Br. J. Pharmacol. (1994), 112, 1216–1222 © Macmillan Press Ltd, 1994

Effects of bipyridylium compounds on calcium release from triadic vesicles isolated from rabbit skeletal muscle

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1 The effects of 1,1'-diheptyl-4,4'-bipyridinium dibromide (DHBP), a viologen for electrochromic memory display agent, on calcium release and ryanodine binding were studied with triad-rich sarcoplasmic reticulum (SR) vesicles isolated from rabbit skeletal muscle.

2 DHBP inhibited the calcium release induced by 2 mM caffeine and $2\,\mu g\,ml^{-1}$ polylysine with an IC_{50} value of $5\,\mu g\,ml^{-1}$ and $4\,\mu g\,ml^{-1}$ respectively.

3 DHBP inhibited [^{3}H]-ryanodine binding in a dose-dependent manner with an IC_{50} of $2.5\,\mu g\,ml^{-1}$ and 90–100% inhibition at $20-30\,\mu g\,ml^{-1}$.

4 Calcium uptake by SR was inhibited in the presence of caffeine and this inhibition was antagonized by concomitant addition of DHBP.

5 The effect of DHBP on muscle twitches was studied on the mouse diaphragm. Muscle twitches elicited by direct electrical muscle stimulation and contractions induced by either 10 mM caffeine or 1 μM ryanodine were blocked by pretreatment with DHBP.

6 Data from this study provided evidence that DHBP blocked the calcium release from SR by direct interaction with the calcium release channel, also known as the ryanodine receptor. A possible use of this agent as a specific inhibitor for calcium release and as a muscle relaxant was suggested.

Keywords: Skeletal muscle; SR; calcium release; bipyridylium

Introduction

Muscle contraction is initiated by membrane depolarization which, by an unknown mechanism, triggers calcium release from the sarcoplasmic reticulum (SR) into the myoplasma and causes the muscle to contract. How transient changes in the T-tubule membrane potential lead to Ca^{2+} release from SR is one of the most important unresolved questions in muscle physiology (for a recent review, see McPherson & Campbell, 1993). SR is the major internal calcium storage site of the muscle cell, the uptake and release of the calcium ion from and into the myoplasma are controlled by two molecules located on SR membrane, namely, Ca^{2+}-ATPase and the calcium release channel (also known as the ryanodine receptor) respectively. Several chemicals have been shown to affect the calcium release process by direct interaction with the calcium release channel (Palade *et al.*, 1989) and defects of the calcium release channel may be associated with some clinical diseases, such as malignant hyperthermia (Mickelson *et al.*, 1988).

Pyridine compounds, such as dihydropyridines and 4-aminopyridine, have been shown to affect the physiological muscle contraction by acting on L-type calcium channels located on the transverse tubule (Romey *et al.*, 1988) and potassium channels on the transverse tubule or Ca^{2+}-ATPase on the SR (Ishida & Honda, 1993), respectively. Nagura *et al.* (1988) have shown that 4,4'-dithiodipyridine binds to certain SH groups of the Ca^{2+}-induced Ca^{2+} release channels in the SR membrane and thus induces Ca^{2+} release. Recently, we have shown that 2,2'2"-tripyridine, a byproduct found in paraquat manufacturing, produced a curare-like action in the mouse phrenic nerve-diaphragm preparation (Lin-Shiau *et al.*, 1992) and blocked the nicotinic acetylcholine receptor channels in embryonic *Xenopus* muscle cells (Hsu *et al.*, 1993).

In this study, we have investigated the effect of several bipyridylium compounds (also known as viologens) widely used as oxidation-reduction indicators in biological systems or as nonselective contact herbicides, on calcium release and muscle contraction. We have found that 1,1'-diheptyl-4,4'-bipyridinium dibromide (DHBP) but not other bipyridylium compounds tested, can effectively block the calcium release from SR vesicles isolated from mammalian skeletal muscle and inhibit the ryanodine binding to its receptor on the SR membrane by a direct interaction with the calcium release channel. Part of this work has been published in an abstract form (Kang *et al.*, 1993).

Methods

Preparation of heavy SR fraction

The triad enriched heavy fraction of SR (HSR) was prepared from rabbit leg and back muscles by differential centrifugation as described by Ikemoto *et al.* (1984) with modifications. Briefly, the muscles were homogenized in three times their volume of ice cold MOPS 20 mM, EDTA 0.1 mM, EGTA 0.1 mM and PMSF 0.2 mM, pH 7.0 buffer and the homogenates were centrifuged at 10,000 *g* for 5 min in a JA-14 rotor (Beckman). The supernatant fraction was filtered through eight layers of cheese cloth and then centrifuged at 17,000 *g* for 50 min. The sedimented fraction was homogenized in a solution containing sucrose 0.3 M, KCl 150 mM, PMSF 0.2 mM and MOPS 20 mM (pH 6.8), and centrifuged at 17,000 *g* for 40 min in a JA-20 rotor (Beckman). The sedimentable fraction was homogenized in the above solution at a final protein concentration of $20-30\,mg\,ml^{-1}$. The preparation was quickly frozen in liquid nitrogen and stored at − 70°C.

