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COMMENTARY

DANTROLENE AND A23187 IONOPHORE: SPECIFIC ACTION ON CALCIUM CHANNELS REVEALED BY THE AEQUORIN METHOD

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Changes of cytosolic Ca^{2+} concentration control many cellular processes such as the exocytosis of packaged neutrotransmitters in axon terminals or of secretion products of a wide variety of gland cells [1–5]. In muscle cells the intracellular Ca^{2+} concentration being low in resting state, the calcium triggering mechanism must involve either an entry of Ca^{2+} from external medium, through the plasma membrane, or a release of Ca^{2+} from intracellular organelles, as indeed shown in striated muscle fibres [6, 7]. In these cells mechanisms of excitation–contraction (E–C) coupling are obscure, particularly the processes whereby depolarization of the plasma membrane and T-system result in the intracellular augmentation of free calcium ions in the cytosol [8–10]. The ^{45}Ca technique for tracing Ca^{2+} movements in muscle cells was shown to be adequate for the study of resting fluxes, but it appeared rather unsatisfactory for the analysis of phasic changes during contraction [11]. The discovery of the photoprotein aequorin [12], which reacts quickly with Ca^{2+}, opened a new approach for rapid microdetermination of Ca^{2+} concentration in biological systems [13].

One way to act specifically on any one of the processes regulating cytosolic Ca^{2+} is to search for chemical compounds which would selectively involve calcium movements in cells. The present paper reviews the mode of action of the calcium ionophore A23187 and of Dantrolene on Ca^{2+} movements in single barnacle muscle fibres [14]. Compound A23187 is a lipid-soluble carboxylic acid antibiotic isolated from *Streptomyces chartreusensis* which appear to be rather selective for divalent cations [15, 16]. In our work, it has proved to be a remarkable tool for the study of steady and phasic calcium fluxes in single muscle fibres [17]. Indeed, combining the estimation of changes in free myoplasmic calcium by the photoprotein aequorin and of ^{45}Ca efflux, we observed a dual action of A23187: at the outer membrane and at sarcoplasmic reticulum (SR) membranes respectively. Dantrolene sodium, 1-(5(*p*-nitrophenyl)-furfurilidene-amino) hydantoin sodium hydrate has muscle relaxant properties and depresses E–C coupling without interfering with either neuromuscular transmission or conduction of the muscle action potential [18–22].

The barnacle giant cell, micro-injected with ^{45}Ca or with the photoprotein [6], has been proved to be an adequate model for the analysis of steady and phasic Ca^{2+} movements in muscle fibres [9]. Indeed barnacle cells loaded with ^{45}Ca allow the estimation of calcium efflux whereas cells loaded with aequorin allow the intracellular micro-determination of the resting light emission (resting glow) as well as the increase of light emission elicited by membrane depolarization (calcium transient).

Many questions arise with respect to A23187- and Dantrolene-induced effects on calcium movements which may involve one or more of the following: (1) the resting Ca^{2+} influx and efflux; (2) the threshold of muscle membrane depolarization for eliciting Ca^{2+} release from the SR; (3) the kinetics of intracellular Ca^{2+}–troponin interaction. One of the conclusion of this review is that A23187 and Dantrolene act rather selectively by changing the electrochemical coupling in single barnacle giant muscle cells.

It is concluded from this review that the photoprotein aequorin is adequate for the study of chemical and pharmacological induced changes of cytosolic Ca^{2+} concentration in various cell types. In the barnacle muscle fibre, A23187 was found to intensify the Ca–Ca exchange efflux component at the outer membrane and to potentiate the electro-chemical coupling by a specific action on the Ca^{2+} channels at the SR membranes. Dantrolene was found to affect more selectively these channels by reducing the steady and phasic leakage of Ca^{2+} into the cell cytosol, without changing the Ca^{2+} fluxes at the plasma membrane. These drugs indeed appear to be fairly selective tools for the discussion, and perhaps for the long term control, of Ca^{2+} regulated cell functions generally.

Because the experimental method has not yet been frequently used in pharmacological research, some technical details are developed in the next section.

METHODS

Dissection and cannulation of the giant fibre. Specimens of the barnacle *Balanus nubilus* (DARWIN) shipped by air from Friday Harbor, Washington State, U.S.A., were kept in oxygenated artificial sea water (ASW) at 9–11°. For each experiment the lateral and rostral pairs of scutal depressor muscles were dissected according to Hoyle and Smyth [14], and single muscle fibres, measuring 2–4 cm in length and 1–2 mm in diameter, were isolated under a binocular microscope. The fibres were cannulated with a pyrex tube of 200 μm diameter [23] which permitted forces in the range 0.5–1.0 kg cm^{-2} to be recorded when the fibre was stimulated [6]. For the ^{45}Ca efflux experiments, a small glass

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weight was attached to the tendon end of the fibre. Injections of aequorin or ^{45}Ca were made with a microsyringe of 0.5 μl maximum capacity that ejected 0.08 μl/cm movement of the barrel. A glass capillary of about 100 μm tip diameter was attached to the needle and inserted into the fibre through the cannula.

