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INHIBITION OF THE INTRACELLULAR RELEASE OF CALCIUM BY DANTROLENE IN BARNACLE GIANT MUSCLE FIBRES

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SUMMARY

1. Ca movements in resting and in activated single giant muscle fibres of the barnacle were analysed before and after exposure to Dantrolene Na, a synthetic hydantoin derivative.

2. In fibres micro-injected with the photoprotein aequorin, the resting rate of light emission (resting glow) reversibly decreased upon exposure to Dantrolene. Similar results were obtained if the fibre had first been equilibrated in a 0 Ca–1 mM-EGTA medium.

3. The influx of ^{45}Ca into resting muscle fibres was not modified by 35 μM Dantrolene which also failed to significantly reduce the influx of ^{45}Ca into muscle fibres which had been depolarized by exposure to external solutions in which K^+ had been increased to 60 or 200 mM.

4. In fibres micro-injected with ^{45}Ca, the calcium efflux was reversibly decreased by Dantrolene. This effect was still observed in 0 Ca medium and in 0 Ca–0 Na medium. A possible effect of Dantrolene on the Na–Ca exchange process at the outer membrane was excluded by showing that when the direction of the Ca^{2+} movement was inverted in aequorin-loaded fibres by the sudden removal of Na^+ from the external medium, a marked increase in the resting glow was recorded which was not affected by exposure to Dantrolene.

5. It is argued that the reduction of Ca^{2+} efflux by Dantrolene does not result from any direct inhibitory effect on the metabolically driven Ca pump at the outer membrane, but that it is rather related to the reduction of the concentration of myoplasmic Ca^{2+} which is indeed demonstrated by the reduced resting glow. This in turn is thought to result from a shift in the balance between Ca^{2+} movements into and out of the intracellular storage sites, and namely the sarcoplasmic reticulum (SR).

6. The Ca^{2+} transient in aequorin-loaded fibres and the force of the isometric contraction elicited by imposed membrane depolarizations were

markedly reduced by Dantrolene. The electrochemical threshold for eliciting intracellular Ca^{2+} release was not significantly modified. The linear relation between membrane depolarization and Ca^{2+} transient became less steep. The process of sequestration of myoplasmic Ca^{2+} back into SR was not significantly affected by Dantrolene which appeared to inhibit rather selectively the Ca^{2+} release from SR into the cytosol.

INTRODUCTION

Ca ions play a unique role in the stimulus-contraction coupling in muscle fibres (Ebashi & Endo, 1968; Weber & Murray, 1973) and in triggering the exocytosis of packaged neurotransmitters in axon terminals or of secretion products in a wide variety of gland cells (cf. Katz, 1969; Rubin, 1970; Rasmussen, 1970; Douglas, 1974; Carafoli, Clementi, Drabikowski & Margreth, 1975). The cytosolic Ca^{2+} concentration being very low in resting cells, the calcium triggering mechanisms must involve either an entry of Ca^{2+} from the external medium or a release of Ca^{2+} from intracellular reservoirs such as endoplasmic reticulum or mitochondria. One way to analyse the phasic and steady processes which interact to regulate cytosolic Ca^{2+} is to search for chemical compounds selectively involving one of these processes in intact cells.

The present paper analyses the mechanism of action of Dantrolene Na, 1-(5-(*p*-nitrophenyl)-furfurylidene-amino) hydantoin sodium hydrate which has muscle relaxant properties and depresses excitation–contraction coupling without interfering with either neuromuscular transmission or conduction of the muscle action potential (Ellis & Bryant, 1972; Ellis & Carpenter, 1972; Putney & Bianchi, 1974; Hainaut & Desmedt, 1974*b*). Giant barnacle muscle fibres (Hoyle & Smyth, 1963) were studied in conjunction with ^{45}Ca kinetics across the plasma membrane and with direct estimation of cytosolic Ca^{2+} by means of the photoluminescent reaction of the (intracellularly injected) photoprotein aequorin (Ashley & Ridgway, 1970; Ashley, Caldwell & Lowe, 1972; Ashley, Ellory & Hainaut, 1974; Desmedt & Hainaut, 1976*a*). The study was designed to enquire whether the Dantrolene effects involved one or more of the following: (1) the Ca^{2+} influx and efflux at the plasma membrane, (2) the electrochemical threshold and kinetics of Ca^{2+} release from the SR, (3) the process of sequestration of myoplasmic Ca^{2+} back into SR. One of the conclusions is that the Ca^{2+} release from SR both in resting and in activated barnacle muscle fibres can be rather selectively inhibited by Dantrolene. Some of the preliminary results have been communicated (Hainaut & Desmedt, 1974*b*).

METHODS

Single giant muscle fibres were isolated from the lateral and rostral depressor muscles of the barnacle, *Balanus nubilus* (Darwin) (Hoyle & Smyth, 1963). The cell appears as 2–4 cm long cylinders with a diameter of 1–2 mm. Three quantitative methods were used: (*a*) the estimation of Ca influx during 3 min exposure of isolated fibres (with insertions on the shell maintained intact) to ^{45}Ca artificial sea water (ASW), the fibres being then washed for 10 min in 0 Ca–0 Na–1 mM-EGTA before scintillation counting; (*b*) the estimation of the Ca efflux after loading with ^{45}Ca by intracellular injection; (*c*) the determination of the free Ca^{2+} concentration in cytoplasm by recording variations in light output of the fibre after an intracellular micro-injection of the photoprotein aequorin. Micro-injections of ^{45}Ca or aequorin were made with a pyrex S.G.E. syringe of 0·5 μl. maximum capacity fitted with a glass capillary (100 μm tip diameter) which was inserted axially from the cut shell insertion end of the fibre. Electrical sealing of this end of the fibre was achieved with petroleum jelly. Ca movements were studied both at rest and during electrical activation eliciting mechanical force responses. A glass capillary probe electrode (Ashley & Ridgway, 1970) equipped with silver and platinum wires insulated with varnish except over appropriate segments was inserted axially into the fibre and served both to deliver electrical pulses and to record membrane potentials via a high input impedance amplifier. The contraction of the fibre was recorded isometrically with an RCA 5734 mechano-electric force transducer fed by a zener-stabilized power supply with temperature compensation. Further details of methods can be found elsewhere (Desmedt & Hainaut, 1976 *a*).