Mouse phrenic diaphragm preparation

Mice (ICR strain) of either sex, weighing 20–25 g, were used. The diaphragm preparation was isolated according to the method of Bülbring (1946). A modified Krebs solution of the following composition (mM) was used: NaCl 130.6, KCl 4.8, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1, pH 7.4, with $CaCl_2$ 2.5 or with EGTA 2.5. The diaphragm prepara-

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tion was placed in a 10 ml organ bath that was constantly gassed with 95% O_2 + 5% CO_2 at 37.0 ± 0.5°C. Contractions of the diaphragm were elicited either by direct stimulation of the muscle with a pulse of 0.5 ms at 0.2 Hz and or by the addition of 10 mM caffeine, 1 μM ryanodine (drug-induced) or 50 mM KCl (depolarization-induced) with no electrical stimulation. The muscle was loaded with a resting tension of 0.5 g, and the changes of tension were recorded via an isometric transducer (Grass FT.03) on a Grass Model 7 polygraph (Grass Instrument Co., Quincy, MA, U.S.A.).

Mitochondria preparation and calcium uptake measurement

Mitochondria were prepared from rat liver according to the method of Cain & Skilleter (1985). The calcium uptake was measured with a dual wavelength spectrophotometer using antipyrylazo III as the calcium probe in a medium containing KCl 10 mM, sucrose 0.25 M, Tris-HCl 10 mM, pH 7.1 and 1 mg ml^{-1} of freshly prepared mitochondria. The reaction was initiated by using succinate (5 mM) as substrate of oxidative phosphorylation reaction and $CaCl_2$ (30 μM) was added after 5 min incubation to measure the calcium uptake by mitochondria. The calcium ionophore, A23187 (2 μM; Sigma), was added at the end of reaction to calculate the total calcium release.

Endoplasmic reticulum preparation

Rat liver endoplasmic reticulum (ER) microsomes were prepared by the method of Alvares & Mannering (1970). Briefly, rat liver was homogenized with four times its volume of 1.15% KCl and centrifuged at 9,000 *g*, 4°C for 20 min and the supernatant was then further centrifuged at 100,000 *g*, 4°C for 1 h. The final pellet (the microsomes) after washing once with 1.15% KCl was resuspended in 0.1 M potassium phosphate buffer, pH 7.4 and stored at − 70°C.

Synaptic plasma membrane (brain plasma membrane)

Synaptic plasma membranes were prepared by the modified method of Hakim *et al.* (1982). About 1.5 g of brain from 8 rats was washed and homogenized in 10 times its volume of sucrose 0.32 M, Tris-HCl 10 mM, pH 7.4 (homogenization buffer) with a motor driven pestle. The homogenate was then centrifuged at 3,000 *g*, 4°C for 20 min and then the supernatant was centrifuged at 40,000 *g*, 4°C for 30 min to obtain a crude microsome pellet. After resuspending in Tris-HCl 10 mM, pH 7.4 (resuspension buffer), the microsome was layered on a discontinuous sucrose gradient consisting of equal volumes of 10%, 28.5% and 34% sucrose and centrifuged with the SW41 rotor (Beckman) at 100,000 *g*, 4°C for 1 h. The purified synaptic plasma membrane which appeared at the interface of 28.5% and 34% gradient layers was removed and centrifuged at 100,000 *g*, 4°C for 1 h after dilution with the resuspension buffer and the pellet resuspended with the homogenization buffer and stored at − 70°C.

Ca^{2+} release assay of SR vesicles

The time course of Ca^{2+} release from SR vesicles was investigated with a calcium sensitive probe, antipyrylazo III, in a dual wavelength spectrophotometer (Model DW-2000, Aminco) with no addition of precipitating agent according to the method modified from Palade (1987a). SR vesicles (0.5 mg ml^{-1}) were actively loaded by adding ATP 0.5 mM into a reaction mixture containing KCl 150 mM, $MgCl_2$ 0.5 mM, antipyrylazo III 100 μM, MOPS 20 mM (pH 6.8); more Ca^{2+} was then added until the SR vesicles were saturated. A release inducer, such as polylysine (2 μg ml^{-1}, mol. wt. = 3,800 Da; Sigma) or caffeine 2 mM (Sigma), was added to induce Ca^{2+} release from SR. For inhibition studies, DHBP (Aldrich) at concentration indicated was added prior to the addition of the calcium release inducer.