Preparation of the aequorin injection solution and assay of light emission. Several thousand *Aequorea forskalea* were collected in the late summers of 1973 and 1975 at the Friday Harbor Laboratories. After extraction from the marginal tissue containing the photogenic organs, the crude aequorin precipitate was transported by air in a freeze safe with dry ice and it was purified in Brussels [12, 13, 29]. The saturated solution of purified aequorin used for the injection was prepared by mixing 1.0 mg of the lyophilized protein with 20 μl 10 mM-Na acetate, 1 μm-EDTA medium, 10 mM-TES, pH 7.0. Generally 0.1–0.2 μl of this aequorin solution was injected into the muscle fibre. The luminescent reaction of aequorin with the intracellular Ca^{2+} emits a blue light that can be detected with a photomultiplier tube and converted to lumen units. The size of the aequorin molecule is too large to cross the cell membrane, but small enough to diffuse through the sarcoplasm within about 1 hr ([24], Fig. 2). This protein did not apparently modify the behaviour of the preparation during the 2–6 hr of experiment. The cannulated aequorin-injected fibre was mounted vertically in a light tight black perspex chamber [6] in front of an EMI 9635 photomultiplier tube, the output of which was displayed on a Tektronix 565 oscilloscope and recorded with a Grass C4-K camera. The chamber was temperature-stabilized by constant circulation of water through its walls.

Dual axial probe-electrode. The glass capillary electrode used to stimulate the fibre and record simultaneously the membrane potential was of the type described by Hodgkin, Huxley and Katz [25] and modified by Ashley and Ridgway [6]. It was inserted longitudinally by way of the cannula so that its tip arrived at about 3 mm from the tendon end. A 50 μm diameter silver wire around the capillary served to deliver constant current pulses. The membrane potential was recorded with an intracellular 25 μm diameter platinum electrode (exposed area 0.5 mm, near the tip of the capillary) which was connected to a high input impedance differential amplifier [26]. A chlorided indifferent silver electrode of 1 mm diameter was placed in the external medium. Resting potentials were determined before and after each experiment by inserting a glass capillary electrode of about 50 μm tip diameter filled with 1 M KCl into the fibre through the cannula. The mean resting potential was measured over the entire length of the fibre, between this probe and a chlorided silver wire of 1 mm diameter in the extracellular medium. The values were not corrected for liquid junction potentials.

Force transducer. The isometric myogram was recorded on a Tektronix 565 oscilloscope with a horizontally mounted RCA 5734 mechano-electric transducer fed by a home-made zener-stabilized power supply with temperature compensation. The anode of this tube was connected to the tendon end of the fibre by a light gold chain and a lever (2:1) system. The weight of the chain was balanced by a counter weight.

Preparation of the isotope and assay of radioactivity. $CaCl_2$ containing $^{45}CaCl_2$ (U.K. Atomic Energy Authority, Amersham) had a specific activity of 1 mc/ml with 72 μg Ca/ml. and 20 μl. were removed and dissolved in 5 μl 50 mM Tris, pH 7.2. Usually 0.1–0.2 μl of this solution was injected into the muscle fibre. The Ca concentration injected did not exceed 2–4 mM, and this did not increase the total fibre Ca concentration by more than 0.05 mmole/kg wet wt. The efflux of ^{45}Ca was followed for a known period of time into a series of small tubes containing 1.5 ml artificial sea water (ASW) or modified ASW at 22° [27]. At the end of the experiment, the fibre was digested in 1.5 ml soluene for several hours and counted. The radioactivity in each tube was assayed by liquid scintillation. The efflux was expressed as a rate constant k: $k(\text{time}^{-1}) = (\text{Counts lost/collection time})/\text{mean counts in the fibre during collection time}$.

Solutions. Artificial sea water (ASW) was prepared as described by Fatt and Katz [28]. In the O Ca experiments, $CaCl_2$ was replaced by NaCl. In the O Ca–O Na experiments, LiCl was used as replacement salt. A23187 (Eli Lilly Inc.) was dissolved at 10^{-3} M in ethanol which was added to ASW to obtain a final concentration chosen from 10^{-7} to 10^{-5} M. The concentration of ethanol in the ASW was therefore 1 per cent or less. Dantrolene (Eaton Labs., Norwich, N.Y.) was directly dissolved in ASW to obtain a final concentration of 35 μM after filtration. Dantrolene depressed the Ca^{2+} aequorin reaction by 15 per cent and the percentage figures quoted were corrected accordingly. An upper correction (mean 5 per cent) was also made when counting the radioactivity by liquid scintillation in the presence of Dantrolene. Solutions were freshly prepared and adjusted to pH 7.3–7.5 before use; they did not modify the resting membrane potential by more than ± 5 mV. When necessary the solution bathing the fibre could be totally replaced in less than 5 sec by another at identical temeperature.