Preparation and micro-injection of ^{45}Ca. $CaCl_2$ containing $^{45}CaCl_2$ was obtained from the U.K. Atomic Energy Authority, Amersham. It had a specific activity of 1 mCi/ml. with 72 μg Ca/ml.; 20 μl. were removed and dissolved in 5 μl. 250 mM-Tris, pH 7·2. Usually 0·1–0·2 μl. of this solution was injected into the muscle fibre. The efflux was followed by putting the fibre for periods of 10 min into a series of small tubes containing 1·5 ml. ASW or modified ASW at 20–22° C. The radioactivity was assayed by liquid scintillation and the efflux was expressed as a rate constant k:

$$k = \frac{\text{(counts lost/collection time)}}{\text{mean counts in the fibre during collection time}} \quad (\text{min}^{-1}).$$

Preparation of the photoprotein aequorin. The protein was extracted from the photogenic organs of several thousands *Aequorea forskalea* and transported by air in a cold container for purification in Brussels (cf. Shimomura, Johnson & Saiga, 1962; Shimomura & Johnson, 1977). Generally 0·1–0·2 μl. of a saturated solution of purified aequorin in 10 mM-Na acetate, 1 μM-EDTA medium, 10 mM-TES (pH 7·0) was micro-injected into the muscle fibre. The aequorin reacts with the intracellular Ca^{2+} to emit a blue light (maximum at about 460 nm) that can be detected with a photomultiplier tube and converted to lumen units (Ashley & Ridgway, 1970). The size of the aequorin molecule is too large for it to cross the cell membrane, but small enough to diffuse throughout the cytoplasm of the fibre within about 1 hr (Fig. 1). Aequorin did not modify the behaviour of the preparation during experiments of 2–6 hr.

Solutions. Artificial sea water (ASW) was prepared as described by Fatt & Katz (1953). In the 0 Ca experiments, $CaCl_2$ was replaced by NaCl. In 0 Ca–0 Na experiments, LiCl was used as replacement salt. Dantrolene Na (Eaton Labs, Norwich, N.Y.) was freshly dissolved in ASW before the experiment to obtain a final concentration of 35 μM in which the fibre was bathed. The solutions with pH adjusted to 7·3–7·5 before use did not modify the resting membrane potential by more than $\pm$ 5 mV. Dantrolene depressed the Ca^{2+} aequorin reaction by 15 % and the percentage

figures quoted were corrected accordingly. An upper correction (mean 5%) was also made when counting the radioactivity by liquid scintillation in the presence of Dantrolene.

RESULTS

^{45}Ca efflux in Dantrolene. The efflux of ^{45}Ca from a micro-injected muscle fibre follows a rather complex pattern initially (Caldwell, 1964; Ashley *et al.* 1974) but becomes roughly exponential after 2–3 hr, the rate constant declining only slowly with time from then on. The latter phase was used as base line (Fig. 1*A*) and we transferred after 3 hr the ^{45}Ca loaded fibre from ASW containing 35 μM Dantrolene. This elicited a consistent but rather small decrease in rate constant (mean of $10{\cdot}6 \pm 3{\cdot}2$% in eight fibres) (Fig. 1*B*). When the fibre was subsequently transferred back into normal ASW, the rate constant returned towards the control level. The maximum inhibition of ^{45}Ca efflux by Dantrolene was observed within the first 10 min exposure to the drug. Smaller reductions were observed in the presence of 10 μM Dantrolene. This reduction of the ^{45}Ca efflux by Dantrolene could be explained either by an effect on Ca^{2+} channels at the outer cell membrane, or by a reduction of the myoplasmic Ca^{2+} concentration.

Effect of Dantrolene on cytosolic Ca^{2+}. A barnacle muscle fibre micro-injected with aequorin emits a resting light (resting glow) which is very high immediately after the injection, but subsequently decreased progressively as the Ca^{2+} released in the cell by mechanical damage from the injection pipette was sequestered, and as the aequorin concentration decreased by radial diffusion from the injection site throughout the cytoplasm. After about 60 min, the resting glow reached a fairly steady level (Fig. 2*A*) corresponding to the normal free Ca^{2+} concentration at rest which in these cells is of the order of 10^{-7} M (Ashley, 1970). This latter phase of resting glow remained stable for many hours (the rate of consumption of injected aequorin is small) and it allowed experimentally induced changes of Ca^{2+} to be quantitated.

Fig. 2*B–F* indicates that the resting glow decreased in the presence of 35 μM Dantrolene. This reduction showed two steps: an initial quick reduction involving about two thirds of the total effect in less than 30 sec and a second much slower decrease during the next 5–6 min (Fig. 2*F*). In this experiment, the maximum decrease of the resting glow after exposure to Dantrolene for 6 min was 30% of control (corrected value, see Methods). When the fibre was subsequently transferred into ASW, a somewhat slow recovery was observed: about half of the phenomenon disappeared after 1 min (Fig. 2*E–F*) but the subsequent phase was very slow and the resting glow rarely recovered to the control level at the end of 1 hr.

Similar results were obtained when the fibre had been first equilibrated in 0 Ca–1 mM-EGTA medium before Dantrolene was added to this saline

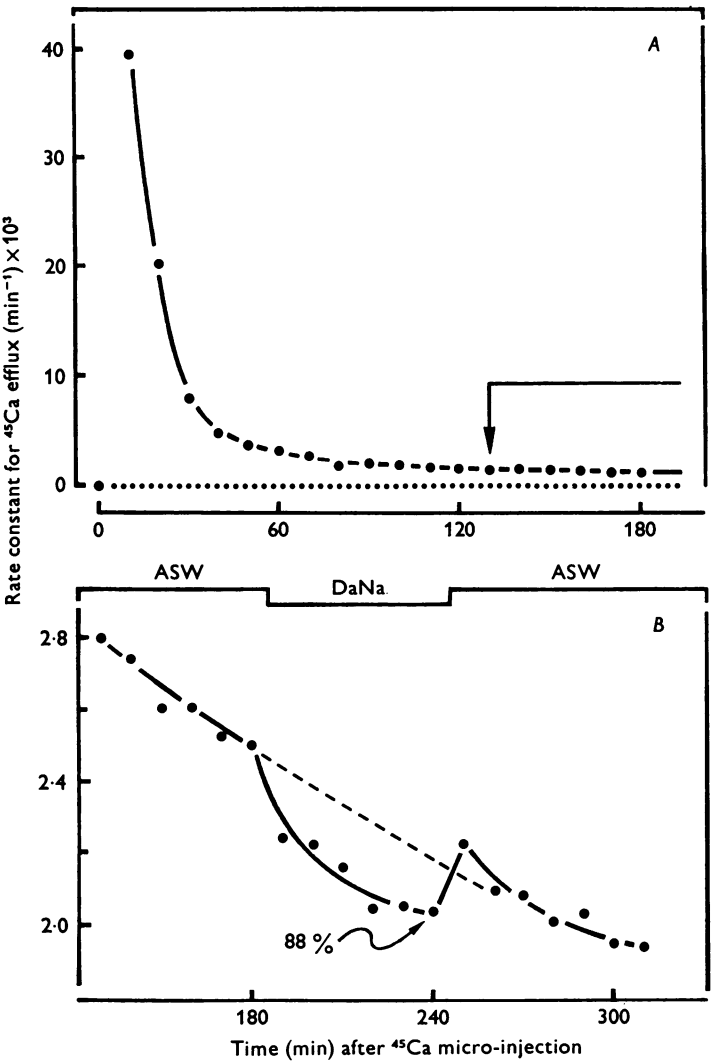

Fig. 1. *A*, initial rapid efflux of ^{45}Ca from a micro-injected barnacle fibre and subsequent slower decline with time after a period of 2–3 hr; the arrow indicates the part used as base line for the experiment illustrated in *B*. *B*, effect of 35 μM Dantrolene (DaNa) on ^{45}Ca efflux from the same cell; the resting efflux function was calculated from the first six experimental data recorded in ASW ($y = 3{\cdot}49 \exp(-0{\cdot}002\,x)$; $r^2 = 0{\cdot}97$). Fibre diameter 1·2 mm. Mean intracellular resting potential − 60 mV. Temperature of the solutions 22° C.

