The External Ca^{2+}-Dependence of Acetylcholine-Induced Contracture in Single Innervated and Denervated Skeletal Muscle Cells in Mice

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ABSTRACT—We examined morphological differences and compared the Ca^{2+}-dependence between acetylcholine (ACh)-contractures in normal and denervated skeletal muscle cells. ACh (1 μM-1 mM) contracted the normal cells into a jackknife-shape. The higher the concentration of external Ca^{2+}, the greater the ratio of responding cells/total cells observed and the sharper the angles of the "jack-knives". ACh contracted the denervated cells into a compression-form, and the contraction was dependent on the external Ca^{2+}. These results indicate that ACh-contractures in both normal and denervated cells are external Ca^{2+}-dependent.

Single muscle cells obtained by dissociating adult muscle preparations are useful for electrophysiological and morphological investigations of membrane properties (1, 2). Microscopic changes in nicotinic acetylcholine (ACh) receptor channels following denervation have been well-documented electrophysiologically by the patch-clamp technique in dissociated adult muscle cells (3, 4). On the other hand, the effect of bath-applied ACh on a denervated muscle preparation is detected macroscopically as a development of muscle tension (5). In the present study, we examined morphological differences and compared the external Ca^{2+}-dependence in ACh-induced contracture in single muscle cells obtained from innervated and denervated skeletal muscles.

Male ddY mice (33–38 g, 7–9 weeks old) were used. Under sodium pentobarbital anesthesia, one leg was denervated by cutting the sciatic nerve located at the thigh, and 2–3 mm of the nerve was removed. This transection denervated the flexor digitorum brevis (FDB) muscles located in the sole of the hindlimb. The contralateral FDB muscles were used as controls. At the 7th day after denervation, denervated and control FDB muscles were isolated in Krebs-Henseleit solution (KHS) saturated with 95% O_2 and 5% CO_2. The composition of the solution was as follows: 136.9 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl_2, 1.2 mM MgSO_4, 15.0 mM NaHCO_3 and 10.0 mM glucose. The high potassium (60, 80 and 154.4 mM) solution was prepared by substituting KCl for NaCl in KHS. The muscles were incubated with 0.2% collagenase (from Wako, for cell dispersion) in KHS at 37°C for 75 min and then with 0.05% trypsin (Sigma, Type III) in KHS without added calcium for 15 min. Following the incubation, the preparation was rinsed in KHS and then triturated gently to obtain single cells. Dispersed muscle cells were transferred to a glass-bottomed chamber and observed under an inverted transmitted-light microscope (Leitz) at 24–26°C, 2 hr after

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the above enzyme treatment. Normal muscle cells were 400–800 μm long and 20–50 μm wide. The dispersed cells obtained had resting membrane potentials in the range of –65 to –80 mV, which was determined by using conventional intracellular microelectrodes.

These normal cells had an endplate (Fig. 1A) and contracted immediately in a jackknife-shape in response to bath-applied ACh (100 μM) (Fig. 1B). This form of contracture was restricted to the endplate membrane. At the 7th day after denervation, denervated muscle cells had resting membrane potentials in the range of –55 to –70 mV, and they had obviously great nuclear bulges on their cell surface, indicating atrophy (Fig. 1C). Such morphological changes were much more marked after the 14th day of denervation (data not shown). Denervated muscle cells were immediately shortened longitudinally by 100 μM ACh into a compression-form (Fig. 1D). The ratio of ACh-responding cells to total cells increased in a concentration-dependent manner (1–1000 μM) in both normal and denervated muscle cells (Table 1). The angle of the jackknife-shape of contracture be-

Fig. 1. Photomicrographs of contractures induced by acetylcholine and high potassium in single flexor digitorum brevis muscle cells obtained from normal and 7 days-denervated muscles of adult mice. A: normal muscle cells without ACh, B: jackknife-type contracture of the same cells as in A bent at the endplate (arrow) by bath-applied acetylcholine (100 μM), C: denervated muscle cells without ACh, D: compression-type contracture of the same cells as in C shortened longitudinally by bath-applied acetylcholine (100 μM), E: normal muscle cells without ACh (2.5 mM K⁺), and F: compression-type contracture of the same cells as in E induced by high potassium solution (154.4 mM K⁺) in which KCl was substituted for NaCl in KHS. Calibration bar = 100 μm.
came more acute as the concentration of ACh increased, but we were unable to quantitate this because the cells detached from the chamber glass. By 1000 \( \mu \text{M} \) ACh, 77.3% of normal muscle cells were contracted, and the rest were silent (not dead), because all the normal muscle cells were always contracted strongly (showing more acute angles of “jackknife” shape) in response to 100 \( \mu \text{M} \) ACh with 5 mM CaCl\(_2\). Bath application of 100 \( \mu \text{M} \) ACh produced depolarization to \(-11.9 \pm 1.9\) mV with 2.5 mM CaCl\(_2\) (mean \(\pm\) S.D. 9 cells) and to \(-11.2 \pm 1.7\) mV with 5 mM CaCl\(_2\) (9 cells). Therefore, ACh-induced strong contracture with 5 mM CaCl\(_2\) was independent of membrane depolarization. Without the addition of CaCl\(_2\), no cells responded to 100 \( \mu \text{M} \) ACh. These results indicate that ACh contracture is dependent on extracellular calcium concentration, demonstrating that the quantity of calcium influx following the opening of ACh receptor channels determines the strength of ACh contracture.

