skeletal muscle (flexor digitorum brevis) fibres of adult mice.

**2** Bath-applied SuCh (0.1–3 $\mu$M, in 2.5 mM $Ca^{2+}$) increased in a concentration-dependent manner the mean open time of ACh-activated channel currents recorded at membrane potentials which cancelled the SuCh-induced depolarizations.

**3** In the presence of 0.5 and 2.5 mM external $Ca^{2+}$, SuCh (3 $\mu$M) applied outside the patch pipette prolonged the mean open time of ACh-activated channel currents in a time-dependent manner (by 45% and 52%, respectively), and simultaneously significantly decreased the single channel conductance (by 14% and 10%, respectively). These SuCh-induced effects did not occur in a nominally $Ca^{2+}$-free extracellular medium.

**4** Under the same conditions, SuCh (3 $\mu$M) augmented the time-dependent decline in the opening frequency of ACh-activated channel currents obtained in nominally $Ca^{2+}$-free medium.

**5** These results suggest that external calcium ions act to modulate nicotinic ACh receptor channel activity, and accelerate desensitization of the receptor.

**Keywords:** Calcium; succinylcholine; desensitization; nicotinic acetylcholine receptor; single channel recording

## Introduction

The mechanisms by which nicotinic cholinergic responses are desensitized by the prolonged application of agonists are not yet well understood. The process of desensitization by the continuous application of acetylcholine (ACh) at the motor endplate has been demonstrated electrophysiologically to involve: (1) a gradual decay of depolarization (Miledi, 1980; Chesnut, 1983), (2) a frequency-dependent depression of responses to iontophoretically applied ACh (Katz & Thesleff, 1957; Anwyl & Narahashi, 1980; Magazanik *et al.*, 1982) and (3) a time-dependent decline in the opening frequency of ACh-activated channel currents (Sakmann *et al.*, 1980; Hopfield *et al.*, 1988; Dionne, 1989).

Succinylcholine (SuCh), like other depolarizing neuromuscular blockers, acts to block the postsynaptic response in part through desensitization of the nicotinic ACh receptor (Durant & Katz, 1982). The rate of onset of desensitization can be accelerated by increasing the intracellular or extracellular free calcium levels (Miledi, 1980; Fiekers *et al.*, 1980; Magazanik & Vyskočil, 1982; Chesnut, 1983). Depolarizing neuromuscular blockers affect the mobilization of intracellular $Ca^{2+}$ (Kimura *et al.*, 1990), and extracellular calcium modulates nicotinic ACh receptor-channel activities in single skeletal muscle fibres (Kimura *et al.*, 1991).

The aim of the present study was to elucidate the effect of extracellular calcium on the activity of single ACh-activated channels in the presence of SuCh at endplates of flexor digitorum brevis (FDB) muscle fibres in adult mice by use of the cell-attached patch-clamp technique.

## Methods

Adult male ddY mice (32–38 g) were used.

[1] Author for correspondence.

### *Intracellular recordings*

Diaphragm muscles were isolated, and maintained in Krebs-Henseleit solution (KHS) saturated with 95% $O_2$ and 5% $CO_2$, at 35–37°C. The composition of the solution was as follows (mM): NaCl 137, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 15 and glucose 10. ACh was applied by iontophoresis (rectangular current pulses; 1 nA, 5 s duration) from a microelectrode (100–200 MΩ) filled with 2 M ACh. To avoid leakage of ACh, a few nA of braking current was applied.

### *Single channel recordings*

The FDB muscles were isolated from the hind feet. Dissection and enzyme treatment were carried out in modified KHS (KCl reduced from 5 to 2.5 mM) which did not contain tetrodotoxin and was saturated with 95% $O_2$ and 5% $CO_2$. The muscles were incubated at 37°C for 75 min in KHS containing 0.2% collagenase, transferred to KHS containing 0.05% trypsin but no $CaCl_2$ for 15 min, rinsed in KHS, and then triturated gently to obtain single fibres. Experiments were performed 2 h after enzyme treatment.