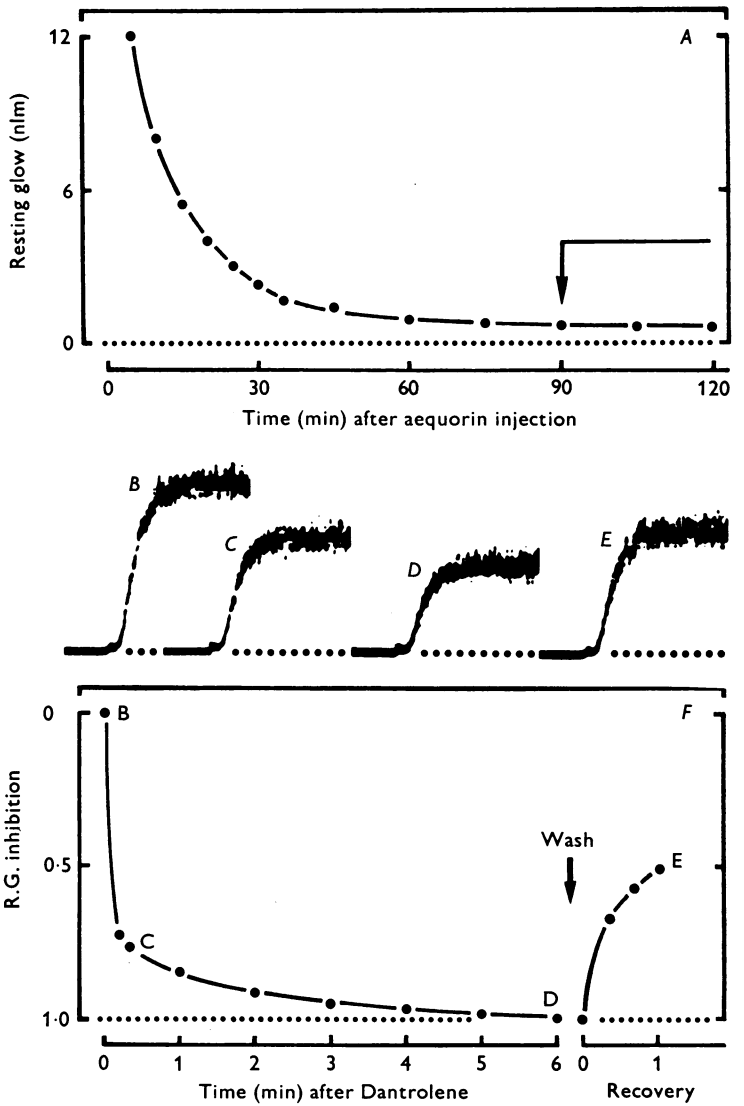

Fig. 2. *A*, initial rapid decrease of the resting light emitted by a barnacle muscle cell micro-injected with aequorin (resting glow). The arrow indicates the subsequent stable phase corresponding to the normal cytosolic Ca^{2+} concentration. *B–D*, effect of 35 μM Dantrolene on the resting glow; these samples were recorded when the photomultiplier was switched on after 20 sec (*C*) or 6 min (*D*) of exposure to Dantrolene. *E*, partial recovery of the resting glow after the fibre had been transferred back to ASW for 1 min. *F*, time course of the resting glow reduction, which reached 30 % (corrected value, see Methods) of the resting glow after 6 min exposure of this cell to Dantrolene. Fibre diameter 1·1 mm. Mean intracellular resting potential −45 mV. Temperature of the solutions 21° C.

(Hainaut & Desmedt, 1974*b*). Thus Dantrolene consistently reduced the level of free Ca^{2+} in the cells whether calcium was present or absent in the external medium.

Effect of Dantrolene on ^{45}Ca influx in resting fibres. The above finding that Dantrolene reduced the resting glow even when the fibre was bathed in 0 Ca does not exclude the possibility that it would in addition decrease the resting Ca influx. This was tested by bathing thirty-five isolated fibres in ^{45}Ca ASW for 3 min and by counting the cell radioactivity after washing out the external ^{45}Ca clinging to the cell surface (see Methods). For half of these fibres, 35 μM Dantrolene was added to the ^{45}Ca ASW. The exposure time exceeded the delay required for the drug to decrease the resting glow markedly (Fig. 2). The ^{45}Ca in each single fibre was expressed in counts/min.cm^{-2} of outer plasma membrane (calculated from measurements of diameter and length, with no correction for clefts; see Hoyle, McNeill & Selverston, 1973). No significant difference ($P > 0{\cdot}1$) was found in ^{45}Ca influx in the presence of 35 μM Dantrolene (Table 1).

TABLE 1. Effect of Dantrolene on ^{45}Ca efflux

	Exposure time (min)	Control in ASW	K^+-ASW	In presence of Dantrolene (35 μM)
^{45}Ca efflux (%) in ASW	10	100	—	$89{\cdot}4 \pm 3{\cdot}2$
^{45}Ca influx (cpm/cm^2 membrane) in ASW	3	509 ± 87 ($n = 20$)	—	471 ± 124 ($n = 15$)
^{45}Ca influx (%) in 60 mM-K^+ ASW	3	100 ($n = 14$)	125 ± 11 ($n = 15$)	123 ± 9 ($n = 15$)
^{45}Ca influx (%) in 200 mM-K^+ ASW	3	100 ($n = 12$)	153 ± 21 ($n = 12$)	155 ± 14 ($n = 12$)

Effect of Dantrolene on ^{45}Ca influx in fibres depolarized by K^+. Exposure to increased external K^+ depolarizes muscle fibres and elicits contractions which involve an intracellular Ca^{2+} release, but also an increased entry of Ca^{2+} from the external medium through the outer membrane (cf. Caldwell, 1972; Ashley *et al.* 1972). The question arises whether Dantrolene would affect the latter increased influx. Experiments on eighty single fibres were carried out as in the preceding section, but in the presence or absence of 60 or 200 mM-K^+. The fibres were exposed to these solutions for 3 min. The ^{45}Ca influx was increased to 125% by 60 mM-K^+ which was insufficient to cause contraction, and to 153% by 200 mM-K^+ which elicited a strong contraction maintained throughout the testing period. When the same tests were repeated on other single fibres exposed to 35 μM Dantrolene, the data were not significantly different ($P > 0{\cdot}4$) (Table 1). Thus the drug failed to influence the increased Ca^{2+} influx at the outer

