Increase in electrically-stimulated Ca^{2+} release and suppression of caffeine response in diaphragm muscle of alloxan-diabetic mice compared with the denervation effect

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Summary. Changes in intracellular Ca^{2+} release in the diaphragm muscle of alloxan-diabetic mice were compared with changes in normal muscles and non-diabetic denervated muscles. We measured Ca^{2+} transient aequorin luminescence by direct electrical stimulation of these muscles. External Ca^{2+} -free solution readily decreased the Ca^{2+} transient in normal muscles but had less of an effect in diabetic muscles. Only when the muscles were pre-injected with EGTA (reducing intracellular levels of free Ca^{2+}) did the Ca^{2+} transients decrease significantly in diabetic muscles, however, there was no effect in denervated muscles. The caffeine-induced increase in Ca^{2+} transients, however, was delayed in both diabetic muscles and non-diabetic denervated muscles. The caffeine response was observed in normal muscles under the external Ca^{2+} -free conditions even after EGTA-pretreat-

ment, whereas it was suppressed, after a brief increase, in both diabetic and non-diabetic denervated muscles. These results demonstrate (1) the insensitivity of intracellular Ca^{2+} mobilization to external Ca^{2+} levels and the ready accumulation of intracellular Ca^{2+} in the cytosol in the diabetic state, (2) increased permeability to Ca^{2+} in the denervated state and (3) impairment of the Ca^{2+} pool which responds to caffeine in both diabetes and the non-diabetic denervated state. Diabetic neuromyopathy thus appears to be a state of abnormal Ca^{2+} -mobilization caused secondarily by high levels of blood glucose.

Key words: Aequorin luminescent- Ca^{2+} transient, caffeine, non-diabetic denervation, Ca^{2+} -free, EGTA intracellular injection.

In our previous paper we demonstrated that alloxan-diabetes reduces Ca^{2+} entry into skeletal muscles. This was determined electrophysiologically on the basis of Ca^{2+} slow action potentials [1]. A significant decrease in Ca^{2+} uptake is observed in skeletal muscle sarcoplasmic reticulum preparations from streptozotocin-induced diabetic rats [2]. Altered Ca^{2+} binding by the sarcolemmal membrane has been reported in the myocardium of the diabetic rat [3]. These reports suggest that diabetes may cause abnormal mobilization of intracellular Ca^{2+} .

Denervation-induced changes in muscles (decreased resting membrane potential and increased density of extra-junctional receptors) are associated with a decrease in the concentration of free intracellular calcium ions [4]. The steady-state component of the inactivation phase of Ca^{2+} currents disappears following denervation of tonic fibers [5]. The diabetic state is considered to be similar to a state of partial denervation because one component of the diabetes syndrome is neuromyopathy.

Cell-injected aequorin is used to quantify Ca^{2+} release in isolated amphibian skeletal muscles [6]. The Ca^{2+} released into the cytosol is believed to be rapidly seques-

tered by the sarcoplasmic reticulum. The term “ Ca^{2+} transient” is frequently used to refer to the transient mobilization of intracellular Ca^{2+} . We previously reported that Ca^{2+} is released into the cytosol from intracellular pools by two kinds of voltage-dependent mechanisms, basal and caffeine-sensitive, using the aequorin luminescence technique in directly stimulated diaphragm muscles of normal mice [7]. Ca^{2+} release in the former is dependent on external Ca^{2+} , whereas in the latter it is external- Ca^{2+} independent [8].

In the present study, the theory that the intracellular mobilization of Ca^{2+} is abnormal in diabetic muscle and in non-diabetic denervated muscle was investigated by comparing the diabetic state with the denervated state with respect to both Ca^{2+} pools. This is the first report on Ca^{2+} transient in the diabetic state and in the denervated state.

Materials and methods

Normal ddY-strain male mice (7–8 weeks old, 29–39 g), alloxan-induced diabetic mice (14–17 days after alloxan administration; 7 weeks old, 36–39 g; blood glucose levels 17.9–38.6 mmol/l), and de-

Fig. 3 A and B. **A** Typical data for the effects of 5 mmol/l caffeine on luminescence amplitudes in external normal Ca^{2+} (upper tracings), in diaphragm muscles stimulated directly in the presence of external Ca^{2+} -free solutions with (lower tracings) and without (middle tracings) intracellular pre-injection of EGTA from (a) normal, (b) diabetic, and (c) non-diabetic denervated mice. Caffeine responses in normal nutrient solution are shown in the upper tracings. EGTA (20 $\mu\text{mol/l}$) solutions were injected into the muscle fibers with aequorin beforehand. Data are shown as functions of time (min). Aequorin signals were transformed by microcomputer (basal noise was subtracted and only peak signals were plotted). The vertical lines represent a luminescence scale of 5 kilocounts per second (kcps), except that 2.5 kcps were used for the bottom portion of (a). Observations were made in 3–5 experiments. **B** Effects of external Ca^{2+} -free solution and intracellular EGTA injection on caffeine-induced changes in log % luminescence amplitude. Data 2–3 min after applying caffeine are shown vs EGTA concentrations. * $p < 0.05$ in the two-tailed t -test; and (*) $p < 0.05$ in one-tailed t -test; and ** $p < 0.01$ in two-tailed t -test

