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Effect of dantrolene sodium on calcium movements in single muscle fibres

DANTROLENE sodium or 1-[5-(*p*-nitrophenyl) furfurylidene amino] hydantoin sodium hydrate (DaNa) is a long-acting drug with skeletal muscle relaxant properties which has recently seemed clinically useful in the treatment of some types of muscle hypertonia[1,2]. DaNa does not affect neuromuscular transmission nor the electrical properties of skeletal muscle[3] and it apparently involves the processes of excitation–contraction coupling[1,3,4]. The mode of action of DaNa has not been studied before on single muscle fibres and here we provide direct evidence that DaNa interferes with the intracellular movements of calcium ions.

Rana temporaria (60 g) kept at 15° C and force fed twice a week with 1–1.5 g chopped beef were used. Single skeletal muscle fibres of the fast twitch type were isolated by microdissection from the dorsal part of the semitendinosus muscle. The fibres diameter 70–150 μm were kept in a standard phosphate Ringer solution[5] at *p*H 7.2 and they maintained their physiological properties (including electrical threshold) for several hours. When dissected, the muscle fibre was transferred into a small (1.5 ml) Perspex chamber and mounted horizontally with one of its tendons hooked to the anode of a RCA 5734 mechano-electrical transducer tube. A home-made Perspex four-way tap allowed the thorough replacement of the chamber fluid within about 1 s. A continuous circulation system with appropriate bypasses maintained the temperature of the experimental solutions and of the chamber at 18° C. Electrical pulses of 0.2 ms duration and 1.2 times threshold were delivered through a pair of bright platinum wires fixed 10 mm apart in the bottom of the chamber at right angles to the fibre. DaNa freshly dissolved in phosphate Ringer was applied at a concentration of 10 μM. This produced a mean depression of the twitch force by 58% (eight experiments) within 30 s and an additional 6% decrease during the next 10 min (Fig. 1*a*). The twitch contraction time and relaxation time were shortened. The action of DaNa was much more rapid for these single muscle fibres than for the whole muscles studied previously. Most of our data were collected within about 6 min of exposure DaNa. The effect of the drug was reversible on return to normal Ringer but a complete recovery of the twitch force took 30–60 min.

When the isometric twitch force was markedly reduced by DaNa, the tetanus force for a 1.5 s supramaximal stimulation at 100^{-1} s was not significantly reduced. The finding of a large reduction of the twitch with little, if any, change in tetanus tension bears out the earlier results on whole muscle[1,4] and indicates that DaNa does not impair the maximum capacity