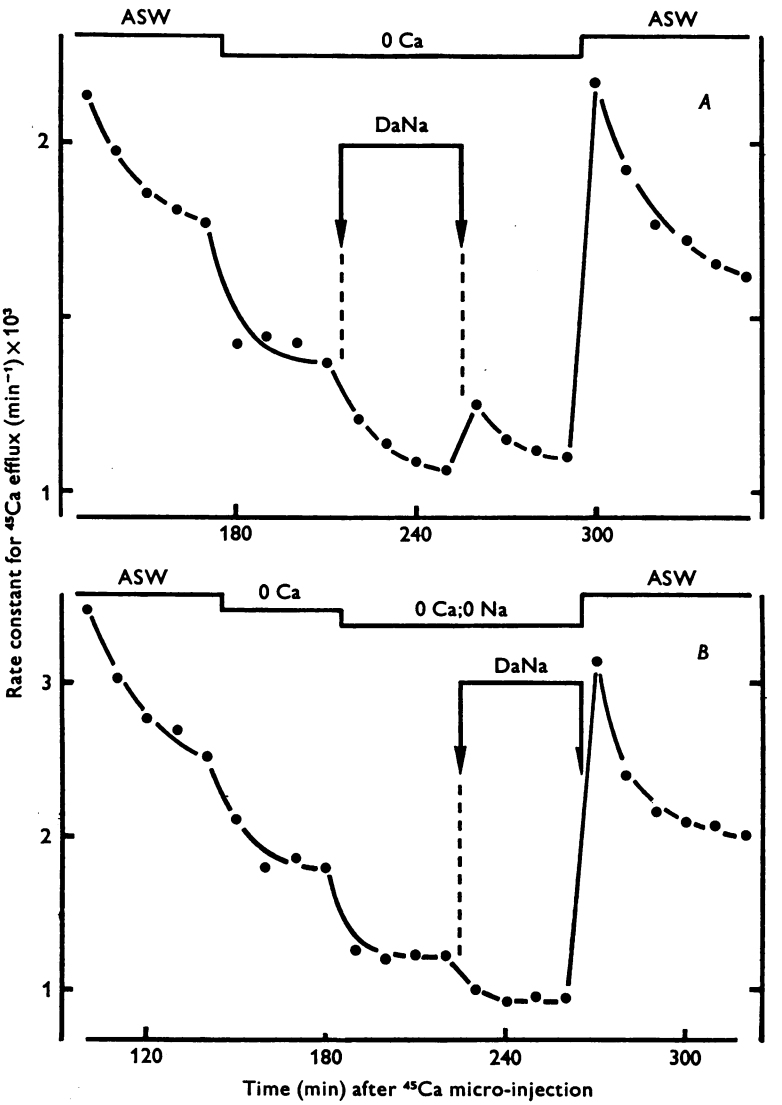

Fig. 3. *A*, effect of 35 μM Dantrolene (DaNa) on the ^{45}Ca efflux after this had first been reduced by suppressing the Ca-Ca exchange diffusion. In 0 Ca medium, Ca^{2+} was replaced with Na^+. Fibre diameter 1·0 mm. Mean intracellular resting potential −48 mV. Temperature of solutions 22° C. *B*, effect of 35 μM Dantrolene on the ^{45}Ca efflux after this had first been reduced by eliminating the Ca-Ca exchange diffusion and the Na-Ca exchange mechanisms. In the 0 Ca–0 Na medium, Ca^{2+} and Na^+ were replaced with Li^+. Fibre diameter 1·3 mm. Mean intracellular resting potential −52 mV. Temperature of the solutions 22° C.

membrane of single muscle fibres which were either subliminally depolarized by K^+ or contracted by 200 mM-K^+.

Effect of Dantrolene on calcium extrusion mechanisms at the plasma membrane. A large electrochemical gradient favours Ca^{2+} entry into the cell while extrusion mechanisms maintain the intracellular Ca^{2+} concentration at a stable low level (Baker, 1972; Ashley *et al.* 1974). Besides a Ca–Ca exchange diffusion process, there is a Na–Ca exchange mechanism which depends on the inward Na^+ electrochemical gradient to extrude Ca^{2+} out of the cell; in addition there is a third metabolically driven component which is responsible for the residual Ca^{2+} efflux recorded in 0 Ca–0 Na external medium (see Ashley *et al.* 1974; Blaustein, 1974). We tried to see whether these components were affected by Dantrolene.

Fig. 3*A* illustrates an experiment in which the Ca–Ca exchange diffusion component was first abolished by transferring the ^{45}Ca-loaded fibre to 0 Ca-ASW. After the Ca^{2+} efflux had stabilized to a lower level the addition of 35 μM Dantrolene elicited a further reduction of the ^{45}Ca efflux which must obviously involve the second and/or the third Ca^{2+} efflux components considered above. These effects were reversible. The overshoot observed in the latter part of Fig. 3*A* upon return from 0 Ca solution to normal ASW had been reported previously (Ashley *et al.* 1974; Russell & Blaustein, 1974) and is not to be related to the Dantrolene exposure.

Fig. 3*B* illustrates another typical experiment in which, after 40 min equilibration in 0 Ca-ASW, the fibre was transferred to a 0 Ca–0 Na medium (Li replaced) in order to also eliminate the Na–Ca exchange mechanism. Exposure to 35 μM Dantrolene produced a definite further inhibition of the residual ^{45}Ca efflux. These findings indicate that the reduction of ^{45}Ca efflux in Dantrolene must be related to a reduction of the Ca^{2+} efflux via the third mechanism considered which persists in 0 Ca–0 Na external medium. It is not possible from these experiments to definitely exclude an additional effect of Dantrolene on the Ca–Ca and Na–Ca exchange components since the reductions in rate constant recorded upon removal of either Ca alone or of Ca and Na from the external medium were somewhat variable from fibre to fibre. One factor for the observed variations may be the complex architecture of the outer membrane in these giant fibres (Hoyle *et al.* 1973).