muscles were exposed to direct electrical stimulation for 5 ms at 0.1 Hz. The evoked (electrically stimulated) Ca^{2+} transients decreased gradually in both normal muscles and non-diabetic denervated muscles (Fig. 1a and 1c). In non-diabetic denervated muscles increases in spontaneous (not electrically-stimulated) Ca^{2+} transients were observed for 3 min beginning 3 min after replacing nor-

mal Krebs solution with Ca^{2+} -free solution, however, no such increases were observed in normal muscle. In denervated muscle, a brief increase in spontaneous Ca^{2+} transients preceded the decrease in evoked Ca^{2+} transients (Fig. 1c), during which the fluctuations in the Ca^{2+} transient showed the same pattern as in smooth muscle [10]. In contrast, there was very little decrease in the evoked Ca^{2+} transients of diabetic muscles in response to the external Ca^{2+} -free solution (Fig. 1b). These results indicate that the intracellular release of Ca^{2+} in diabetic muscles is comparatively insensitive to external Ca^{2+} .

The mobilization of loosely bound Ca^{2+} was suppressed by pre-injecting EGTA into the muscles. EGTA at higher concentrations than 50 $\mu\text{mol/l}$ apparently caused some damage to normal and diabetic muscle cells, since the aequorin luminescence corresponding to twitch tensions was no longer detected. The concentration of EGTA used was less than 20 $\mu\text{mol/l}$, except in the case of non-diabetic denervated muscles.

In normal muscles, the effect of Ca^{2+} -free solution was slightly altered by pre-injecting the cells with 5 and 20 $\mu\text{mol/l}$ EGTA. In diabetic muscles, the Ca^{2+} -free solution-induced suppression of Ca^{2+} transients was clearly demonstrated by pre-injecting the muscles with 5 and 20 $\mu\text{mol/l}$ EGTA in a concentration-dependent manner, as shown in Figure 2. In non-diabetic denervated muscles, no Ca^{2+} -free solution-induced suppression of the Ca^{2+} transient was observed in response to pre-injection of 5, 20, or even 200 $\mu\text{mol/l}$ EGTA.

These results suggest that only when readily mobilized or cytosol-free Ca^{2+} is removed by EGTA pre-injection do diabetic muscles exhibit the same pattern of Ca^{2+} release as normal muscles.

Effect of caffeine on intracellular release of Ca^{2+} after external treatment with Ca^{2+} -free solution

The caffeine-induced Ca^{2+} release was found to be selective. The properties of the caffeine-sensitive Ca^{2+} release were compared in the normal, diabetic, and non-diabetic denervated muscles. With normal nutrient Ca^{2+} solution, 5 mmol/l caffeine induced an increase in the Ca^{2+} transient in all muscles. The Ca^{2+} release was prolonged in normal muscle, brief in non-diabetic denervated muscle, and delayed and brief in diabetic muscle. These small differences in the caffeine-induced increase in Ca^{2+} transient were enhanced by pretreatment with external Ca^{2+} -free solution. Even after pretreatment, the caffeine-induced increase in aequorin luminescence was maintained in normal muscles as shown in Figure 3a (a) (upper and middle) and Figure 3b (a). However, the increase was brief and finally decreased both in non-diabetic denervated and diabetic muscles, as shown in Figure 3a (b and c) (upper and middle) and Figure 3b (b and c).

Effect of external Ca^{2+} -free solution on caffeine-induced Ca^{2+} release in diabetic muscles intracellularly pre-injected with EGTA

In normal muscles the caffeine-induced increase was altered only slightly or not at all even by 5 $\mu\text{mol/l}$ EGTA pre-injection as shown in Figure 3a (a) (lower) and Figure 3b (a). These data have already been reported [6]. In diabetic muscles, intracellular pre-injection of 20 $\mu\text{mol/l}$ EGTA after external pretreatment with Ca^{2+} -free solution abolished the caffeine-induced increase in the Ca^{2+} transient or slightly inhibited it in the case of external Ca^{2+} -free conditions as shown in Figure 3a (b). The effects of caffeine on diabetic muscles were measured quantitatively up to 5 $\mu\text{mol/l}$ EGTA in Figure 3b (b). In non-diabetic denervated muscles, the caffeine-induced Ca^{2+} release after treatment with Ca^{2+} -free solution increased slightly for a brief time and then was abolished completely. Pre-injection with 20 $\mu\text{mol/l}$ EGTA enhanced this pattern as shown in Figure 3a (c) (lower) and Figure 3b (c). A higher concentration (200 $\mu\text{mol/l}$) of EGTA produced stronger inhibition (data not shown).

In non-diabetic denervated muscles in external Ca^{2+} -free solution, the suppressed Ca^{2+} transient response did not recover, even after the caffeine solution was washed out. However, recovery did occur after exposure to normal Ca^{2+} solution. The same tendency was observed in diabetic muscles (data not shown).