Effect of A23187 and Dantrolene on the resting cytosolic Ca^{2+} concentration

In the aequorin-injected barnacle muscle fibre the rate of light emission related to the intracellular ionized

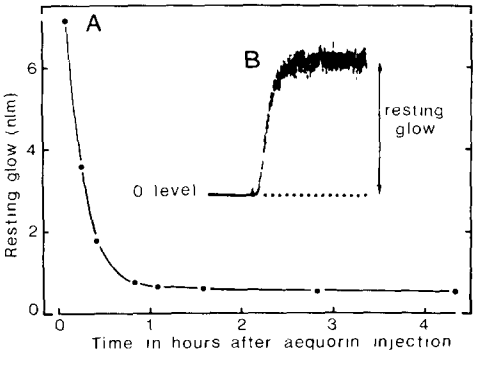

Fig. 1. Illustration of the resting light emitted by a giant Barnacle muscle fibre loaded with 0.1 μl aequorin. (A): rate of light emitted (resting glow) plotted against the time after aequorin injection. (B): sample of the resting glow level that can be recorded from the oscilloscope on a strip of film when the photo-multiplier is switched on. Fibre diameter 1.1 mm. Mean resting potential 58 mV. Temperature 22°.
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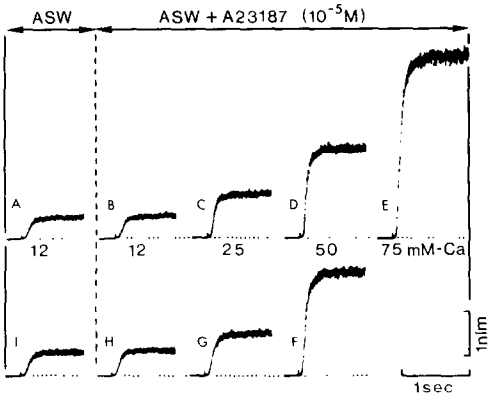

Fig. 2. Effect of A23187 (10^{-5} M) on the resting glow of an aequorin-injected fibre in the presence of different extracellular calcium concentrations. The results illustrated were obtained by switching on the high voltage of the photo-multiplier. Each sample was recorded within 15–45 sec after exposure to the following external medium without returns to artificial sea water (ASW) the sequence being A–I. Fibre diameter 1.0 mm. Mean resting potential: – 60 mV. Temperature of solutions 22°.
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calcium is high initially, and then decreases to become stable after about one hour. This resting light emission (resting glow) is illustrated by Fig. 1. Under these conditions, changes in the level of cytosolic Ca^{2+} concentration can be studied by recording the resting glow increase or decrease in response to changes in the extracellular medium composition [24].

When the cell is exposed for only a few minutes to A23187 whereby the ionophoretic effect appears largely restricted to the surface membrane, the Ca^{2+} influx is reversibly increased when the external calcium

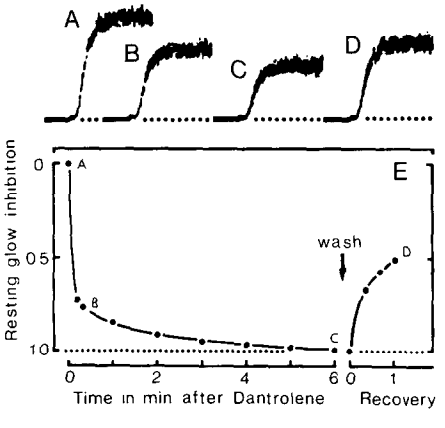

Fig. 3. A–D: effect of 35 μM Dantrolene on the resting glow of an aequorin-injected cell; these samples were recorded when the photo-multiplier was switched on after 20 sec (B) or 6 min (C) of exposure to Dantrolene. D: partial recovery of the resting glow after the fibre had been transferred back to artificial sea water (ASW) for 1 min. E: time course of the resting glow which reached 30 per cent of the control resting glow after 6 min exposure of this cell to Dantrolene. Fibre diameter 1.1 mm. Mean resting potential: – 45 mV. Temperature of solutions: 21°.
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ions concentration is augmented (Fig. 2, A–I). In control experiments in the absence of the ionophore, the resting glow is not increased by high external calcium. The A23187-induced entry of calcium into the cell occurs rapidly within seconds after the exposure to the ionophore and must involve that part of the myoplasm which underlies the outer membrane. The augmented rate of light emission illustrated by Fig. 2, A–E, is indeed only recorded provided the injected aequorin is given enough time to diffuse radially throughout the section of the muscle cell [29]. Another argument is that the resting glow increased by a factor of up to 9 in high external Ca^{2+} (Fig. 2, E) without there being any mechanical force produced by the muscle fibre. In similar experiments carried out in OCa–1mM-EGTA to exclude any Ca influx, the rate of light emission only increases slowly, with a delay of several minutes after exposure to the ionophore. Such an increased level of intracellular Ca^{2+}, recorded after about 20 min, can only be the result of an induced Ca^{2+} leakage from intracellular reservoirs. It was already known that A23187 can increase Ca^{2+} leakage from SR vesicles and mitochondria after they had been isolated by ultracentrifugation [15, 16]. Thus our finding shows that A23187 readily crosses the sarcolemma in an intact barnacle muscle cell to act on the membranes of intracellular organelles.

