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IN VITRO BINDING OF DANTROLENE TO SARCOPLASMIC RETICULUM OF RABBIT SKELETAL MUSCLE

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Abstract—Dantrolene upon binding to microsomes containing sarcoplasmic reticulum of rabbit thigh muscle exhibits a fluorescence with emission at 490 nm, which shows a blue shift of 35 nm compared with its fluorescence in ethylacetate. Using fluorescence techniques, dantrolene binding to microsomes isolated from rabbit thigh muscle was investigated. From Scatchard plots of binding studies, the association constant (K_{ass}) and the number of binding sites of dantrolene to sarcoplasmic reticulum were calculated, which was found to be $9.6 \times 10^4\ M^{-1}$ and 1.71 μmole/g of membrane proteins, respectively. In the presence of verapamil (1.25×10^{-4} M), another calcium antagonist, the binding of dantrolene to microsomes was enhanced. However, at a high concentration of verapamil (3.75×10^{-4} M), the Scatchard plot of dantrolene binding was found to be biphasic.

It has been shown that studing the interaction of a variety of fluorescent probes with membranes of various cell organelles can provide valuable data concerning the states of membrane and the membrane bound enzymes [1, 2]. Dantrolene sodium, a skeletal muscle relaxant [3] exhibits fluorescence in hydrophobic media (a mixture of nitropropane and heptane), with an excitation of 395 nm and a fluorescence of 530 nm [4]. It also has been proposed that the action of dentrolene on skeletal muscle is mediated by decreasing the availability of calcium channels of cell membranes, including the sarcoplasmic reticulum, for the release of Ca^{2+} during activation of the muscle fibres [5, 6]. Using a potassium depolarized rabbit jejunum, it has been shown [7] that dantrolene inhibits the calcium induced contractions of jejunum indicating that its effect on smooth muscle is also mediated via inhibition of calcium movement across the membrane.

In the present work we have investigated the binding of dantrolene with one of its possible sites of action, the sarcoplasmic reticulum, and the effect of another calcium antagonist, verapamil, upon this binding.

MATERIALS AND METHODS

Chemicals. The stock solution of dantrolene sodium (Dantrium®), 10^{-3} M was prepared in an alcohol–water (50–50) mixture and that of verapamil (Isoptin®) in Tris (0.05 M) buffer, pH 7.4. All other reagents were of reagent grade or higher.

Microsomes containing sarcoplasmic reticulum. Albino rabbits of either sex weighing 1–2 kg were used. The animals were killed by a blow on the head. Microsomes were prepared according to the method described by Martonosi [8], by homogenizing rabbit thigh muscle with 4 volumes of 5 mM Tris–0.1 M KCl buffer, pH 7.4. The myofibrils were removed by centrifugation at 1000 *g* for 20 min. The supernatant was then centrifuged at 8000 *g* for 20 min. This step was repeated once more. The microsomal fraction was obtained by centrifugation of the supernatant at 28000 *g* for 60 min. The microsomes were then resuspended in the Tris–KCl (pH 7.4) buffer to attain desirable protein concentration.

Fluorescence probe techniques. Dantrolene sodium from stock solution was added to the buffer solution (5 mM Tris, pH 7.4) in a quantity to obtain a concentration of 0.6 μg/ml. Next dantrolene was extracted into ethylacetate from buffer solution (5 mM Tris, pH 7.4). The excitation and emission spectra of dantrolene (0.6 μg/ml) in aqueous solution and in ethylacetate was recorded at room temperature using an Aminco-Bowman spectrofluorometer.

Dantrolene sodium, in a final concentration of 2.5×10^{-5} M, was added to microsomal membranes in the Tris–KCl (pH 7.4) buffer, (microsomal protein, 1.0 mg/ml). Setting the excitation wave length at 390 nm, the emission spectra of the mixture, microsomal preparation alone, and dantrolene alone in buffer were recorded.

Binding studies. The binding of dantrolene to microsomes containing sarcoplasmic reticulum was studied according to the method described by Bashford *et al.* [1]. Rabbit sarcoplasmic reticulum membrane (0.1–1.75 mg of protein/ml) were titrated with 5×10^{-6} M dantrolene and the 'limiting fluorescence' at infinite membrane concentration was estimated from a double-reciprocal plot. Next dantrolene (1.25–25 μM) was titrated at constant membrane concentration (1 mg of protein/ml), and the number of binding sites (n) and association constant (K_{ass}) were determined by Scatchard analysis [1. 9]. Alternatively, the number of binding sites and association constant of dantrolene binding to sarcoplasmic reticulum were determined in the presence of verapamil ($1.25 \times 10^{-4} - 3.75 \times 10^{-4}$ M).

Protein determinations were carried out according to biuret method [10] using bovine serum albumin as standard and correction was made using a blank containing only Tris buffer.

RESULTS

Microsomal preparation. Microsomal protein yield was found to be 3.6 mg/g of rabbit thigh muscle. While, there may be a possibility of contamination with other non-sarcoplasmic reticulum components, Martonosi [8] has shown that this preparation has a high adenosinetriphosphatase activity indicating a high content of sarcoplasmic reticulum membranes.