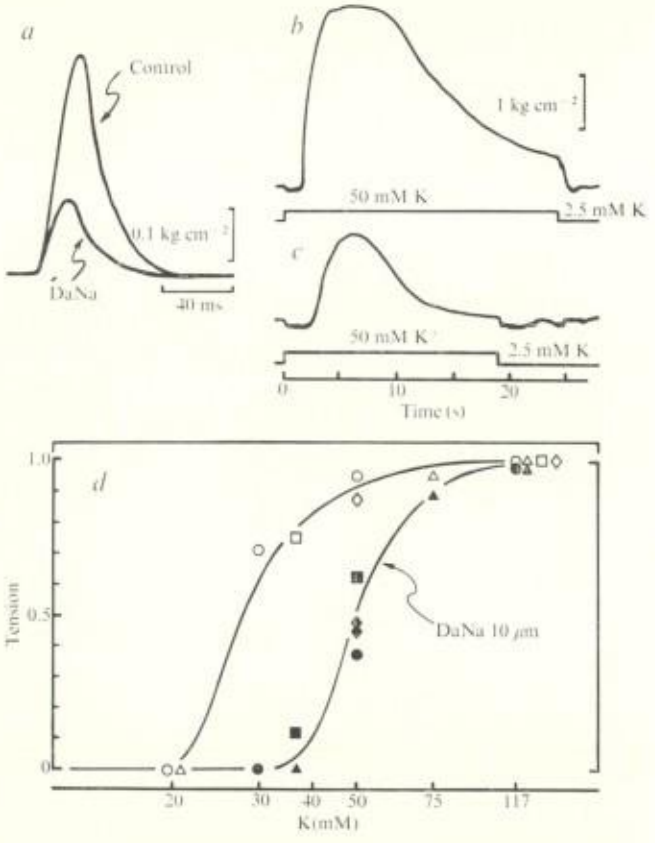

Fig. 1 Effect of DaNa on single muscle fibre. *a*, Superimposed isometric twitches elicited by supramaximal pulses before and in presence of DaNa; *b* and *c*, contractions elicited by a rapid increase in external K from 2.5 to 50 mM (see step), *b*, before and, *c*, in the presence of DaNa. The vertical calibration is expressed in kg force output per cm² cross sectional area of the muscle fibre. *d*, Relationship between peak contracture force and the increase of the external K (log scale). ○, □, △, ◇, before DaNa; ●, ■, ▲, ♦, in presence of 10 μM DaNa. Each different symbol corresponds to a different muscle fibre: ○ ● diameter 86 μM, maximum tension 3.1 kg cm^{-2}; □ ■ diameter 74 μM, maximum tension 3.4 kg cm^{-2}; △ ▲ diameter 98 μM, maximum tension 3.7 kg cm^{-2}; ♦ ◇ diameter 118 μM, maximum tension 2.5 kg cm^{-2}. Temperature of solutions 18° C.

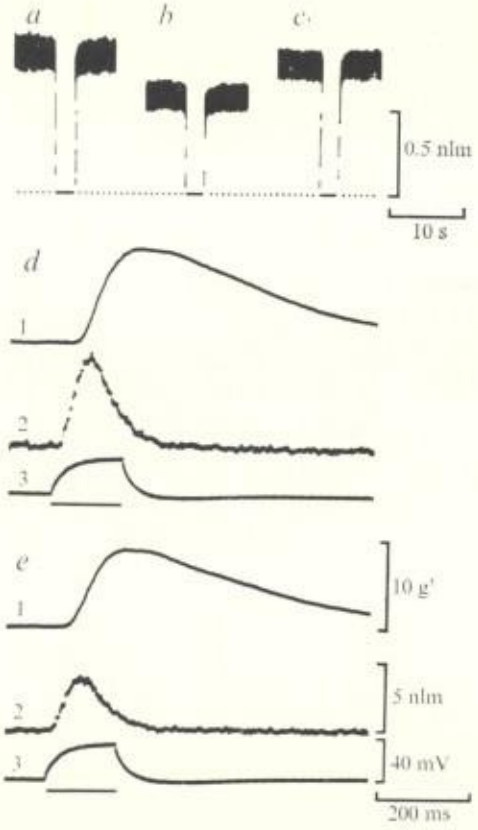

Fig. 2 *a–c*, Effect of 35 μM DaNa in 0 Ca–1 mM ethyleneglycol-*bis*-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) artificial seawater on the resting glow of an isolated barnacle muscle fibre injected with aequorin. The horizontal dotted line represents the 0 level. *a*, Resting glow after 3 min equilibration in 0 Ca–EGTA; *b*, decrease of the resting light emission after 30 s in DaNa; *c*, recovery in 0 Ca–EGTA seawater. Fibre diameter 1.1 mm; mean intracellular resting potential −55 mV. *d* and *e*, Result of applying a single electrical stimulation of 150 ms duration to a barnacle muscle fibre injected with aequorin, before, *d*, and after, *e*, 6 min exposure to DaNa 35 μM in the external medium E. Trace 1, isometric tension; trace 2, calcium mediated light emission; trace 3, membrane response. Fibre diameter 1.8 mm. Mean intracellular resting potential −59 mV. Temperature of solutions 21° C.

of the muscle fibre to produce force. The recorded reduction of the twitch force by DaNa must therefore be related to an inhibition of some step in the process of mechanical activation, as the membrane spike generation is not impaired[3].