Single channel currents were recorded from the endplate of each muscle fibre in KHS at 24–26°C in the cell-attached patch configuration (Hamill *et al.*, 1981) with a patch-clamp amplifier (Axopatch-1D; Axon Instruments, CA, U.S.A.). Currents were low-pass filtered at 2 kHz and stored in a PCM data recorder (RP-880; NF Electric Instruments, Japan). The sampling frequency was 56.88 kHz. The data were analysed with a histogram analyser (QC-111J; Nihon Kohden, Japan). Data replayed from a PCM data recorder were sampled at 100 $\mu$s per point and stored on a memory oscilloscope (VC-11, Nihon Kohden, Japan). The traces of channel currents shown were drawn by a pen-recorder from the stored data. The threshold for detecting opening and closing transitions was set at a current amplitude of 2 pA, which corresponded to a value of 35–47% of the mean channel current amplitude in both the control and test recordings. Only data from one channel in

the patch were adopted. The mean open time and mean current amplitude were determined at the resting membrane potential in a non-depolarized state, or at various membrane potentials with various pipette potentials to cancel the degree of depolarization, from 400–1600 events in each membrane patch. The opening frequency (the number of events per second) was determined simultaneously. Channel conductance was obtained from the slope of the current-voltage relationship, using the ACh reversal potential of 0 mV determined from the measurement of ACh-activated channel currents at various pipette potentials in the cell-attached patch configuration. ACh (1 $\mu$M)-activated channel currents were recorded continuously at an endplate for 20 min immediately after patch-seal formation. In these experiments, SuCh (3 $\mu$M) was applied in the bath after a control recording period of 3 min. At the end of the experiments, the fibres were impaled with the patch pipette in order to measure directly the intracellular membrane potentials. The membrane potential during the patch recordings was calculated as the sum of the pipette potential and the membrane potential measured directly at the end of the recordings.

Patch pipettes were prepared from capillary glass tubing (Pyrex, U.S.A.) which was pulled to a tip diameter of less than 1 $\mu$m. The resistance of pipettes filled with KHS containing 1 $\mu$M ACh was 15–20 MΩ. When small-tipped patch-pipettes were used, multi-level channel openings were not observed frequently at the resting membrane potential.

### *Statistics*

The paired or unpaired $t$ test and Student's range test (the analysis of variance with a subsequent multiple comparisons test) or one-way analysis of variance (ANOVA) were used, and $P < 0.05$ or $P < 0.01$ were accepted as significant.

### *Drugs*

Acetylcholine chloride (Dai-ichi, Japan), succinylcholine chloride (Nacalai tesque, Japan), collagenase (for cell dispersion; Wako Pure Chemical Industries, Japan) and trypsin (Type III; Sigma, St. Louis, MO, U.S.A.) were used.

## Results

The iontophoretic application of a prolonged pulse of ACh evoked endplate potentials which gradually declined with the desensitization of the nicotinic ACh receptors. After the application of 0.2 $\mu$M SuCh for 30 min, the peak of the ACh potential was reduced and the ACh potential decayed more rapidly than without SuCh (Figure 1).

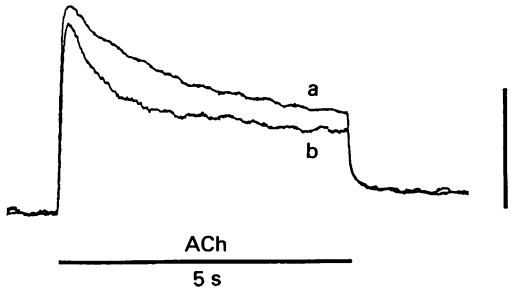


**Figure 1** The effect of succinylcholine (SuCh, 0.2 $\mu$M) on the decay phase of depolarization produced by a prolonged acetylcholine (ACh) pulse at an endplate in mouse diaphragm muscle. Depolarizations generated by iontophoretically-applied ACh were recorded from the same fibre before (a) and 30 min after the application of SuCh (b). The horizontal bar represents the duration of the ACh pulse. The vertical calibration bar represents 7 mV in (a) and 5 mV in (b).

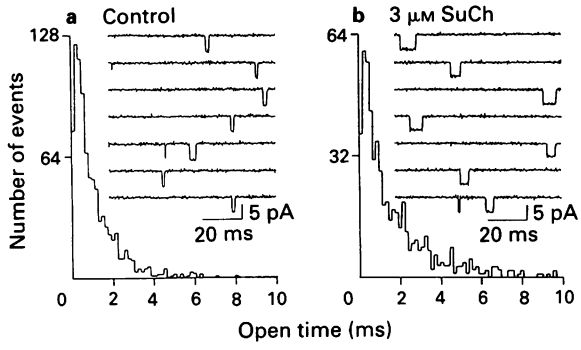


**Figure 2** Frequency distribution histograms for the open time and typical data of acetylcholine (ACh, 1 $\mu$M)-activated channel currents recorded from the endplates of single FDB muscle fibres from mice at a resting membrane potential (−73 mV, which was a value directly measured at the end of the recording) before (a) and at a membrane potential (−75 mV, which was a sum of a membrane potential of −35 mV directly measured at the end of the recording and a pipette potential of 40 mV hyperpolarization during a patch recording) 10 to 15 min after bath application of 3 $\mu$M succinylcholine (SuCh) (b). (a) The mean open time and opening frequency without SuCh were 1.13 ms ($n$ = 895) and 6.86 Hz respectively; (b) the mean open time and opening frequency with SuCh were 1.81 ms ($n$ = 568) and 5.11 Hz respectively. The records are analysed quantitatively in Table 1.