[^{3}H]-ryanodine binding measurement

Ryanodine binding was measured according to the method described by Pessah *et al.* (1986). In order to mimic the calcium release conditions, triadic membranes (0.5 mg ml^{-1}) were incubated at 37°C for 2 h in a medium containing KCl 250 mM, NaCl 15 mM, [^{3}H]-ryanodine 10 nM (NEM, 87 Ci $mmol^{-1}$), Tris 20 mM, pH 7.1 with the test compounds at the concentration indicated in each experiment. Non-specific binding was measured in the presence of 1 μM cold ryanodine (Calbiochem). At the end of the incubation, 100 μl of each reaction mixture was withdrawn and added to 5 ml of ice cold buffer to quench the reaction and rapidly filtered through the Whatman GF/B glass filter and rinsed once with 5 ml ice cold buffer. A value of 2 pmol mg^{-1} SR protein for [^{3}H]-ryanodine binding to SR was obtained for our SR vesicles. The data shown are the average of triplicate determinants of at least two different preparations.

Cation-dependent ATPase activity

ATPase activity was determined with a coupled-enzyme spectrophotometric ADP-release assay (Warren *et al.*, 1974) by measuring the oxidation of NADH at 340 nm in a medium containing MOPS 20 mM, pH 6.8, NADH 0.3 mg ml^{-1}, $MgCl_2$ 5 mM, EGTA 0.2 mM, PEP 0.45 mM, pyruvate kinase 5 units ml^{-1} and lactate dehydrogenase 10 units ml^{-1} (the assay mixture). Mg^{2+}-ATPase activity was measured by incubating 5–10 μg of protein in a 1 ml assay mixture at 37°C for 5 min, ATP (1 mM) was then added to start the reaction. Ca^{2+}-ATPase was measured with the addition of $CaCl_2$ (0.2 mM) and calcium ionophore, A23187 (2 μM). Na^+,K^+-ATPase was measured in the presence of NaCl 100 mM and KCl 20 mM with ouabain 1 μM used as the inhibitory control. To unmask the latent Na^+,K^+-ATPase activity, the reaction was carried out in the presence of saponin 0.2 mg ml^{-1} (or 0.003% SDS).

Lipid peroxidation measurements

Thiobarbituric acid (TBA) test described by Begin & Ells (1992) was used for lipid peroxidation determination. Microsomes (ER, 10 μg; SR, 200 μg) were incubated with NADPH 6.25 mM in 250 μl 0.1 M K-phosphate buffer, pH 7.4 at 37°C with or without the test compounds. After the reaction was stopped by addition of 1 ml of 15% TCA at the time indicated and centrifuged at 15,000 *g* for 15 min, 1 ml of supernatant was withdrawn and reacted with 1 ml of 0.67% 2-thiobarbituric acid-0.25 N HCl at 85°C for 10 min. The reactant was cooled and degassed before absorbance measurement at 532 nm.

Protein determination

Protein was determined by the method of Lowry *et al.* (1953) with bovine serum albumin used as standard.

Results

Effects of DHBP on calcium release from SR vesicles

Figure 1 shows a typical experiment using a metallochromic calcium indicator dye, antipyrylazo III, to monitor the calcium uptake and release by isolated membrane vesicles, e.g. triad rich heavy sarcoplasmic reticulum (HSR) and mitochondria in this study. As shown in Figure 1a, calcium in the reaction medium was rapidly and actively translocated into the SR upon addition of Mg^{2+} ATP by the Ca^{2+}-ATPase located on the SR membrane, as indicated by the decrease of

optical absorbance difference at 710 nm and 790 nm; aliquots of 10 nmol $CaCl_2$ were added sequentially to the reaction medium until a near saturated level of calcium was loaded (usually 80–100 nmol Ca^{2+} mg^{-1} protein) and further addition of $CaCl_2$ would have initiated a Ca^{2+}-induce Ca^{2+} release. Addition of 2 $\mu g\ ml^{-1}$ polylysine (trace 1, Figure 1a), a calcium release inducer (Cifuentes *et al.*, 1989), after loading induced a rapid release of 80% of the loaded calcium and total release was obtained upon addition of the calcium ionophore, A23187 (2 μM). This polylysine-induced calcium release from HSR was blocked by prior addition of 2 μM ruthenium red (trace 6, Figure 1a).

DHBP blocked the calcium release induced by 2 $\mu g\ ml^{-1}$ polylysine in a dose-dependent manner (traces 2–5, Figure 1a) with an IC_{50} of 5 $\mu g\ ml^{-1}$. This inhibition was not due to the interaction of DHBP with antipyrylazo III or calcium ion chelation by DHBP since the optical signal of total released calcium was not affected when the calcium ionophore was added. Unlike ruthenium red, which can also block calcium uptake by mitochondria (trace 1, Figure 1b), DHBP (50 $\mu g\ ml^{-1}$) did not affect the calcium uptake by mitochondria through the uniporter (trace 3, Figure 1b).