A useful way to approach this problem is by recording single fibre contractions to known increases in the external potassium concentration whereby Hodgkin and Horowicz mechanical threshold[6] can be estimated. Figure 1*b* shows a near maximum contraction of a fresh muscle fibre suddenly exposed to 50 mM K. Four min after bathing the fibre in 10 μM DaNa (Fig. 1*c*) a second exposure to 50 mM K elicited a contraction which was reduced by more than 50%. The latency of this contraction was increased and its rate of rise reduced while the subsequent spontaneous relaxation during the maintained K exposure was quicker than in Fig. 1*b*. These effects of DaNa were reversible. The graph of Fig. 1, *d* shows similar data for different concentrations of K in four different muscle fibres. The peak force of each K contraction is expressed with respect to the maximum force recorded in the same fibre during exposure to 117 mM K. These maximum contractions were not significantly affected by 10 μM DaNa in the present single fibre experiments and we think the reduction reported by others[4] must have been related to their use of whole muscle preparations[6]. The effect of lower K concentrations, however, was markedly reduced and the curve was shifted to the right by a factor of about 1.7. The mean threshold for K contractions was approximately 20 mM in untreated fibres and 35 mM K after 1 min in 10 μM DaNa.

The observation that DaNa reduces both submaximal potassium contractions and twitch force without significantly affecting the maximum potassium contractions or the tetanus force could be explained by an inhibitory effect on some intracellular step in excitation–contraction coupling. To analyse this possibility, single muscle fibres from the barnacle *Balanus nubilus*[7] were injected with aequorin, the calcium-sensitive bioluminescent protein[8]. Several thousands of the jellyfish *Aequorea forskalea* were collected at Friday Harbor Laboratories during summer, 1973, and the extracted aequorin was purified in Brussels, using standard techniques[9]. Each barnacle muscle fibre of 1.0–2.0 mm diameter was cannulated with a Pyrex tube of 200 μm diameter and mounted vertically in a light-tight black Perspex chamber[8]. The calcium-mediated light output was recorded on a Tektronix oscilloscope with an EMI 9635 photomultiplier tube. When the injected muscle fibre was placed in front of the photomultiplier, the rate of light emission related to the intracellular ionised calcium (resting glow) showed a rapid decrease during the first 30 min and then stabilised at a rather steady level as the $[Ca]_i$ is very low in the resting state and the rate of consumption of the injected aequorin is small. In these conditions, the replacement of the normal seawater by DaNa seawater was followed by a decrease of the resting glow to a lower level which depends on the drug concentration.

This reduction of the intracellular Ca concentration by DaNa could result either from a reduced resting Ca^{2+} influx, or from an increased resting Ca^{2+} efflux or from a direct action involving the intracellular Ca stores. To distinguish these possibilities, the aequorin-injected muscle fibres were tested in 0 Ca–1 mM EGTA artificial seawater, and DaNa 35 μM still reversibly reduced the resting glow by about 20% (Fig. 2*a–c*) which suggests that the DaNa effect is not dependent on any reduction in resting Ca^{2+} influx. The second alternative could also be excluded in other experiments in which the leakage of ^{45}Ca into external seawater was estimated in barnacle muscle fibres loaded by an intracellular injection of 0.1–0.2 μl ^{45}Ca in Tris buffer at *p*H 7.2 (ref. 10). The resting calcium efflux was actually decreased by 35 μM DaNa which implies that the reduced intracellular calcium cannot result from an increased loss.

Therefore it would seem that DaNa acts primarily on the intracellular Ca storage sites to reduce the level of free sarcoplasmic calcium. This third alternative is indeed supported by studies of the calcium transient recorded during single contractions of barnacle fibres injected with aequorin. The fibre was stimulated at intervals of at least 20 s through an intracellular silver wire with a constant current pulse of 150 ms while the membrane potential was recorded with another intracellular platinum electrode connected to a high input impedance amplifier[8]. Although the actual membrane depolarisations thus elicited were identical before and after DaNa (third trace in Fig. 2*d* and *e*), the mechanical force (first trace) produced by the stimulated muscle fibre was reduced by 20% after DaNa 35 μM while the transient increase in aequorin luminescence (second trace) decreased by about 30% in this example. These results were regularly recorded in the six different barnacle muscle fibres studied, each one being tested with various combinations of stimulus durations and intensities.

In conclusion, DaNa depresses the mechanical force output of single muscle fibres by an inhibitory effect involving the calcium movements at intracellular storage sites, both at rest and during electromechanical activation.

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