Fluorescence characteristics of dantrolene. While dantrolene does not exhibit any fluoresence in the aqueous solutions, in the hydrophobic media, e.g. ethylacetate, it exhibits a strong fluorecence with excitation wavelength of 390 nm and emission wavelength of 525 nm (Fig. 1). The fluorescence intensity versus dantrolene concentration is linear and is not affected by addition of verapamil at concentrations up to 6×10^{-4} M. When dantrolene 2.5×10^{-5} M was added to the microsomal suspension (1 mg of protein/ml), an emission spectrum similar to that of dantrolene in ethylacetate was observed. But in this case the emission wavelength was 490 nm showing a shift of peak to the right (Fig. 2).

Binding studies. The binding of a probe, in this case dantrolene, to a membrane system can be characterised by the analysis of two different titrations [1]. Titration of the membrane at a constant concentration of dentrolene can be used to determine the 'limiting fluorescence' at infinite membrane concentration (when all dantrolene will be bound) by a double reciprocal analysis (Fig. 3). The 'limiting fluorescence' is characteristic for each probe in a particular membrane system and can be used to calculate the fraction of the probe bound at every point of a titration curve of the same probe at constant membrane concentration [1]. Then analysis by Scatchard plot [9] will give the number of binding sites and the association constant for the probe–membrane interaction (Fig. 4). Figures 3 and 4 and Table 1 show the results of such an experiment for the interaction of dantrolene with microsomes containing sarcoplasmic reticulum.

The effect of verapamil upon dantrolene binding is shown in Table 1 and Fig. 4. Verapamil at concentration of 1.25×10^{-4} M increases the number of dantrolene binding sites without significant change in affinity of dantrolene towards its binding sites. However at a higher concentration (3.75×10^{-4} M), the effect of verapamil is more complicated. The Scatchard plot of dantrolene became biphasic and the parameters calculated from the second linear

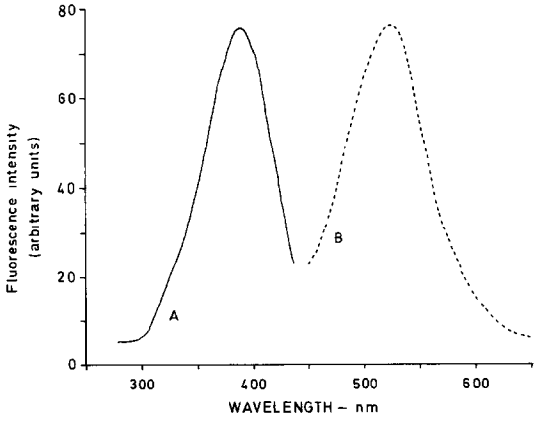

Fig. 1. Fluorescence characteristics of dantrolene in ethylacetate. Dantrolene concentration is 0.6 μg/ml. (A) Solid line, excitation spectrum with emission wavelength fixed at 525 nm; and (B) broken line, emission spectrum with excitation wavelength fixed at 390 nm.

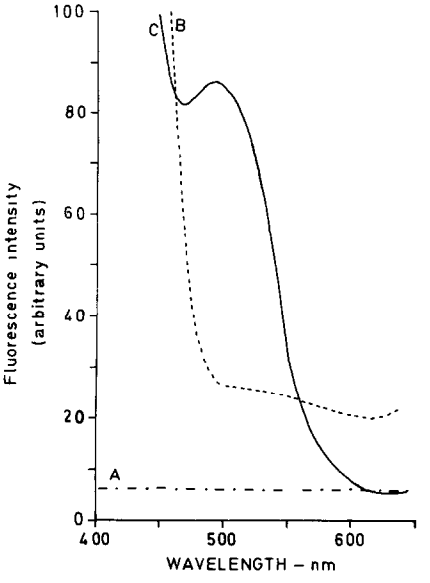

Fig. 2. Emission spectrum of dantrolene upon binding to sarcoplasmic reticulum membranes. Excitation wavelength was fixed at 390 nm. (A) Dotted line, dantrolene (2.5×10^{-5} M) in buffer; (B) broken line, microsomes containing sarcoplasmic reticulum alone (1 mg/ml); and (C) solid line, microsomes (1 mg/ml) plus dantrolene (2.5×10^{-5} M).

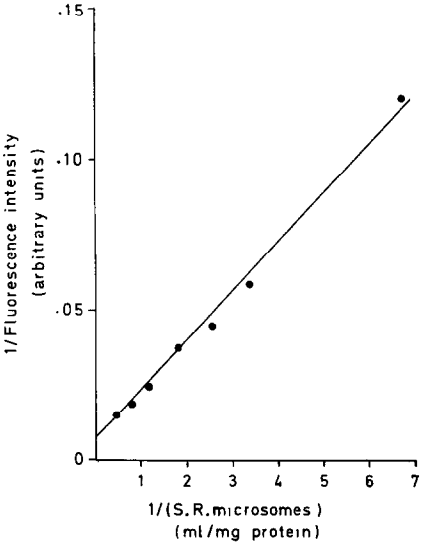

Fig. 3. Double-reciprocal plot of dantrolene binding to sarcoplasmic reticulum membranes. Fluorescence intensity of 5×10^{-6} M of dantrolene was measured in the presence of different amounts of microsomal proteins. Excitation was at 390 nm and emission was measured at 490 nm. The experiment was carried out at room temperature. Each point is the mean of four determinations.