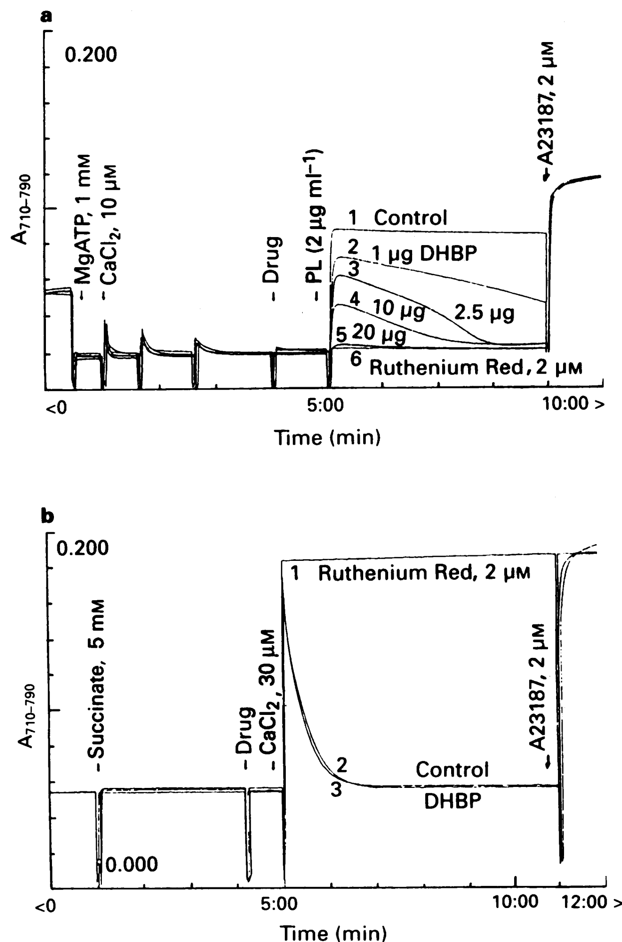

Figure 1 Effects of DHBP on calcium release of SR and uptake of mitochondria. In (a) SR vesicles (0.5 $mg\ ml^{-1}$) were loaded with calcium as described in the text and the calcium concentration was monitored by the absorbance difference at 710 nm and 790 nm according to the procedure outlined in Methods. Trace 1, calcium release induced by polylysine (PL) without any addition of drug; traces 2–5, calcium release induced by polylysine with prior addition of DHBP at the concentration indicated; trace 6, calcium release with prior addition of 2 μM ruthenium red. In (b) mitochondria (1 $mg\ ml^{-1}$) were incubated for 5 min with succinate (5 mM) to initiate the respiratory chain reaction. Ruthenium red (trace 1) and DHBP (50 $\mu g\ ml^{-1}$) (trace 3) was then added prior to addition of $CaCl_2$ (30 μM). Trace 2 was the control with no drug addition.

DHBP can also block calcium release induced by 2 mM caffeine (Figure 2) and 5 μM silver ion (data not shown) in a dose-dependent manner with IC_{50} of 4 $\mu g\ ml^{-1}$ and 6 $\mu g\ ml^{-1}$, respectively.

Effect of DHBP on ryanodine binding

Since both polylysine (Cifuentes *et al.*, 1989) and caffeine (Palade, 1987a) induced calcium release from SR through a direct interaction with the ryanodine receptor and this induced calcium release was blocked by DHBP, suggesting that DHBP might exert its inhibitory effect by direct interaction of ryanodine receptor resulting in a blockade of the release channel. We then tested the DHBP effect on ryanodine binding of HSR. DHBP inhibited the ryanodine binding in a dose-dependent manner as shown in Figure 2. An IC_{50} of 2.5 $\mu g\ ml^{-1}$ DHBP was observed with 90–100% inhibition of ryanodine binding at 20–30 $\mu g\ ml^{-1}$.

Effects of other dipyridyl analogues on calcium release and ryanodine binding

The effects of other dipyridyl analogues, including diethyl bipyridinium (DEBP), dibenzyl bipyridinium (DBBP), dimethyl bipyridinium (paraquat), 4,4'-dipyridine and 2,2'-dipyridine, on calcium release and ryanodine binding were tested. No significant effects on either calcium release or ryanodine binding were observed with all compounds tested at concentrations up to 100 $\mu g\ ml^{-1}$, suggesting that DHBP effects were specific.