Figure 3 indicates that the resting glow decreases in two steps when aequorin-loaded fibres are exposed to 35 μM Dantrolene in the extracellular medium (Fig. 3, E). In this experiment, the maximum decrease of the resting glow after exposure to Dantrolene for 6 min is 30 per cent of control. When the fibre is subsequently transferred into ASW, a somewhat slow recovery is observed: about half of the phenomenon disappears after 1 min (Fig. 3, D–E) but the subsequent phase is very slow and the resting glow rarely recovers to the control level at the end of 1 hr. Similar results are obtained when the fibre is first equilibrated in OCa–1mM-EGTA medium (in order to eliminate any Ca^{2+} influx through the outer membrane) before Dantrolene is added to this saline [24]. Thus Dantrolene consistently reduces the level of free Ca^{2+} in the cells whether calcium is present or absent in the external medium. This reduction of the resting cytosolic Ca^{2+} concentration by Dantrolene can result either from an increased Ca^{2+} efflux or from a direct effect involving the intracellular Ca stores.

Ca^{2+} *fluxes in the presence of A23187 or Dantrolene*

Although the SR membranes and the myofibrils play the major role in determining the cytosolic Ca^{2+} concentration in muscle cells, there is also a contribution of the calcium fluxes through the plasma membrane. The electro-chemical gradient of Ca^{2+} is maintained by two distinct extrusion mechanisms: besides Ca–Ca exchange diffusion, there is a transport of calcium ions depending upon the downhill movement of sodium ions [29] and another Na-independent ATP-driven Ca extrusion; the latter mechanism is difficult to assess in muscle fibres, since ATP cannot be experimentally excluded because it is required to maintain the intracellular Ca stores in the SR membranes, and prevent leakage of Ca through SR membranes into the myoplasm. Could the above observation be related to some changes of the calcium fluxes at the outer membrane? A

dual experimental methodology using aequorin- and ^{45}Ca-loaded fibres, was combined to examine this question. It is well known that the efflux of ^{45}Ca from a micro-injected muscle fibre follows a rather complex pattern initially [11, 29], but becomes roughly exponential after 2–3 hr, the rate constant declining only slowly with time from then on. The latter phase is used as base line for estimating the resting calcium efflux in the presence or in the absence of A23187 or Dantrolene in the extracellular medium.

The ^{45}Ca efflux experiments require rather long exposures to the ionophore, so that both the intracellular organelles and the outer membrane are involved in the A23187 effects. On the other hand, with prolonged actions of A23187, the effects are also found to be less reversible upon return to normal ASW. For a muscle cell loaded with ^{45}Ca in normal ASW, the addition of A23187 (10^{-5} M) for 30–50 min increases the ^{45}Ca efflux to as much as 350 per cent of the resting efflux curve in normal ASW. At this time, the resting glow is about double its control value before A23187. This large calcium efflux and the increased resting glow resulting from prolonged exposure to A23187 (cf. previous section) suggest that the cytosolic Ca^{2+} concentration is augmented through ionophore potentiation of both calcium influx and calcium leakage from SR. It is interesting that, in high external calcium, the resting glow could be further augmented (Fig. 2), but not the ^{45}Ca efflux, probably because of saturation of the extrusion process. Further evidence showing that A23187 directly potentiates resting leakage of Ca^{2+} from intracellular organelles, is provided by the markedly increased (up to 200 per cent) calcium efflux from muscle cells loaded with ^{45}Ca and placed in O Ca saline. In this experiment such ^{45}Ca efflux into O Ca solution can only be related to an augmented cytosolic Ca^{2+} concentration resulting from intracellular Ca^{2+} release [30].

When Li is substituted for both Na and Ca in the external medium, the Ca–Ca and Na–Ca exchange processes are blocked, but A23187 still increases the ^{45}Ca efflux to about 180 per cent. Thus A23187 does not affect to a significant extent the Na–Ca exchange, as further shown by experiments on aequorin loaded cells, when Li is substituted for external Na while keeping a normal extracellular Ca concentration. The Na electrochemical gradient being then reversed, Ca entry via the Na–Ca exchange is promoted, as indeed shown by a very rapid and marked increase in the resting glow. This light response to O Na is not at all modified in the presence of A23187. These data emphasize that the ionophore does not promote Na movements or affect the Na–Ca pump. They document the rather selective ionophoretic action for divalent cations, as suggested on the basis of other evidence by Reed and Lardy [15].