ACh (1 $\mu$M)-activated single channel currents in a FDB muscle fibre were recorded 10 to 15 min after the bath application of SuCh (0.1–3 $\mu$M). Bath-applied SuCh at concentrations greater than 0.1 $\mu$M depolarized the membrane in a concentration-dependent manner. The SuCh-induced depolarization was then cancelled with a pipette potential of 10 mV hyperpolarization at 0.3 $\mu$M SuCh and 40 mV hyperpolarization at 1 and 3 $\mu$M SuCh, and the open time was measured. SuCh prolonged the mean open time of ACh currents in a concentration-dependent manner, but did not significantly alter opening frequency (Figure 2 and Table 1).

ACh (1 $\mu$M)-activated single channel currents were recorded continuously at an endplate for 20 min immediately after patch-seal formation, with a patch pipette containing various concentrations of $Ca^{2+}$ (nominally free, 0.5 and 2.5 mM). With 2.5 mM $Ca^{2+}$ in the patch pipette, the opening frequency of ACh-activated channel currents was decreased time-dependently from 7.3 ± 0.8 Hz (mean ± s.e.mean, $n$ = 6) at 0–3 min after the seal, to 4.0 ± 0.2 Hz at 10–15 min, and simultaneously the mean open time was significantly increased from 1.21 ± 0.07 ms to 1.39 ± 0.08 ms ($n$ = 6, $P < 0.01$; paired $t$ test). Nominally $Ca^{2+}$-free solution and 0.5 mM $Ca^{2+}$ in the patch pipette also decreased the opening frequency time-dependently from 6.6 ± 0.9 Hz to 4.4 ± 0.6 Hz ($n$ = 6) and from 8.4 ± 1.0 Hz to 4.8 ± 0.5 Hz ($n$ = 6), respectively. $Ca^{2+}$, 0.5 mM also significantly prolonged time-dependently, the mean open time from 0.92 ± 0.05 ms to 1.07 ± 0.03 ms ($n$ = 6, $P < 0.05$; paired $t$ test). Nominally $Ca^{2+}$-free medium in the patch pipette did not change the mean open time measured 10–15 min after a control recording, but the values for the mean open time were significantly lower than those recorded in 0.5 and 2.5 mM $Ca^{2+}$ (Table 2).

ACh (1 $\mu$M)-activated single channel currents were recorded continuously at an endplate in the presence of various concentrations of $Ca^{2+}$ (nominally 0, 0.5 and 2.5 mM) in the extracellular medium. SuCh (3 $\mu$M) was applied in the bath after a control recording for 3 min. SuCh induced a maintained depolarization which was independent of the $Ca^{2+}$ concentration (Table 2). In 2.5 mM $[Ca^{2+}]_o$, SuCh prolonged the mean open time by 52% 10–15 min after its bath application (Figure 3a) and in 0.5 mM $[Ca^{2+}]_o$ SuCh prolonged the mean open time by 45%. However, in nominally $Ca^{2+}$-free medium, SuCh failed to prolong the mean open time 10–15 min after its bath

**Table 1** Effects of bath-applied succinylcholine (SuCh) on acetylcholine (ACh)-activated channel activities and resting membrane potentials

| *SuCh* (μM) | n | *Membrane potential* (mV) (pipette potential, mV) | *Mean open time* (ms) | *Opening frequency* (Hz) |
|---|---|---|---|---|
| 0 | (7) | −73.3 ± 0.9 (0) | 1.17 ± 0.04 | 6.52 ± 0.79 |
| 0.1 | (6) | −73.0 ± 0.6 (0) | 1.19 ± 0.04 | 6.44 ± 0.82 |
| 0.3 | (6) | −61.8 ± 2.3** (10) | 1.40 ± 0.05** | 5.44 ± 1.07 |
| 1 | (7) | −35.6 ± 1.3** (40) | 1.48 ± 0.02** | 4.91 ± 0.44 |
| 3 | (8) | −32.6 ± 1.5** (40) | 1.95 ± 0.06** | 4.77 ± 0.51 |

ACh (1 μM)-activated channel currents were recorded 10 to 15 min after the bath application of SuCh. The mean open time and opening frequency were estimated at membrane potentials with various pipette potentials (hyperpolarizing pulse in parentheses) to cancel the SuCh-induced depolarization. Significant differences (** $P < 0.01$) were detected by an unpaired $t$ test between the values with and without SuCh. The values are means ± s.e.mean ($n$ = number of cells).