Effects of DHBP on calcium uptake and ATPase activity

The effects of DHBP on active calcium uptake and Ca^{2+}-ATPase of SR were also tested. The amounts of calcium sequestered by SR vesicles without and with 10 $\mu g\ ml^{-1}$ DHBP measured by the use of $^{45}Ca^{2+}$ as described in Methods were 70 ± 5 nmol mg^{-1} and 72 ± 5 nmol ng^{-1} respectively. The uptake capacity was greatly reduced to 25 ± 2 nmol mg^{-1} in the presence of 2 mM caffeine with no added DHBP due to the activation of the calcium release channel on the SR by caffeine. DHBP at 10 $\mu g\ ml^{-1}$ can effectively antagonize the effect of caffeine and restore the

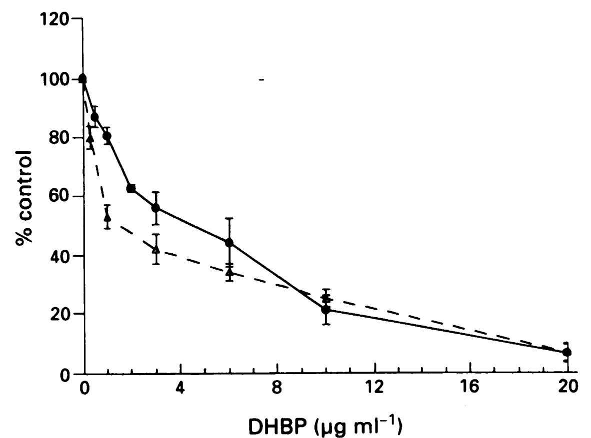

Figure 2 Dose-response of DHBP on SR calcium release and [^{3}H]-ryanodine binding: (●) total calcium release of SR induced by 2 mM caffeine with addition of DHBP at concentration indicated. Data are presented as percentage of control with at least three duplicates of three different preparations. IC_{50} value of 4 $\mu g\ ml^{-1}$ was determined by fitting $y = b + a\log(x)$. (Δ) DHBP effect on the specific binding of 10 nM [^{3}H]-ryanodine (2 pmol mg^{-1}) according to the procedure outlined in Methods. An IC_{50} of 2.5 $\mu g\ ml^{-1}$ DHBP was observed with 90–100% inhibition of ryanodine binding at 20–30 $\mu g\ ml^{-1}$.

calcium uptake capacity of the SR to 72 ± 2 nmol mg^{-1} when added at the same time as caffeine.

When ATPase activity of the SR was measured by a coupling assay, DHBP at concentrations up to 50 μg ml^{-1} neither inhibited nor stimulated Ca^{2+}-ATPase activity (data not shown), but at higher concentrations (> 100 μg ml^{-1}), a slight activation (~ 10%) was observed, suggesting that the effect of DHBP on calcium uptake at low concentration was not due to the stimulation of Ca^{2+}-ATPase.

No effects of DHBP on ATPase activity from other sources, such as Na^+,K^+-ATPase of synaptic plasma membrane and Mg^{2+}-ATPase of mitochondria, was observed at 100 μg ml^{-1} DHBP (data not shown).

Lipid peroxidation of SR of skeletal muscle and of liver microsomes

Recently, Dinis *et al.* (1993) have reported that lipid peroxidation of the SR membrane can affect the Ca^{2+} pump activity through alteration of physical properties in the lipid phase or lipid-protein interface. By using thiobarbituric acid-reactive substances (TBARs) production as an indicator (Begin & Ells, 1992), the effect of DHBP on the level of lipid peroxidation of SR membrane and liver microsome was examined. Unlike paraquat and CCl_4, both of which enhance the level of lipid peroxidation of liver microsomes, DHBP at concentrations up to 100 μg ml^{-1} did not enhance or produce lipid peroxidation of liver microsomes. All the compounds tested did not produce lipid peroxidation of the SR membrane suggesting that the effect of DHBP seen on the calcium channel was not due to alteration of lipids in the SR membrane.

Effect of DHBP on muscle contraction of mouse diaphragm

The effects of DHBP on muscle contraction were tested on the mouse diaphragm preparation as described in Methods. As shown in Figure 3a, biphasic contraction (a phasic contraction followed by a slowly developing tonic contraction) was induced upon addition of 10 mM caffeine in the presence of 2.5 mM calcium. The tonic contraction was greatly reduced (> 90%) leaving a small and slow relaxing tonic contraction upon removal of the external calcium (Figure 3b) while the phasic contraction was only partially reduced (~ 25%) suggesting that the tonic contraction was induced by the influx of extracellular calcium and the phasic contraction was induced mainly by the calcium released internally from the SR. The remaining small tonic contraction might be the consequence of incomplete removal of the external calcium such as in the lumen of the T-tubule since this tonic contraction can be prevented by using a higher concentration of EGTA but this also accelerated the inactivation of the muscle. Pretreatment with DHBP 0.1 mg ml^{-1} inhibited the phasic contraction induced by caffeine with little effect on the tonic contraction (Figure 3c). Muscle contraction induced by ryanodine 1 μM in the presence of 2.5 mM calcium (Figure 4a, upper trace) was also blocked by pretreatment with DHBP 0.1 mg ml^{-1} (Figure 4a, lower trace).