Dantrolene reversibly reduces the ^{45}Ca efflux. After eliminating the Ca–Ca exhange diffusion component by placing the cell in O Ca, the exposure to Dantrolene still depresses the efflux reversibly. Similar observations are made when the fibre is placed in O Ca–O Na solution which eliminates in addition the contribution of the Na–Ca exchange. The observation of a sizable Dantrolene effect after inactivation of the Na–Ca exchange offers a contrast with the effect of Lanthanum which selectively inhibits the fraction of ^{45}Ca efflux depending on the presence of external Na in squid axons [31, 32] and in barnacle muscle fibres [29]. Other evidence that Dantrolene does not affect the Na–Ca exchange is found in aequorin-loaded fibres placed in O Na, thus with an inversed Na gradient. Under these conditions the passive efflux of Na ions drives Ca^{2+} into the cell and increases the resting glow. This response in O Na is not modified by Dantrolene [22] whereas it is decreased by lanthanum [29]. This is a new application of the aequorin technique for tracing changes in cytosolic Ca^{2+} concentration which are localized to the peripheral cytoplasm directly underlying the plasma membrane (cf. Fig. 4).

The question arises whether Dantrolene might also block the ATP-driven calcium pump at the plasma membrane. If this is the case, Dantrolene should increase the cytosolic Ca^{2+} concentration of the fibre in normal ASW, much in the same way as blocking the ATP-dependent Na pump by ouabain [33] is followed by an increase in the intracellular Na concentration. This possibility can be excluded since Dantrolene rather elicits a definite *decrease* of the resting glow in normal external Na and Ca. The reduction of ^{45}Ca

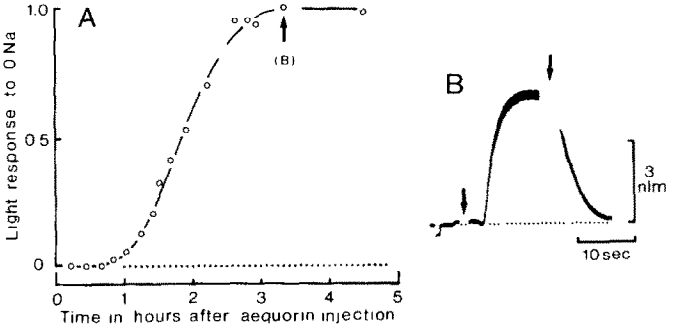

Fig. 4. Illustration of the light response to sudden exposure to a ONa medium. A, progressive increase of the response as the aequorin diffused more completely through the myoplasm and reached the outer cell membrane. B, sample of the maximal light response recorded after complete diffusion of the aequorin. The first arrow indicates the moment when the normal artificial sea water (ASW) was replaced by ONa medium; and the second arrow indicates when artificial sea water was restored in the external medium. The initial resting glow of the fibre is indicated by the dotted line. Fibre diameter 1.8 mm. Mean resting potential: − 49 mV. Temperature of solutions: 22°.

efflux by Dantrolene in O Ca–O Na ASW must therefore be related to the decreased cytosolic Ca^{2+} concentration observed from aequorin experiments and illustrated by Fig. 3. The Ca extrusion pump should indeed become less efficient when there is less free calcium in the cell and the Ca^{2+} electrochemical gradient across the plasma membrane is thus decreased. The next problem is to inquire whether the Dantrolene-induced reduction of the cytosolic Ca^{2+} concentration is related (1) to a reduced resting calcium influx down the electro-chemical gradient, or (2) to augmented sequestration of cell Ca^{2+} into the intracellular stores. The first alternative has been excluded by the finding that ^{45}Ca influx per cm^2 of plasma membrane was not changed during the first 3 min of exposure to Dantrolene, thus at a time when the resting glow was reduced by the drug (Fig. 3). Furthermore Dantrolene also failed to modify the recorded ^{45}Ca influx in cells depolarized by 60–200 mM-K. It is concluded that Dantrolene acts primarily at the internal calcium stores. Dantrolene is indeed poorly soluble in water [20], but its liposolubility would explain the quick penetration into the cell and also the slow dissipation of the effects after removal of Dantrolene from the external medium (Fig. 3) [34].

Thus A23187 and Dantrolene penetrate the cell and exert a direct effect on the resting Ca^{2+} leakage from the intracellular reservoirs into the cytosol.

Specific Actions of A23187 and Dantrolene on phasic intracellular Ca^{2+} release

Stimulus–secretion and stimulus–contraction coupling involve transient increases of the cytosolic Ca^{2+}. In striated muscle cells, the depolarization of the plasma membrane elicits the release of Ca from SR membranes [10, 35–38]. The kinetics of rapid increase in cytosolic Ca^{2+} and in the subsequent re-uptake by SR are directly demonstrated by the phasic increase in light output in aequorin-loaded fibres [6, 17, 21]. Figure 5 illustrates the possibility to tell apart the electro-chemical step of E–C coupling from the mechano-chemical events when an aequorin-loaded barnacle cell is electrically stimulated. This method was found adequate to analyse the following processes involved in E–C coupling: (1) the electro-chemical threshold; (2) the kinetics of Ca^{2+} release from SR; (3) the process of sequestration of myoplasmic Ca^{2+} back into SR [22, 30].