**Table 2** Effects of succinylcholine (SuCh) and calcium applied to the non-patched membrane on single acetylcholine (ACh)-activated channel currents

| *SuCh* (μM) | $CaCl_2$ (mM) | *Membrane potential* (mV) (pipette potential, mV) | *Conductance* (pS) 10–15 min | | *Mean open time* (ms) Pre | *Mean open time* (ms) 10–15 min | | *Opening frequency* 10–15 min/Pre (%) | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | −74.5 ± 1.4 (0) | 75.0 ± 0.9 | NS | 0.66 ± 0.02 | 0.69 ± 0.04 | NS | 69.2 ± 5.9 | |
| 0 | 0.5 | −75.3 ± 1.4 (0) | 71.9 ± 1.0 | ** | 0.92 ± 0.05 | 1.07 ± 0.03* | * | 57.8 ± 5.0 | |
| 0 | 2.5 | −73.5 ± 0.7 (0) | 65.9 ± 1.0 | | 1.21 ± 0.07 | 1.39 ± 0.08** | | 58.4 ± 7.0 | |
| 3 | 0 | −33.8 ± 0.8 (40) | 73.4 ± 1.4 | | 0.69 ± 0.03 | 0.68 ± 0.04 | ** | 68.8 ± 7.3 | † |
| 3 | 0.5 | −33.5 ± 2.2 (40) | 61.9 ± 1.2 | ** | 0.87 ± 0.04 | 1.26 ± 0.07** | | 44.5 ± 4.7 | † |
| 3 | 2.5 | −33.9 ± 0.7 (40) | 59.2 ± 0.7 | | 1.22 ± 0.05 | 1.86 ± 0.08** | | 44.9 ± 5.3 | |

ACh (1 μM)-activated channel currents were recorded continuously at an endplate, with a patch pipette containing various concentrations of $CaCl_2$ (0, 0.5 and 2.5 mM). A solution of SuCh (3 μM) containing the same concentration of $CaCl_2$ as in the pipette was applied to the non-patched membrane after a control recording period for 3 min (Pre); 10–15 min after the application of 0 or 3 μM SuCh, the mean open time, the opening frequency and the mean current amplitude were estimated at membrane potentials with various pipette potentials (hyperpolarizing pulse in parentheses) to cancel the SuCh-induced depolarization. Conductance was determined as described in Methods. Significant differences: * $P < 0.05$ and ** $P < 0.01$ were detected by a paired $t$ test between the values at Pre and 10–15 min; * $P < 0.05$ and ** $P < 0.01$ by an unpaired $t$ test between the values with and without SuCh, and † $P < 0.05$ by a Student's range test. NS: not significant. The values are means ± s.e.mean of 6 cells.

application (Figure 3b). In 0.5 and 2.5 mM $[Ca^{2+}]_o$ SuCh augmented the decrease of opening frequency, and this was accompanied by a prolongation of the mean open time and by a decrease of channel conductance. These effects were not produced by SuCh in nominally $Ca^{2+}$-free extracellular medium.

## Discussion

This paper describes the effect of extracellular calcium on ACh-activated channel activities in the presence of SuCh in skeletal muscle fibres of adult mice. At concentrations higher than 0.2 μM, SuCh accelerated nicotinic ACh receptor desensitization, and concentration-dependently prolonged the mean open time at a membrane potential which cancelled the SuCh-induced depolarization. This effect may reflect an increased affinity of the receptor for ACh during the process of desensitization (Burgermeister *et al.*, 1977; Heidmann *et al.*, 1983). In single channel recordings, the desensitization of nicotinic ACh receptors induced by ACh is manifested as a time-dependent decline in the opening frequency of ACh-activated channel currents (Hopfield *et al.*, 1988; Dionne, 1989) or as the occurrence of 'bursts' of single channel currents observed at irregular intervals (Sakmann *et al.*, 1980; Colquhoun & Ogden, 1988). We showed that the opening frequency of ACh-activated channel currents is reduced independently of the