Discussion

In a previous study [11] we reported the electrical properties of skeletal muscle membranes in diabetic mice. The diabetic state was accompanied by reduced resting membrane potentials and resting membrane conductance. In the present study, the abnormal properties of two kinds of diseased muscle (diabetic muscle and non-diabetic denervated muscle) were compared in terms of their re-

sponse to two kinds of voltage-dependent Ca^{2+} sources (basal and caffeine-reacting), using external Ca^{2+} -free solution and reduced amounts of intracellular Ca^{2+} (with EGTA pre-injected).

Three kinds of Ca^{2+} transients have been previously reported in detail [7]. One is spontaneous, and the others are voltage-dependent. The voltage-dependent Ca^{2+} transients are classified as basal and caffeine-sensitive. Borgers et al. [12] reported the existence of exchangeable sarcolemma associated Ca^{2+} in red skeletal muscles. Yoshioka and Somlyo [13] reported that caffeine releases Ca^{2+} in the terminal cisternae in frog muscles. Caffeine-induced responses in muscles are resistant to external Ca^{2+} -free solution, even after intracellular pre-injection of EGTA [8], which lowers free myoplasmic Ca^{2+} levels and chelates the binding Ca^{2+} of sarcoplasmic reticulum [14, 15]. The previous results, therefore, suggest two kinds of voltage-dependent Ca^{2+} pools in skeletal muscles (basal external Ca^{2+} -sensitive and caffeine-reacting external Ca^{2+} -insensitive).

In diseased muscles, the abnormality of the Ca^{2+} pool was demonstrated more clearly under external Ca^{2+} -free conditions after pretreatment with intracellular EGTA. The final EGTA concentration in the muscle fibers could not be quantified, but was estimated to be roughly less than one-tenth of the concentrations injected [16]. The different properties of the two kinds of voltage-dependent Ca^{2+} pools could also distinguish under external Ca^{2+} -free conditions in the presence of reduced amounts of intracellular Ca^{2+} .

Ca^{2+} transients induced by direct stimulation were decreased in the presence of external Ca^{2+} -free solution in denervated muscles just as in normal muscles [6]. The plasma membrane of denervated muscles may be more permeable to external Ca^{2+} than in normal muscles. This was supported by the fact that spontaneous fluctuations in the Ca^{2+} transients of denervated muscles followed the same pattern as in smooth muscle, in which Ca^{2+} readily flows in [10]. In denervated muscles the Ca^{2+} transients were insensitive to EGTA pretreatment, suggesting less internal buffering of Ca^{2+} .

In contrast, in diabetic muscles, the directly-stimulated Ca^{2+} transient was insensitive to external Ca^{2+} -free solution. Our previous results demonstrate that Ca^{2+} entry (Ca^{2+} slow action potential amplitudes) is reduced in the diabetic state [1]. Only when the muscle was pretreated with EGTA (meaning lower levels of free intracellular Ca^{2+}), did the transients become sensitive. These results indicate that by increasing intracellular Ca^{2+} buffering by means of EGTA they behave normally, suggesting ready accumulation of intracellular Ca^{2+} in the cytosol.

In both types of diseased muscles, the caffeine-induced Ca^{2+} transients became increasingly more sensitive under both the external Ca^{2+} -free and intracellular less Ca^{2+} conditions, in contrast to their insensitivity in normal muscles. In diabetic muscles this tendency was greater than in denervated muscles. The caffeine response itself initially showed a brief increase in Ca^{2+} transients and then an opposite, inhibitory effect. The caffeine-sensitive Ca^{2+} pools can be easily depleted in diabetic muscles and non-diabetic denervated muscles.

Although three weeks after the administration of alloxan, insulin administration restored blood glucose levels, resting membrane potentials and resting membrane conductance remained abnormal [11]. Our findings in the diabetic muscle did not reflect a lasting effect of alloxan directly because similar effects were also caused by streptozotocin (data not shown). Therefore, the abnormal properties of Ca^{2+} transients may be changed irreversibly and secondarily by hyperglycaemia.

Hyperglycaemia gives rise to an abnormal metabolic pathway in the peripheral neuropathy syndrome, i.e. activation of the sorbitol pathway, inhibition of myo-inositol uptake, a decrease in inositol phosphates (representing a decrease in inositol triphosphate), and finally suppression of Ca^{2+} mobilization. The above abnormality in Ca^{2+} transients may be related to polyol pathway hyperactivity and disorders in myo-inositol-inositol metabolism.

In conclusion, these results demonstrate the insensitivity of intracellular Ca^{2+} mobilization in response to external Ca^{2+} , the ready accumulation of intracellular Ca^{2+} in the cytosol in the diabetic state, increased Ca^{2+} permeability in the denervated state and impairment of the caffeine-responding Ca^{2+} pool in both diabetes and the non-diabetic denervated state. Diabetic neuromyopathy thus appears to be a secondary and an abnormal state of Ca^{2+} -mobilization.

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