On the 7th day after denervation, single muscle cells still had morphologically distinguishable endplates. Bath-applied ACh did not induce an usual jackknife-shape, but caused a compression-form of contracture in denervated muscle cells. Denervated muscle cells were ten times more sensitive with respect to the appearance of ACh contracture than normal ones. Both the ratio of ACh-contracting cells and the extent of compression increased in a concentration-dependent manner (1–100 \( \mu \text{M} \)). The denervation-induced change results from an increase in sensitivity to ACh over the entire muscle membrane (6, 7). The change by denervation from local contracture in a jackknife-shape to whole cell contracture in a compression-form reflects the increase in ACh sensitivity.

In normal and denervated muscle cells, high potassium solution caused the same form and time course of contracture as the ACh response in denervated muscle cells (Fig. 1, E and F). Only 9.1% of the normal cells contracted in response to 154.4 mM K\(^+\) (Table 1). This low sensitivity to potassium may arise from a decline in the resting tension by disconnection from the tendon in a dispersed cell. The ratio of potassium-contracting cells to total cells became greater after denervation. In rat skeletal muscle, denervation increases the voltage-dependent Ca channels in addition

### Table 1. External Ca\(^{2+}\)-dependent incidence of contracture induced by acetylcholine, high potassium and A23187 in single muscle cells isolated from innervated and denervated mouse muscles

|                  | Incidence of contracture |
|------------------|--------------------------|
|                  | Innervated | Denervated* |
| Acetylcholine, 1 \( \mu \text{M} + \text{CaCl}_2\), 2.5 mM | 0/76 (0) | 14/69 (20.3)\(^b\) |
| 3 + 2.5          | 10/42 (23.8) | 22/38 (57.9) |
| 10 + 2.5         | 21/56 (37.5) | 37/47 (78.7) |
| 100 + 0          | 0/43 (0) | 0/24 (0) |
| 100 + 2.5        | 59/92 (64.1) | 44/46 (95.7) |
| 1000 + 2.5       | 49/49 (100) | 34/44 (77.3) |
| K\(^+\) 60 mM + 2.5 mM | 0/36 (0) | |
| 80 + 2.5         | 2/42 (4.8) | |
| 154.4 + 2.5      | 5/55 (9.1) | 26/33 (78.8) |
| A23187 3.8 \( \mu \text{M} + \) 2.5 mM | 24/24 (100) | |

\(^{a}\)7 days-denervation. \(^{b}\)Values represent the ratio of number of contracting/tested cells with the percentage in parentheses.
to nicotinic ACh receptors (8). The hypersensitivity of potassium-contracture following denervation may, thus, be the result of increasing voltage-dependent Ca channels. A23187 (3.8 μM), a divalent cation ionophore, always induced an irreversible, slow and compression-type contracture in normal muscle cells within 20 min after bath application, but in FDB muscle preparations, it did not induce any morphological changes over a 60-min period. Normal muscle cells were contracted immediately and irreversibly by 10 μM A23187.

The external Ca$^{2+}$-dependence of ACh-induced tension in denervated muscles has been demonstrated (5). The present results indicate that compression-type contracture is Ca$^{2+}$-dependent as is the jackknife-type one. Muscle contraction was blocked by very low extracellular Ca$^{2+}$ concentration, a condition obtained with high concentrations of EGTA, suggesting the presence of calcium in a “protected” extracellular compartment (9). Intracellular Ca$^{2+}$ release in directly stimulated muscles is dependent on external Ca$^{2+}$ concentration (10). ACh contracture of single muscle cells obtained by enzyme treatment was highly dependent on extracellular Ca$^{2+}$ concentration. Enzyme treatment removes cholinesterase from the endplate (11), which is presumably accompanied by the partial disappearance of the basement membrane and the extracellular compartment. The local concentration of ACh in the synaptic cleft of skeletal muscles is considered to be 300 μM immediately after its release (12). One hundred micromolar ACh used in the present study may be sufficient under physiological conditions to activate the endplate at the neuromuscular junction. In intact muscle preparations, local contracture at the endplate regions may induce the development of whole muscle tension because tendons exist in these areas.

In conclusion, both ACh-induced jackknife-type contracture in normal cells and compression-type contracture in denervated cells are external Ca$^{2+}$-dependent.

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