Another test can be used to directly evaluate a possible effect of Dantrolene on the Na–Ca exchange process. When the Na^+ gradient across the membrane is experimentally reversed in zero external Na, but in the presence of external Ca in normal concentration, the operation of the second component is also reversed and Ca^{2+} is now driven into the cell (Baker, 1972; Ashley *et al.* 1974). The rapid influx of Ca^{2+} thus elicited can be directly visualized in aequorin-injected muscle cells and the resting

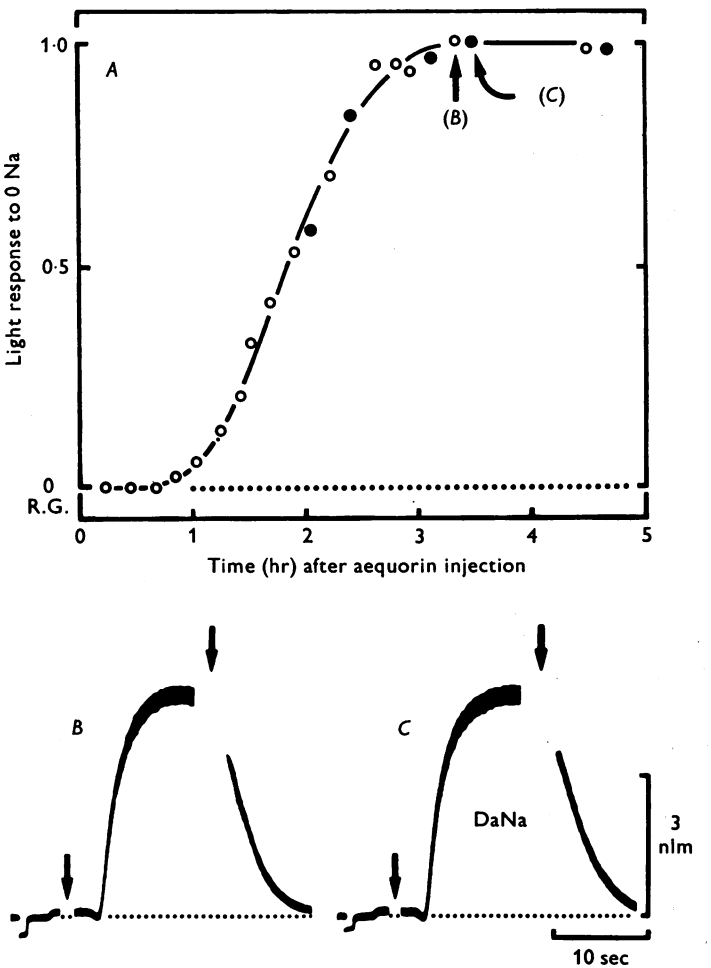

Fig. 4. *A*, progressive increase of the light response to sudden exposure to 0 Na solution, as the aequorin diffused more completely through the myoplasm and reached the outer cell membrane (open circles); this response was also regularly tested in the presence of 35 μM Dantrolene (filled circles). *B–C*, samples of the light response recorded on sudden exposure to 0 Na either in the absence (*B*) or in the presence (*C*) of Dantrolene. In each oscilloscope record the first arrow indicates the moment when the normal ASW was replaced by the 0 Na medium; and the second arrow indicates when normal ASW was restored in the external medium. The initial resting fibre glow is indicated by the dotted line. Fibre diameter 1·8 mm. Mean intracellular resting potential −49 mV. Temperature of the solutions 22° C.

glow indeed increased by a factor of about 10 upon removal of external Na in the experiment of Fig. 4*B*. It must be emphasized that this method only gives meaningful results if the photoprotein injected axially into the cell has been given adequate time to diffuse throughout the cytoplasm up to the regions directly underlying the plasma membrane. As shown in Fig. 4*A* the phasic light output elicited by the sudden removal of the external Na is only fully developed about 3 hr after the micro-injection of aequorin in this large muscle cell of 1·8 mm diameter. We found lesser delays of about 1½ hr in the smaller cells of 1 mm diameter. After such time, consistent responses to 0 Na could be elicited for several hours. This direct test indicates that Dantrolene does not significantly influence the Na–Ca exchange mechanism at the outer cell membrane. The very rapid increase of the resting glow in 0 Na and the lack of concomitant mechanical force output confirm that the effect must be restricted to the portions of cytoplasm directly underlying the fibre plasma membrane; any comparable increase in resting glow which would have involved the entire cross-section of the fibre would definitely have produced a contraction response (see Fig. 5). This application of the aequorin method indeed appears remarkably adapted to reveal rapid increases of Ca^{2+} influx upon inverting the Na^+ gradient, even when the increase of the concentration of cytosolic Ca^{2+} is quite localized to the peripheral myoplasm.

Dantrolene and intracellular Ca release. In nine experiments, aequorin-injected fibres were stimulated with 75–100 msec square electrical pulses of various intensities through the axial intracellular electrode. The resulting membrane depolarization, the phasic increase in cytosolic Ca^{2+} (Ca^{2+} transient) and the mechanical response (cf. Ashley & Ridgway, 1970) were simultaneously recorded on 3-channel FM tape recorder and cathode-ray oscilloscope system for detailed analysis. Fig. 5 illustrates typical results for identical stimulation intensities, before (*A*) and 20 min after exposure to 35 μM Dantrolene-ASW (*B*). The membrane depolarizations produced by the current were not significantly changed by Dantrolene whereas the simultaneously recorded mechanical force output was decreased to 56% of the control value in ASW. The area of the photoprotein response or Ca^{2+} transient was decreased to 50% (corrected value, see Methods), but its duration was not significantly changed.

When the fibre was subsequently returned into ASW, these modifications recovered slowly and took about 30 min to reach half control level. By contrast the initiation of the Dantrolene inhibitory effect reached half its maximum in only about 30 sec (Fig. 5*C–E*). After this first phase, the depression increased more slowly over the next 20 min. The reduction of both the peak value and the area of the Ca^{2+} transient by Dantrolene cannot be explained by an increased rate of Ca^{2+} re-uptake by the