Dose-dependence of DHBP inhibition of caffeine- and potassium-induced muscle contraction are summarized in Figure 5, IC_{50} values being 54 μg ml^{-1} and 1 mg ml^{-1}, respectively. DHBP blocked the potassium-induced contraction at a much higher concentration, with only 20% inhibition at 0.25 mg ml^{-1}, suggesting a higher sensitivity of DHBP to calcium-induced (caffeine) or drug-induced (ryanodine) calcium release than to depolarization-induced (high potassium) calcium release; this was supported by the fact that a high concentration of DHBP (2 mg ml^{-1}) was needed to block completely the twitches induced by electrical stimulation (Figure 4b). Addition of a high dose of ryanodine after the twitch had been completely blocked (Figure 4b) was unable to induce contraction suggesting a complete blockade of the calcium release channel. This twitch inhibition at a high concentration of DHBP was not due to the inhibitory effect on contractile proteins since permeabilization of the sar-

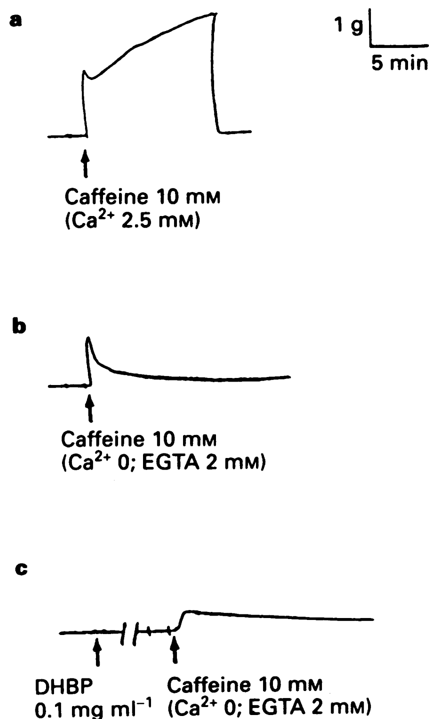

Figure 3 Effects of DHBP on muscle contraction of mouse diaphragm induced by caffeine. Mouse diaphragm was prepared as described in Methods and equilibrated in calcium 2.5 mM (a) or calcium-free (b) medium for 30 min before addition of caffeine. In (c) DHBP (0.1 mg ml^{-1}) was added prior to addition of caffeine.

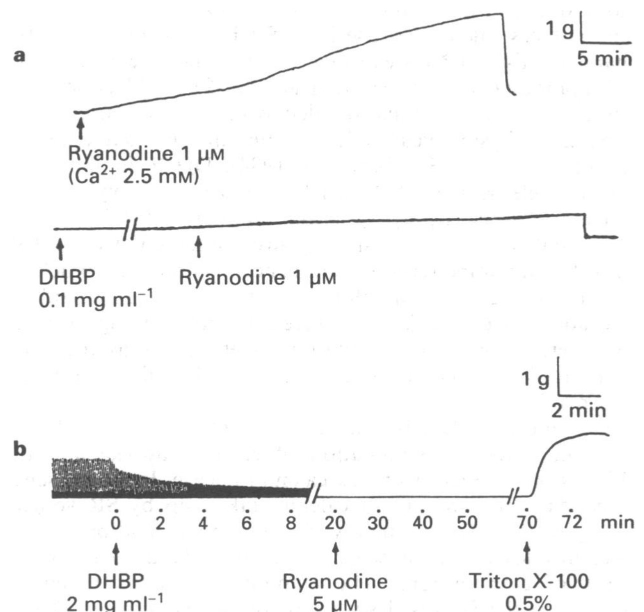

Figure 4 Effects of DHBP on muscle contraction of mouse diaphragm induced by ryanodine or direct electrical stimulation. Mouse diaphragm was prepared as described in Methods and equilibrated in Krebs solution containing 2.5 nM calcium for at least 30 min. In (a) ryanodine (1 μM) was added without (upper trace) or with (lower trace) preincubation with DHBP (0.1 mg ml^{-1}). In (b) DHBP, at a high concentration of 2 mg ml^{-1} was added after the twitch became stable and ryanodine (5 μM) and 0.5% Triton X-100 were added after the twitch had been completely blocked.