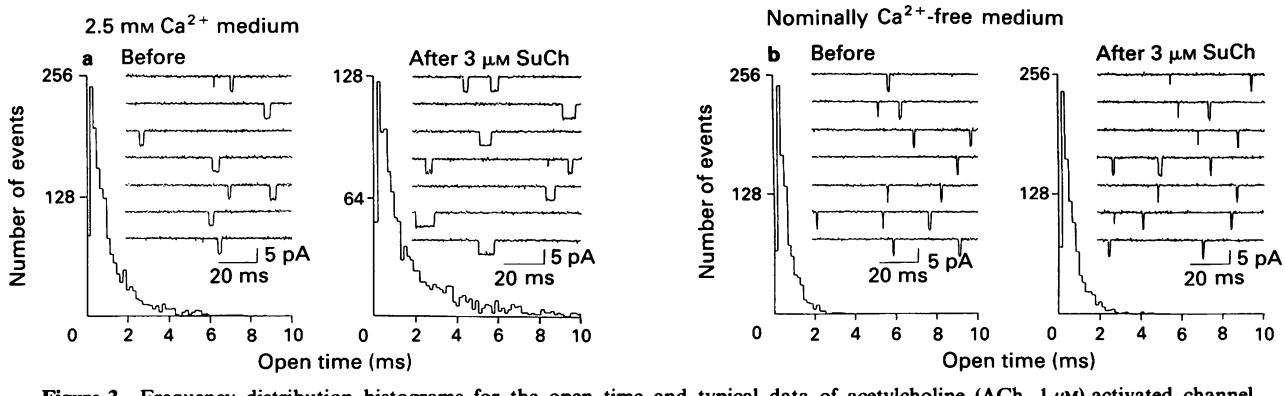


**Figure 3** Frequency distribution histograms for the open time and typical data of acetylcholine (ACh, 1 μM)-activated channel currents recorded from the endplates of single muscle fibres at a resting membrane potential before (left) and at a membrane potential of −73 mV (a) and −74 mV (b) 10 to 15 min after (right) the application of 3 μM succinylcholine (SuCh) to the non-patched membrane in 2.5 mM $Ca^{2+}$ (a) and nominally $Ca^{2+}$-free (b) medium. The mean open time and opening frequency were 1.17 ms ($n$ = 1599) and 8.88 Hz respectively without SuCh and 1.86 ms ($n$ = 1172) and 3.91 Hz respectively with SuCh in (a), and 0.67 ms ($n$ = 982) and 8.18 Hz respectively without SuCh and 0.70 ms ($n$ = 938) and 5.21 Hz respectively with SuCh in (b). The records are analysed quantitatively in Table 2.

presence of external calcium, and that the mean open time is prolonged by increasing the extracellular calcium concentration. These results indicate that calcium is certainly not necessary for the desensitization of nicotinic ACh receptors by ACh alone, and that $Ca^{2+}$ influx induced by nicotinic ACh receptor-activation (Miledi *et al.*, 1980) may prolong the mean open time.

Desensitizing concentrations of SuCh applied to the membrane outside the patch pipette augmented the time-dependent prolongation of the mean open time, accelerated the time-dependent decline of the opening frequency, and decreased the conductance of single ACh-activated channel currents. All these effects were dependent on the extracellular calcium concentration. Prolongation of the mean open time of the ACh-activated channel has been observed during hyperpolarization (Anderson & Stevens, 1973; Aracava *et al.*, 1984; Greenberg *et al.*, 1985). The effects of SuCh are not associated with SuCh-induced depolarization, because SuCh induced a constant depolarization which was independent of changes in the extracellular calcium concentration. These results indicate that the indirect effects of SuCh on the nicotinic ACh receptor channel are triggered by the SuCh-induced $Ca^{2+}$ influx (Kimura *et al.*, 1990). In a previous paper, we showed that increasing the calcium concentrations in a patch pipette reduces both the conductance and the opening frequency of ACh-activated channels, and prolongs their mean open time (Kimura *et al.*, 1991). In the presence of desensitizing concentrations of SuCh, channel activities may be modulated by external calcium ions with the consequence that SuCh amplifies the time-dependent changes in the ACh-activated channel activities.

However, our data are at variance with those of Eusebi *et al.* (1987) in chick myotubes, where the decreases in both channel opening frequencies and conductance induced by ACh applied to the membrane outside the patch pipette also occurred when the non-patched membrane was exposed to nominally $Ca^{2+}$-free extracellular medium.

In conclusion, the modulating effects of SuCh on ACh-activated channel activities probably resulted from an increase of intracellular $Ca^{2+}$ during nicotinic ACh receptor-activation, because these effects were not produced when calcium was not present in the extracellular medium. These results suggest that extracellular calcium ions modulate nicotinic ACh receptor channel activity, and accelerate desensitization of the receptor.

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