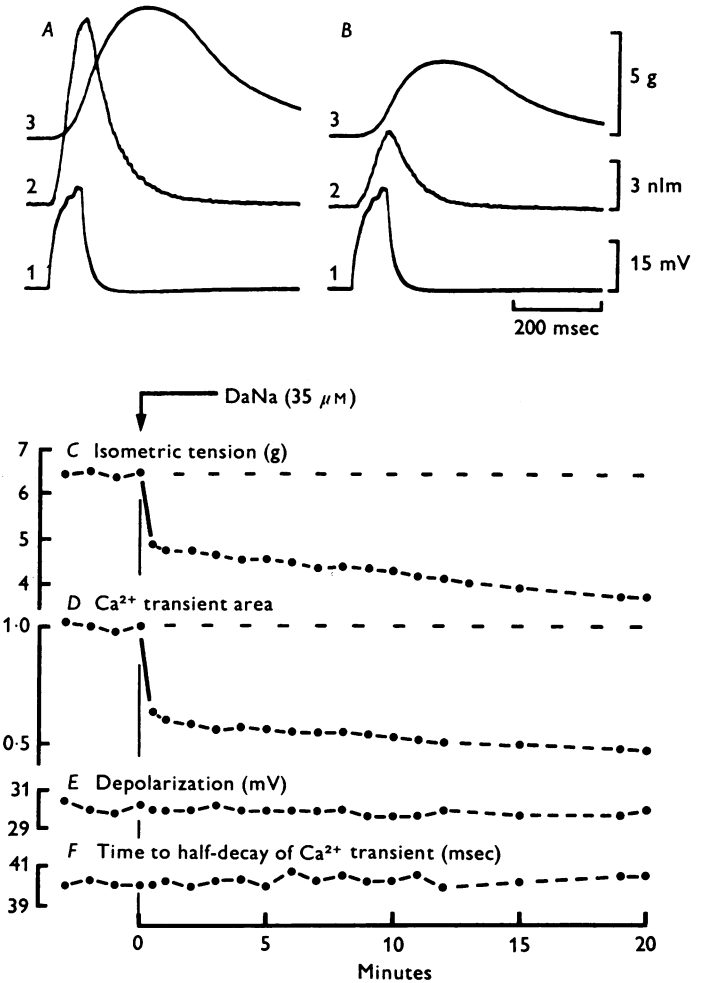

Fig. 5. *A–B*, effect of applying a single electrical stimulation of 75 msec duration to a barnacle muscle fibre injected with aequorin, before (*A*) and after (*B*) 20 min exposure to Dantrolene (35 μM) in the external medium. Trace 1, membrane depolarization. Trace 2, Ca^{2+} transient; trace 3, isometric peak force. *C–F*, time course of the effect of Dantrolene (35 μM) on several processes involved in stimulus contraction coupling of the same muscle fibre. Abscissa, time in min. *C*, isometric peak force in g. *D*, Ca^{2+} transient (relative to the initial resting level of light output). *E*, membrane depolarization in mV. *F*, time from peak to half-decay of the Ca^{2+} transient in msec. Fibre diameter 1·6 mm. Mean intracellular resting potential − 56 mV. Temperature of the solutions 22° C.

intracellular storage sites, since the time to half-decay of the Ca^{2+} transient was not reduced by Dantrolene (Fig. 5*F*; Table 2) (cf. Hainaut & Desmedt, 1974*b*, 1975).

TABLE 2. Significance of results in ASW and in Dantrolene

	Exposure time (min)	Control in ASW	In presence of Dantrolene (35 μM)	*t* test
Area of Ca^{2+} transient (arbitrary units)	20	$801{\cdot}1 \pm 18{\cdot}9$ ($n = 10$)	$477{\cdot}8 \pm 12{\cdot}2$ ($n = 10$)	$P < 0{\cdot}005$
Rate of rise of Ca^{2+} transient (normalized) (arbitrary units)	20	$1{\cdot}10 \pm 0{\cdot}10$ ($n = 24$)	$1{\cdot}08 \pm 0{\cdot}09$ ($n = 17$)	$P > 0{\cdot}25$
Time to half-decay of Ca^{2+} transient (msec)	20	$43{\cdot}2 \pm 1{\cdot}5$ ($n = 24$)	$43{\cdot}5 \pm 2{\cdot}3$ ($n = 17$)	$P > 0{\cdot}15$

The next question is whether the reduction by Dantrolene of intracellular Ca^{2+} release results either from a reduced sensitivity of the reticulum reservoir to the plasma membrane depolarization, for example by an elevation of the threshold of the electrochemical coupling process, or from some other action on the release mechanism itself. This problem can be analysed directly by imposing different levels of transient depolarization to the cell membrane and by estimating the corresponding Ca^{2+} transient (Desmedt & Hainaut, 1976*a*). Consistent results were obtained in each of the four fibres which were investigated in detail (Fig. 6). The different stimulus intensities were applied in a random sequence at interval of 10–20 sec and it was further checked that the resting light emission had returned to its basal level between the successive depolarizations. A complete run lasted for about 6 min. The fibre was then left for 10 min in ASW with 35 μM Dantrolene in order to reach stable conditions before starting another series of stimuli. In the control series before drug, the linear relation between membrane depolarization (range not exceeding about 30 mV) and area of Ca^{2+} transient was confirmed (Desmedt & Hainaut, 1976*a*). In the presence of Dantrolene, the relation was also linear but its slope was definitely reduced (even after correction for the drug effect on the Ca^{2+}-aequorin reaction). If the two divergent lines are extrapolated to zero ordinate, they become very close to each other which suggests that the membrane depolarization threshold for intracellular Ca^{2+} release had been little, if at all, affected by Dantrolene. Thus the sensitivity of the Ca^{2+} releasing sites to small membrane depolarizations is not appreciably depressed by Dantrolene, a finding which appears interesting since one might have supposed that this threshold would rather be elevated, if only because the level of resting glow is reduced by

Dantrolene (Fig. 2). The data also suggest that the drug involves either the kinetics of electrochemical coupling for supraliminal depolarizations, or the Ca^{2+} release sites at the reticulum membranes directly.