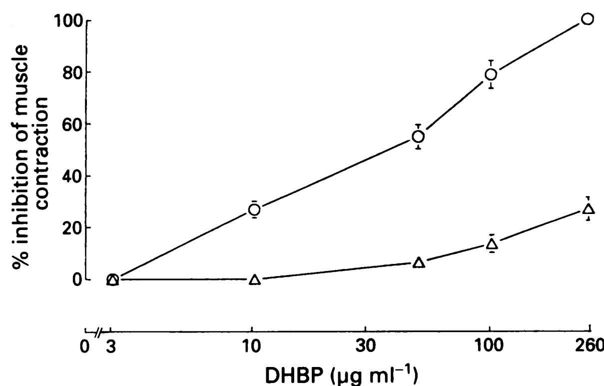

Figure 5 Dose-response to DHBP of muscle contraction induced by caffeine or high potassium. DHBP at concentrations indicated was added and incubated with muscle in calcium-free medium for 15 min before challenge with 10 mM caffeine (O) or 50 mM KCl (Δ). Data are presented as percentage inhibition relative to control. Data are presented as means ± s.e.mean ($n \geq 6$).

colemma by 0.5% Triton X-100 still caused the muscle to contract in response to the influx of external calcium.

Discussion

In this study, we have presented evidence showing that DHBP can inhibit the calcium release from skeletal muscle induced by caffeine, polylysine, ryanodine and low concentrations of silver ion. These calcium release inducers have been shown to activate specifically the calcium release channel, also known as the ryanodine receptor, of the SR. For example, polylysine at low concentrations has been shown to induce calcium release from SR by a direct interaction with the ryanodine receptor (Cifuentes *et al.*, 1989; Kang *et al.*, 1992), while at high concentrations, polylysine actually blocked the calcium release (Palade, 1987b). Caffeine, not only induces calcium release from SR but also modulates the channel activity (Rousseau *et al.*, 1988) and alters the K_d of [^{3}H]-ryanodine binding (Pessah *et al.*, 1987). Silver ions, at low concentrations, induced calcium release by acting at the apparent physiological release site in the sarcoplasmic reticulum (Salama & Abramson, 1984). DHBP inhibited the calcium release from SR vesicles induced by polylysine or caffeine as well as [^{3}H]-ryanodine binding to SR in a dose-dependent manner, suggesting a direct interaction of DHBP with the ryanodine receptor and hence resulting in a blockage of the channel and an inhibitory effect on ligand binding. The addition of blocker concurrently with the triggering of Ca^{2+} release producing inhibition as effectively as the addition of blocker before triggering, would indicate a direct action of DHBP.

The effect of DHBP on calcium uptake correlated well with the extent of stimulation of calcium uptake and the abilities of the agent to block calcium release. In the presence of caffeine, the amount of calcium taken up by SR vesicles was greatly reduced, mainly due to the activation of the calcium release channel which resulted in the decrease of net influx of calcium ions. The amount of calcium taken up actively by the SR vesicles was greatly increased when DHBP was added at the same time as caffeine and stimulation of transport by DBHP was due to closure of the channel activated by caffeine. This conclusion is consistent with the finding that DHBP does not alter Ca^{2+}-ATPase activity.

Recently, Dinis *et al.* (1993) have reported that lipid peroxidation of the SR membrane can affect the Ca^{2+} pump activity through alteration of physical properties in the lipid phase of lipid-protein interface. Nonselective contact herbicides, such as paraquat and related bipyridylium compounds, produce toxicity through oxygen-derived radicals formed by the reaction of oxygen with the bipyridylium radical cations (DeGray *et al.*, 1991) and cause lipid peroxidation (Steffen & Netter, 1979; Ruiz-Gutierrez *et al.*, 1991) or release of iron from ferritin (Thomas & Aust, 1986). By using TBARs as the indicator of lipid peroxidation, we have found that DHBP did not induce lipid peroxidation of SR membrane or liver microsomes, suggesting that the effect of DHBP on SR calcium transport is not due to the alteration of physical properties of the SR membrane. It is interesting to note that although CCl_4 induced an increase of lipid peroxidation in liver microsomes it had no effect on SR, suggesting that SR might not contain metabolic enzymes, such as cytochrome P450, and this could explain why none of the bipyridylium compounds, including paraquat, affected the Ca^{2+}-ATPase activity of SR.

Caffeine contraction is biphasic in calcium-containing bathing solution and several sites of action have been suggested (Endo, 1977; Røed, 1991). We showed that, in a calcium-free buffer, caffeine induced a small phasic contraction of mouse diaphragm through direct action of caffeine at the terminal cisternae of the SR (Kumbarachi & Nastuk, 1982) resulting in calcium release from the SR. Pretreatment with DHBP can effectively block this caffeine-induced contraction suggesting that DHBP can enter the muscle cell and interact with the calcium release channel located on the terminal cisternae of SR resulting in a blockade of calcium release. The IC_{50} value of DHBP for blockade of caffeine-induced contraction of the diaphragm is higher than that for blockade of calcium release from SR vesicles. It is possible that this apparent discrepancy is due to the differences in the experimental conditions between the *in situ* (e.g. the use of EGTA) and *in vivo* studies or it could reflect the different sensitivity of preparations to DHBP or protein and lipid concentration. Although easy diffusion of drug into the muscle strip of the diaphragm makes it the preparation of choice in most pharmacological studies, a large amount of drug was always needed compared to the dose tested with isolated membrane vesicles, possibly due to the presence of a large amount of proteins and multimembrane barriers which made it difficult to estimate the free drug concentration, especially when a hydrophobic compound was used. Blockade of the ryanodine-induced muscle contraction by DHBP further suggested a direct effect of DHBP on ryanodine receptor.