This section provides evidence that A23187 and Dantrolene strongly influence the Ca^{2+} transients elicited by standard membrane depolarization. Consistent results were obtained for a rather extensive range of subthreshold depolarizations of 75–150 msec duration thus allowing us to plot the relations between membrane potentials, calcium transients and mechanical force output [30]. In the presence of A23187, the force production by the single giant fibre was markedly and reversibly increased, as well as the area and the peak amplitude of the transient light output (Ca^{2+} transient) of the aequorin-injected cell. Ashley and Ridgway [6]

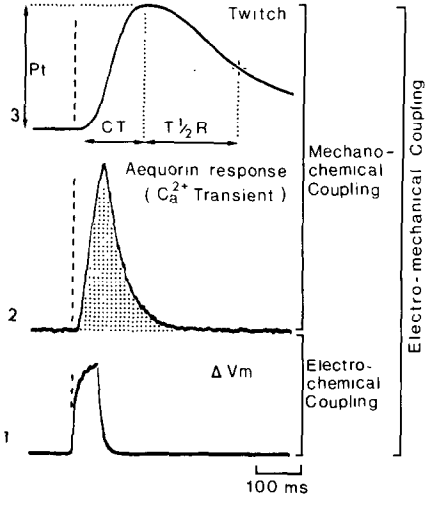

Fig. 5. Illustration of the use of aequorin method to analyze the excitation–contraction (E–C) coupling in single giant barnacle muscle cells. Trace 1, membrane depolarization, trace 2, Ca^{2+} transient; trace 3, isometric peak force (Pt).

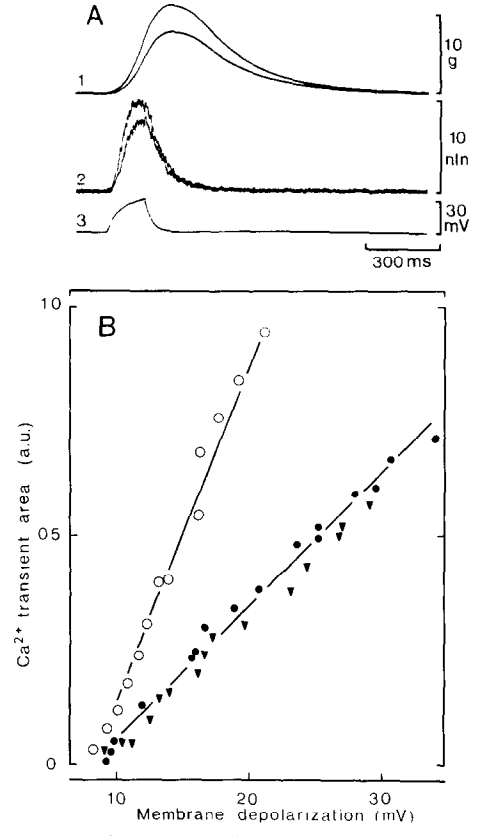

Fig. 6. Result of applying a single electrical stimulation of 150 msec duration to a barnacle muscle fibre injected with aequorin. Two traces are superimposed in each record: one before the application of A23187 (10^{-5} M) serving as control and the other after the ionophore had been present for 10 min. Trace 1, isometric tension; trace 2, calcium mediated light emission; trace 3, membrane response. Fibre diameter: 1.9 mm; mean resting potential: −54 mV; temperature of solutions: 23°. Reproduced with the courtesy of Nature. B, Electrochemical relation recorded for different depolarizations (mV abscissa). Ordinate, area of the Ca^{2+} transient in arbitrary units: (●), in artificial sea water (ASW); (○), after 7–11 min exposure to A23187 (10^{-5} M) in ASW; (▼), after return to ASW for 15 min. The pulse duration was 75 msec. Fibre diameter: 1.8 mm; mean resting potential: −61 mV; temperature of solutions: 22°.
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reported that larger depolarizations elicited Ca^{2+} transients with a steeper rise and an earlier onset (their Fig. 7). The potentiation by A23187 is of a different kind since, for identical depolarizations of the plasma membrane, A23187 accelerates the rate of the Ca^{2+} transient without there being any change in its time onset (Fig. 6A). Low concentrations of A23187 (10^{-6} M) do not modify the rate of fall of the Ca^{2+} transient (Fig. 6A), which suggests a lack of effect on the sequestration of the released Ca^{2+}. This indeed points to a rather selective action of A23187 only involving the dynamic release process from the SR membrane. As a matter of fact, the resting glow of the muscle fibre was not changed when the above mentioned observations were made. Larger concentrations of A23187 (10^{-5} M) induce larger (and still reversible) effects which however are associated with a prolongation of the Ca^{2+} reuptake.

Excitation–concentration (E–C) coupling processes were analysed by plotting the peak mechanical tension of the aequorin-injected fibre and the area of the Ca^{2+} transient (cf. Fig. 5) for graded depolarizations, with and without ionophore in the external medium. The electro-mechanical threshold is reached for about 10 mV depolarization and it is not significantly affected by A23187 which however considerably increases the slope of the function ([30], Fig. 7).