Table 1. Parameters of dantrolene binding to sarcoplasmic reticulum *in vitro* in the absence or presence of verapamil

Verapamil concentration (M)	K_{ass} ± S.D. ($\times 10^4$ M^{-1})	Number of binding sites ± S.D. (μmoles/g protein)
Control	9.6 ± 0.57	1.71 ± 0.058
1.25×10^{-4}	10.5 ± 1.24*	2.49 ± 0.143†
3.75×10^{-4}	22.7 ± 1.39†‡	2.13 ± 0.037†‡

Parameters were calculated using the data of Fig. 4. Altogether four sets of observations were made.

K_{ass}, association constant.

* No statistically significant difference with that of control.

† Statistically significant difference with that of control ($P < 0.001$).

‡ Parameters were calculated using only the linear part of curve 3 in Fig. 4.

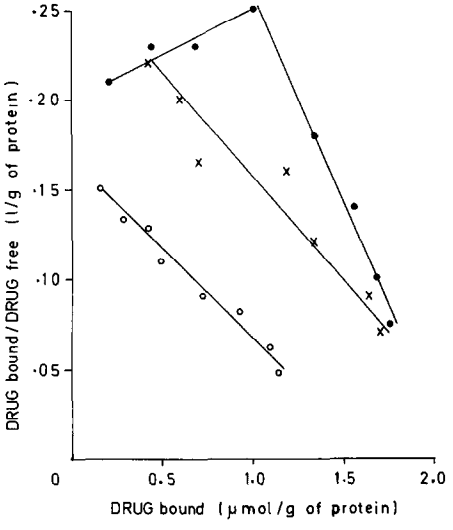

Fig. 4. Scatchard plots of dantrolene binding to sarcoplasmic reticulum membranes. The concentration of microsomes was 1 mg protein/ml. Fluorescence was observed as indicated in Fig. 3. The fraction of dantrolene bound per g of protein was calculated from the fluorescence enhancement. The plots are that of dantrolene, (○——○), and dantrolene in the presence of verapamil at 1.25×10^{-4} M (×——×), or 3.75×10^{-4} M (●——●). Each point is the mean of four determinations.

phase, show that the number of dantrolene binding sites is slightly reduced compared with lower verapamil concentration (1.25×10^{-4}), but the affinity of dantrolene towards its binding sites increases (Table 1).

DISCUSSION

These data suggest that dantrolene sodium is bound to the membrane of sarcoplasmic reticulum of rabbit thigh muscle. This finding is consistent with pharmacological observations that dantrolene acts by inhibition of calcium movement across the cell membranes [5, 6]. Dantrolene emission spectrum shows a blue shift when it has been dissolved in a more polar solvent; the dantrolene emission wave length in ethylacetate is 525 nm compared to 530 nm reported when a heptane–nitropropane mixture is used as the solvent [4]. Since the emission wavelength of dantrolene upon binding to the membrane is found to be 490 nm, one may conclude that it binds to relatively polar constituents of the membrane.

The number of dantrolene binding sites (1.71 μmole/g of membrane protein) is many orders of magnitude lower than what has been reported for non-specific membrane probes such as *N*-phenyl-1-napthylamine [1, 2]. This may indicate a higher degree of selectivity of dantrolene towards sarcoplasmic reticulum membranes. But, it also has a lower affinity towards membranes compared to *N*-phenyl-1-naphthylamine [1, 2]. Its dissociation constant ($1/K_{ass}$) is 1.04×10^{-4} M which is higher than its ED_{50} in skeletal muscle, 7.4×10^{-7} M [1], but is comparable to its ED_{50} in smooth muscle 7.9×10^{-5} M [7]. It has been proposed [5] that dantrolene may also act by interfering with the voltage-dependent charge movement in the T-system. Therefore either dantrolene has a higher affinity towards T-tubes or fluorescence techniques show only the binding of dantrolene to some other receptors, i.e. 'silent receptors'.

The overall effect of verapamil upon dantrolene binding was that of enhancement (Table 1, Fig. 4). It should be mentioned that in spite of dantrolene, verapamil as a calcium antagonist is more potent toward the smooth muscle [7]. Verapamil effect on skeletal muscle is complicated, it causes $^{45}Ca^{2+}$ release and contracture as well as depression of the twitch of field stimulated muscle [12]. It also has been reported [13] that verapamil can cause shape changes in erythrocytes in concentrations of $1 \times 10^{-4} - 4 \times 10^{-4}$ M. Therefore, it is not clear whether verapamil's effect on dantrolene binding to sarcoplasmic membranes is due to its specific effect on dantrolene binding to sarcoplasmic membranes or a non-specific change in the membrane structure.

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