An even higher concentration of DHBP was needed to block the potassium-induced contraction (also in calcium-free solution), suggesting a different sensitivity of DHBP to different calcium release mechanisms. Endo (1977) has suggested that the two classes of Ca^{2+} release, namely, Ca^{2+}-induced Ca^{2+} release including drug-induced Ca^{2+} release and depolarization-induced Ca^{2+} release are governed by different mechanisms, as indicated by the fact that they differ in several criteria, such as Ca^{2+}-loading requirements and sensitivities to Mg^{2+}, ATP, sugars and procaine. In contrast to this observation, Antoniu *et al.* (1985) found that procaine inhibited both Ca^{2+}-induced and depolarization-induced Ca^{2+} release in isolated SR vesicles. We have not tested the DHBP effect on depolarization-induced Ca^{2+} release in isolated SR vesicles; it is therefore difficult to compare the sensitivity of DHBP on these two types of calcium release inducing mechanism. Data in this study, however, have shown a different sensitivity to DHBP of calcium-induced (caffeine) or drug-induced (ryanodine) and depolarization-induced (high potassium) muscle contraction. This is further supported by the fact that a high concentration (2 mg ml^{-1}) of DHBP was needed to block completely the muscle twitch induced by direct electrical stimulation.

Effects of other bipyridylium compounds, including positively charged bipyridylium analogues (e.g. DEBP, DBBP, paraquat) and neutral bipyridines (e.g. 2,2'-bipyridine, 4,4'-bipyridine), on calcium release and ryanodine binding were compared and found to be ineffective both on release blockade and ligand binding inhibition. This result suggested,

beside the specificity of DHBP, that a positive charge on the nitrogen atom of pyridine and a long alkyl chain are both needed to produce the inhibitory effects on the calcium release channel thus resembling the action of procaine on caffeine-contracture proposed by Bianchi (1968, cf. Figure 10). Several unrelated cations, such as neomycin, and spermine (Palade, 1987b), ruthenium red (Miyamoto & Racker, 1982) and Mg^{2+} (Nagasaki & Kasia, 1981) have been shown to inhibit calcium release from SR. We cannot conclude; at this point, whether these cations and DHBP interact at the Ca^{2+}-regulatory domain of the ryanodine receptor which binds Ca^{2+} with μmolar affinity for gating the Ca^{2+} channel and is inhibited by millimolar Mg^{2+} by direct competition for the activator site as Mg^{2+} (Pessah *et al.*, 1987) or other ligand binding domains.

Although ruthenium red is an effective calcium release blocker of skeletal muscle (Miyamoto & Racker, 1982) by reacting with the calcium release channel (Antoniu *et al.*, 1985), it was reported to inhibit functions of many different systems: e.g., the spontaneous miniature endplate potential at synaptic junctions (Rahamimoff & Alnaes, 1973), mitochondrial Ca^{2+} transport (Reed & Bygrave, 1974), and SR Ca^{2+} binding (Vale & Carvalho, 1973). In contrast to ruthenium red, which blocks both the calcium release from SR (trace 6, Figure 1a) and calcium uptake by mitochondria (trace 1, Figure 1b), DHBP did not inhibit the calcium uptake by mitochondria (trace 3, Figure 1b). DHBP at concentrations tested also had no effect on Ca^{2+}-ATPase of SR, Na^{+},K^{+}-ATPase of synapsomal membrane and Mg^{2+}-ATPase of mitochondria. These results further suggested a specific effect of DHBP on the ryanodine receptor of skeletal muscle and that DHBP may be a more useful pharmacological agent than ruthenium red for intact tissue studies, besides its specificity, since unlike ruthenium red, it is membrane permeable.

Data in this study provided evidence that DHBP blocked the calcium release from SR and the ligand binding through a direct interaction with the ryanodine receptor. Use of this agent as a specific antagonist for the calcium release channel of skeletal muscle for biochemical study and possibly as a muscle relaxant clinically is suggested.

This work was supported by research grant from the National Science Council, Republic of China (NSC-82-0412-B-002-492).

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*(Received November 29, 1993
Revised April 11, 1994
Accepted April 20, 1994)*