The threshold depolarization for eliciting a Ca^{2+} transient is about 7.5 mV, thus somewhat smaller (as indeed expected) than the threshold for the force production. The Ca^{2+} transient area varies linearly with the depolarization ($r_{xy} = 0.99$). A23187 markedly and reversibly increases the slope of this linear relation without modifying the electro-chemical threshold (Fig. 6B). The latter observation is again in agreement with the lack of change in resting glow under these conditions. The data on Ca^{2+} transient are in line with the view that A23187 potentiates the calcium channels at the SR membranes thereby increasing the release of myoplasmic Ca^{2+} for a standard plasma membrane depolarization. Additional observations have shown that A23187 regulates intracellular Ca^{2+} release but does not interfere with Ca^{2+}–troponin interaction ([30], Fig. 9).

On the other hand, Dantrolene not only reduces the steady leakage of Ca^{2+} from intracellular stores (previous section) but it markedly inhibits the Ca^{2+} transient and decreases the mechanical force of the contraction (Fig. 7 A,B). The actual depolarization produced by a given electrical current delivered through the intracellular electrode across the plasma membrane is not changed by Dantrolene. A quick inhibitory effect of Dantrolene appears after a few seconds and further increases at a slow rate over the next few minutes, not unlike the pattern recorded for the reduction of resting glow Fig. 3.

The reduction of Ca^{2+} transient by Dantrolene no doubt accounts for the simultaneously recorded depression of the mechanical force of the muscle fibre but the nature of the Dantrolene inhibition of phasic Ca^{2+} movements remains to be considered. As a matter of fact, the linear relation between the subthreshold membrane depolarization and the area of the corresponding Ca^{2+} transient presents a reduced slope in the presence of Dantrolene, while the threshold for just eliciting Ca^{2+} release is not changed (Fig. 7C). The possibility that Dantrolene might facilitate the pumping process whereby myoplasmic Ca^{2+} is moved back into SR is excluded by the finding that the time to half-decay of the Ca^{2+} transient is not shortened in the presence of Dantrolene [39]. Since the drug does not affect the sequestration of cytosolic Ca^{2+} back into the SR, it is proposed that Dantrolene reduces the availability of calcium channels for the release of Ca^{2+} during activation of the muscle fibre. The observation that the normalized rate of rise of the Ca^{2+} transient is not significantly depressed would be in line with the reduced availability of Ca^{2+} releasing channels at SR membranes in the presence of Dantrolene. In experiments on single frog muscle fibres Hainaut and Desmedt [21] observed 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. These data suggest that, for strong depolarizations, Dantrolene does not reduce the amount of Ca^+ release below the quantity required for maximal activation on

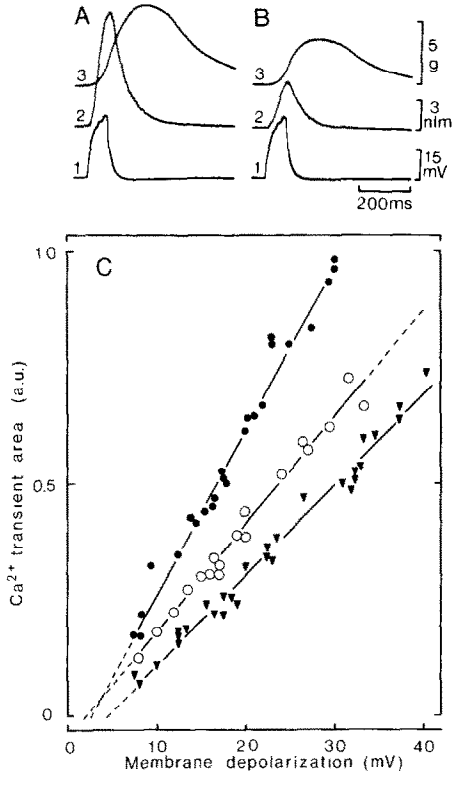

Fig. 7. A, B: effect of applying a single electrical stimulation of 75 msec duration before (A) and after (B) 20 min exposure to Dantrolene (35 μM) in the external medium. Trace 1, membrane depolarization; trace 2, Ca^{2+} transient; trace 3, isometric peak force. Fibre diameter: 1,6 mm; mean resting potential: − 56 mV; temperature of solutions 22°. C: relation between cell membrane depolarization produced by graded subliminal currents and the area of the corresponding Ca^{2+} transient (arbitrary units): (●), control in artificial sea water (ASW); (○), after exposure for 10 min to 35 μM Dantrolene; (▲), after exposure for 60 min to 35 μM Dantrolene. Fibre diameter: 1.9 mm; mean resting potential: − 62 mV; temperature of solutions: 22°.

the contractile proteins. The overall evidence clearly documents the view that Dantrolene is a potent drug which inhibits the electro-chemical coupling for sub-maximal membrane depolarizations and which reduces the number of Ca^{2+} releasing sites at the SR membranes activated by a given depolarization.