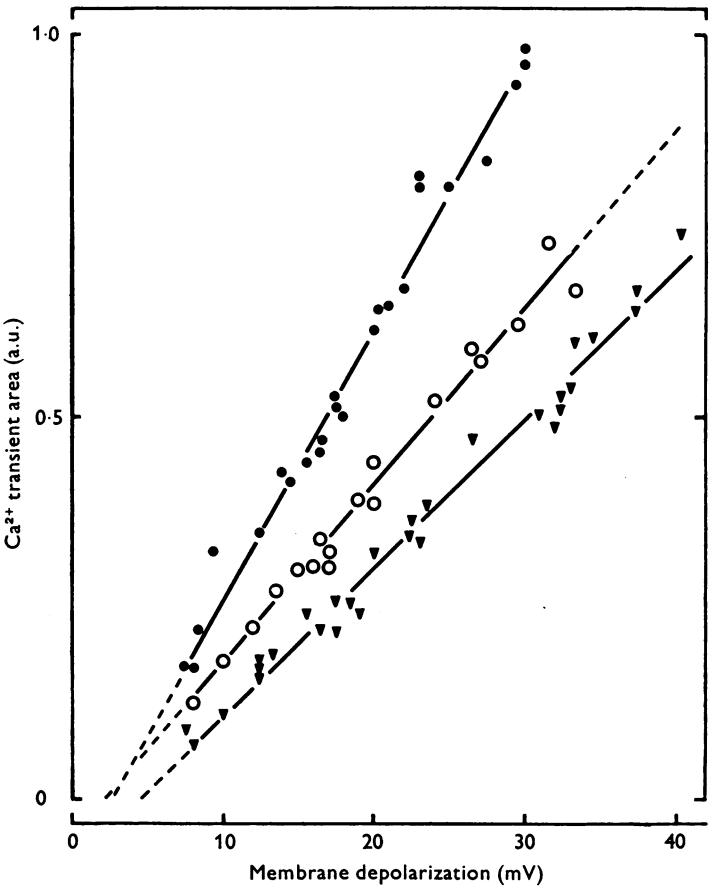

Fig. 6. Relation between the cell membrane depolarization produced by graded subliminal currents and the area of the corresponding Ca^{2+} transient (arbitrary units). A linear regression has been calculated for each set of data. Filled circles, control in normal ASW ($y = 0{\cdot}35, x - 0{\cdot}89$; $r^2 = 0{\cdot}98$). Open circles, after exposure for 10 min to 35 μM Dantrolene ($y = 0{\cdot}23, x - 0{\cdot}54$; $r^2 = 0{\cdot}99$). Triangles, after exposure for 60 min to 35 μM Dantrolene ($y = 0{\cdot}19, x - 1{\cdot}00$; $r^2 = 0{\cdot}97$). Fibre diameter 1·9 mm. Mean intracellular resting potential -62 mV. Temperature of the solutions 22° C.

DISCUSSION

Many cellular functions are affected by the processes which regulate Ca movements either at the outer membrane of the cell or at the membranes of the intracellular storage sites (see Carafoli *et al.* 1975). Most current studies of Ca movements are based on the kinetics of ^{45}Ca but their time resolution is limited and they cannot resolve rapid shifts of Ca^{2+} such as those involved in the phasic physiological regulation of secretion or contraction events. Moreover, the investigation of ^{45}Ca kinetics at the outer cell membrane provides no direct evidence about the actual changes in the cytosolic Ca^{2+} concentration. The intracellular aequorin method offers a tool for estimating both rapid and slow changes in cytosolic Ca^{2+} directly, and it considerably increases the scope of cell Ca^{2+} studies when used in conjunction with ^{45}Ca kinetics. The present results illustrate this approach in giant muscle fibres and identify a potentially useful chemical tool for Ca studies in other types of cells.

The micro-injection of known amounts of ^{45}Ca into a giant muscle fibre allows the direct study of the Ca efflux which was found to be reversibly reduced by exposure to Dantrolene (Fig. 1*B*). As is well known the electrochemical gradient of Ca^{2+} promotes its entry into the cell and the normal low level of cytosolic Ca^{2+} is maintained by at least two extrusion mechanisms (Baker, 1972; Ashley *et al.* 1974). When the Ca–Ca exchange diffusion component has been removed by placing the cell in 0 Ca, the remaining ^{45}Ca efflux involves a Na–Ca exchange which depends on the energy provided by a passive Na^+ influx down its own electrochemical gradient, and another ATP-dependent Ca^{2+} extrusion process (Ca pump) which still operates when Ca and Na are both removed from the bathing solution. Exposure to Dantrolene still depressed the ^{45}Ca efflux reversibly when the fibre was placed in 0 Ca (Fig. 3*A*) or in 0 Ca–0 Na (Fig. 3*B*) solutions. It is not easy to decide whether the effects were significantly different under either conditions. The observation of a sizeable Dantrolene effect after such experimental inactivation of the exchange processes offers a contrast, for example, with the effect of lanthanum which inhibits the fraction of ^{45}Ca efflux depending on the presence of external Na in squid axons (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Baker, 1972) and in barnacle muscle fibres (Ashley *et al.* 1974). That Dantrolene does not affect the Na–Ca exchange process has directly been shown on aequorin-injected fibres placed in 0 Na, under which conditions the Na^+ electrochemical gradient is inverted with respect to normal and the passive outflux of Na^+ drives Ca^{2+} into the cell and thus increases the resting glow; this reaction was not modified by Dantrolene (Fig. 4) whereas it is decreased by lanthanum (Ashley *et al.* 1974). This particular application of the aequorin

technique for tracing increases of the Ca^{2+} concentration which are localized to the peripheral cytoplasm directly underlying the plasma membrane has been discussed in the Results section.

The observed reduction of ^{45}Ca efflux by Dantrolene even in the absence of external Ca and Na (Fig. 3*B*) could be conceived as resulting from an inhibitory effect on the metabolically driven Ca pump (the third efflux component considered). On this hypothesis the fibre placed in normal external Na and Ca should present an increase of the concentration of cytosolic Ca^{2+} when exposed to Dantrolene, much in the same way as the blocking of the Na pump by ouabain is followed by an increase of the intracellular Na concentration (Skou, 1965). However, this possibility is excluded since Dantrolene rather elicited a definite *decrease* of the resting glow in aequorin-injected fibres (Fig. 2*B–D*). The reduced ^{45}Ca efflux recorded in Fig. 3*B* must therefore be related to another factor which we think is the decreased cytosolic concentration of Ca^{2+}, for which there is indeed direct evidence since aequorin-injected fibres placed in normal Na and Ca ASW presented a decrease in the resting glow when exposed to Dantrolene (Fig. 2). The Ca extrusion pump should indeed become less active when there is less free Ca^{2+} inside the cell and the Ca^{2+} electrochemical gradient across the plasma membrane is thus increased.

Two possibilities can be considered for the reduction by Dantrolene of the concentration of Ca^{2+} in the cytosol and they were tested separately: either the resting Ca influx down the electrochemical gradient is reduced or a larger fraction of the cell Ca^{2+} is sequestered into the intracellular reservoirs.

The first alternative has been directly excluded by the finding that the influx of ^{45}Ca per cm^2 of outer plasma membrane was not significantly changed during the first 3 min of exposure to Dantrolene, thus at a time when the resting glow of aequorin-injected fibres was already found to be clearly reduced by the drug (Table 1). The point whether more prolonged exposures to Dantrolene might eventually affect the resting influx of Ca^{2+} is left open in the present discussion. Furthermore, when the study of ^{45}Ca influx was repeated in single muscle fibres which were either subliminally depolarized by 60 mM-K^+ or strongly contracted by 200 mM-K^+ in the external medium, Dantrolene also failed to significantly modify the recorded Ca^{2+} influx (Table 1).

On the other hand, evidence for the second alternative has been obtained by recording the resting glow of aequorin-injected fibres studied in a 0 Ca medium containing 1 mM-EGTA to eliminate any Ca influx through the cell membrane. Under such conditions, the addition of Dantrolene still reduced the resting glow (Hainaut & Desmedt, 1974*b*) to an extent comparable to that shown in ASW in Fig. 2*B–D*. It can thus be concluded that

the recorded changes of ^{45}Ca efflux are primarily related to an effect of Dantrolene, not at the outer cell membrane, but at the intracellular Ca storage sites. The liposolubility of this compound should indeed allow its rapid penetration into the cell, and this is in line with the very short latency of the recorded effects.

Dantrolene is poorly soluble in water (cf. Putney & Bianchi, 1974). The concentration of 35 μM used in most of our experiments corresponds to the maximum hydrosolubility. We also tested higher concentrations of (unfiltered) solutions of Dantrolene on muscle fibres and found a still larger inhibitory effect on the resting glow. This suggests that the undissolved drug suspended in the external medium may cling to the fibre membrane and penetrate into the cell (Desmedt & Hainant, 1976*b*).

The liposolubility would also explain the somewhat slow dissipation of the effects after removal of Dantrolene from the external medium (Fig. 2*F*). The mode of action of Dantrolene is thus quite different from that of the Ca ionophore A23187 (Reed & Lardy, 1972) which potentiates the Ca movements both at the plasma membrane and at the membrane of intracellular storage sites (Hainaut & Desmedt, 1974*a*; Desmedt & Hainaut, 1976*a*).

Phasic increases of the cytosolic Ca^{2+} can be elicited by adequate activation of stimulus secretion or stimulus contraction coupling. In the case of muscle fibres, the depolarization of the plasma membrane is known to elicit the release of Ca from the SR (Schneider & Chandler, 1973; Bezanilla & Horowicz, 1975; Franzini-Armstrong, 1975; Endo & Thorens, 1975; Ebashi, 1976). The rapid increase in myoplasmic Ca^{2+} and its subsequent re-uptake by the SR can be analysed quantitatively from the phasic increase in light output or Ca transient in aequorin-loaded fibres (Ashley & Ridgway, 1970). We used depolarizing step functions not exceeding 40 mV and no regenerative action potentials were triggered (Hagiwara & Naka, 1964) which would have introduced a complicating factor. Moreover, the range of mechanical responses studied was such as to avoid too strong contractions which could distort the geometric relations of the probe electrode in the fibre during the experiment. Consistent and stable results were obtained and the use of quite a range of subthreshold intensities allowed to plot the relation between passive membrane depolarizations and the corresponding Ca transient (Fig. 6) (Desmedt & Hainaut, 1976*a*).

Dantrolene was found to markedly inhibit the Ca^{2+} transient and to decrease the mechanical force of the corresponding contraction (Fig. 5*A–B*). The actual depolarization produced by a given current delivered across the outer membrane was not changed. These effects appeared after a few seconds in Dantrolene and further increased at a slower rate over the next few minutes (Fig. 5 *C–D*), not unlike the pattern recorded for the reduction of resting glow in Fig. 2*F*. The reduction of the Ca^{2+} transient

no doubt accounts for the simultaneously recorded depression of the mechanical force output. More relevant to the present discussion is the question of the nature of the Dantrolene inhibitory effect on phasic Ca release. There is normally a linear relation between the subthreshold membrane depolarizations and the area of the corresponding Ca transient (Desmedt & Hainaut, 1976*a*). Dantrolene consistently reduced the slope of that relation but the depolarization threshold for just eliciting calcium release was not significantly changed by the drug. Therefore Dantrolene appears to achieve a proportional depression of the electrochemical coupling process of Ca^{2+} intracellular release without however affecting its threshold. The depression of release appears to be genuine and the observations could not be explained by assuming that the Ca release mechanism would be unaffected while the re-uptake of myoplasmic Ca^{2+} would be accelerated by the drug, since time to half-decay of the Ca^{2+} transient was not shortened (Fig. 5*B*, *F* and Table 2). This indicates that the processes of sequestration of cytosolic Ca^{2+} into the SR was not significantly affected by Dantrolene. If so, the recorded reduction of Ca^{2+} phasic liberation upon membrane depolarization must depend on a pharmacological inhibition involving either the electrochemical coupling process at the junction between fibre membrane and reticulum cisternae, or the Ca^{2+} release sites of the reticulum membrane directly. If the latter hypothesis was to be considered one could further ask whether the Ca^{2+} flow through each activated reticulum membrane site might be slowed down, or whether the number of such sites activated by a given depolarization might be reduced. The finding that the normalized rate of rise of the Ca^{2+} transient is not significantly depressed in barnacle single muscle fibres (Table 2) would be in line with the latter possibility postulating a reduced availability of Ca^{2+} releasing sites under Dantrolene. On the other hand experiments with K^+ contractures on single frog muscle fibres have indicated that Dantrolene does not reduce the maximum force elicited by large external K^+ concentrations, whereas it definitely elevates the threshold of the K^+ contracture and shifts to the right the relation between external K^+ concentration and submaximal contracture force (Hainaut & Desmedt, 1974*b*). The latter data suggest that, for strong depolarizations, Dantrolene does not reduce the amount of Ca^{2+} release from SR. Although the argument can obviously not be pressed, the over-all evidence seems to point to Dantrolene as a potent drug which inhibits electrochemical coupling for submaximal activations and reduces the proportion of Ca^{2+} releasing sites at the SR membrane which can be activated by depolarization.

In resting muscle cells, the reduction by Dantrolene of the Ca^{2+} concentration in the cytosolic compartment (Fig. 2) could be related to a shift in the balance between Ca movements respectively into and out of the

intracellular storage sites. The Ca reservoirs include both the sarcoplasmic reticulum and the mitochondria and the question arises whether Dantrolene affects both, or only one of them. This question is difficult to answer from data on resting intact cells. However, the results with graded depolarizations (Fig. 6) make it clear that Dantrolene inhibits the phasic Ca^{2+} release from SR. It is indeed well known that in muscle cells, the membrane depolarization activates Ca releasing sites at the SR membranes, thereby allowing a massive Ca^{2+} flow into the myoplasm (cf. Weber & Murray, 1973). On the other hand there is no evidence at present for any mechanism whereby phasic activating signals could be transmitted from the plasma membrane to the mitochondria so as to possibly trigger a rapid transient release of Ca^{2+} from the latter. The finding that, in activated fibres, Dantrolene does not interfere with the metabolically driven uptake of myoplasmic Ca^{2+} back into the reticulum (Fig. 5*F* and Table 2) but rather selectively inhibits the phasic Ca^{2+} release makes it likely that it acts at the same sites in the resting fibres, thereby presumably reducing a steady leakage of Ca^{2+} from the reticulum into the cytosol compartment. Such effect in resting cells together with the probable normality of the Ca^{2+} sequestration mechanism into SR would shift the steady state of intracellular Ca^{2+} movements and reduce the concentration of free Ca^{2+} in the myoplasm (Fig. 2) which in turn would reduce the activity of the Ca extrusion pump. A rather consistent model of Dantrolene action can thus be proposed on the basis of the present experimental results. The question whether, in addition, Dantrolene also influences Ca exchange equilibrium at mitochondrial membranes must remain open. Besides contributing to the study of Ca^{2+} kinetics in muscle fibres, the data draw attention to this new class of synthetic hydantoin derivatives of which Dantrolene is an example as potentially useful for the investigation, and perhaps for the long term control, of Ca-dependent functions in cells generally.

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