When now considering resting barnacle fibres in the absence of applied membrane depolarization, the reduction by Dantrolene of the cytosolic Ca^{2+} concentration could be related to a shift in the steady-state equilibrium between calcium movements respectively into and out of the intracellular reservoirs. The calcium sequestering organelles include both the sarcoplasmic reticulum and the mitochondria. The interesting question arises whether Dantrolene might affect either, both or only one of these intracellular compartments. Although this question is rather difficult to settle on the basis of observations made on intact fibres, the results obtained with graded depolarizations make it clear that Dantrolene inhibits the phasic Ca^{2+} release from SR membranes. Whereas it is well known in striated muscle fibres that the membrane depolarization triggers calcium releasing sites at the SR membranes [10], there is no evidence at present that this phasic activating signal could also be transmitted to the mitochondria so as possibly to trigger a rapid release of Ca^{2+} from these organelles. The finding that in activated intact cells, Dantrolene does not interfere with the metabolically driven uptake of cytosolic Ca^{2+} into the SR [39], but selectively inhibits the phasic Ca^{2+} release, makes it likely that the drug acts at the same sites in resting cells. Our proposal is therefore that Dantrolene reduces the (small) resting leakage of Ca^{2+} from SR into myoplasm without inhibiting the ATP-linked process of uptake of myoplasmic Ca^{2+} back into SR. Such an effect would indeed shift the steady state equilibrium of intracellular Ca^{2+} movements and thus reduce the concentration of free calcium ions in the cytosol. A secondary effect of the lower concentration of myoplasmic Ca^{2+} would be to reduce the activity of the calcium extrusion pump of the muscle fibre plasma membrane. A rather consistent model of Dantrolene-induced changes in internal Ca^{2+} concentration can thus be suggested.

CONCLUDING REMARKS

New developments in biochemical pharmacology can be related to the use of new methods and to the introduction of new drugs with fairly selective modes of action, whereby molecular mechanisms of drug action can be better understood. As shown by recent work on the sodium channel of nerve and muscle membranes [40–46] both the use of tetrodotoxin and the recent recording of gating current have indeed initiated considerable progress both in pharmacology and in molecular physiology of the action potential mechanisms. The present paper reviews the potentialities of the photoprotein aequorin to detect rapid changes in cytosolic Ca^{2+} concentration and to disclose the mechanism of action of A23187 and Dantrolene on calcium movements.

Aequorin is a photoprotein of molecular weight 31.000 [47] (20.000 according to Blinks, Prendergast and Allen [48]) which can be extracted from the photogenic organs of the luminescent jellyfish *Aequorea forskalea* [12], that can be found in great abundance during the summer along the Pacific coast of North America. The photoprotein extracted from the marginal ring of the animal can be purified according to several biochemical procedures [13, 24, 48]. When a saturated solution of aequorin is micro-injected into the cytosol, the free calcium ions react with the protein to emit a blue light, of an intensity related to the Ca^{2+} concentration and which can be measured by photomultiplier techniques. Aequorin provides unique possibilities to estimate the free calcium levels in different cells, i.e. crustacean giant muscle fibres [6]; squid axons [49]; gland cells [50]; urchin eggs [51].

The sensitivity and the time resolution of the aequorin-Ca^{2+} reaction has been proved to be adequate for the micro-determination of physiological Ca^{2+} concentrations [13, 52], as well as for the dynamic analysis of the Na–Ca efflux component at the plasma membrane of nerve and muscle cells [28, 49]. More recently, it was shown that the aequorin method was of practical importance to detect induced changes of the cell resting cytosolic Ca^{2+} concentration (estimated from the resting glow) as well as the processes whereby the membrane depolarization triggers transient Ca^{2+} release from endoplasmic reticulum [53]. The aequorin method supplements and considerably extends the ^{45}Ca technique of study of Ca^{2+}-controlled cell functions because of the possibility to: (1) directly estimate the intracellular Ca^{2+} concentration; (2) visualize the Ca^{2+} influx and the Na–Ca exchange channel; (3) analyze the phasic intracellular Ca^{2+} release during excitation–reaction coupling in the cell.

The present review of the mechanisms of action of A23187 ionophore and Dantrolene put forward the rather selective effects of these new drugs on calcium movements. A23187 was found to increase the Ca–Ca exchange component of the plasma membrane calcium fluxes without affecting the Na–Ca component. During phasic activities of the cell, the ionophore considerably potentiates the electro-chemical coupling by a rather specific action on the calcium channels at the endoplasmic reticulum membranes. Dantrolene acts specifically on these calcium channels by reducing the steady and phasic leakage of Ca^{2+} into the cytosol, without changing the calcium fluxes at the plasma membrane. These compounds indeed appear to be fairly selective for the study and perhaps for a long term control of Ca^{2+}-regulated cell function generally.

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