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Emodin-induced muscle contraction of mouse diaphragm and the involvement of Ca^{2+} influx and Ca^{2+} release from sarcoplasmic reticulum

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1 The effects on skeletal muscle of emodin, an anthraquinone, were studied in the mouse isolated diaphragm and sarcoplasmic reticulum (SR) membrane vesicles.

2 Emodin dose-dependently caused muscle contracture, simultaneously depressing twitch amplitude. Neither tubocurarine nor tetrodotoxin blocked the contraction suggesting that it was caused myogenically.

3 The contraction induced by emodin persisted in a Ca^{2+} free medium with a slight reduction in the maximal force of contraction. The contraction induced by emodin in the Ca^{2+} free medium was completely blocked when the internal Ca^{2+} pool of the muscle was depleted by ryanodine. These data suggest that the contraction caused by emodin is due to the release of Ca^{2+} from the intracellular ryanodine-sensitive pool.

4 In contrast to the effect seen in the Ca^{2+} free medium, emodin induced a small but consisted contraction in the ryanodine-treated muscle in Krebs medium. The contraction was blocked in the presence of dithiothreitol and was partially blocked by nifedipine, suggesting that oxidation of a sulphhydryl group on the external site of dihydropyridine receptor is involved.

5 Emodin dose-dependently increased Ca^{2+} release from actively loaded SR vesicles and this effect was blocked by ruthenium red, a specific Ca^{2+} release channel blocker, and the thiol reducing agent, DTT, suggesting that emodin induced Ca^{2+} release through oxidation of the critical SH of the ryanodine receptor.

6 [^{3}H]-ryanodine binding was dose-dependently potentiated by emodin in a biphasic manner. The degree of potentiation of ryanodine binding by emodin increased dose-dependently at concentrations up to 10 μM but decreased at higher concentrations of 10–100 μM.

7 These data suggest that muscle contraction induced by emodin is due to Ca^{2+} release from the SR of skeletal muscle, as a result of oxidation of the ryanodine receptor and influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels of the plasma membrane.

Keywords: Skeletal muscle; SR; calcium release; ryanodine receptor; sulphhydryls; emodin; anthraquinones

Introduction

Uncontrolled rises in intracellular Ca^{2+} concentrations activate a number of biochemical pathways which can precipitate rapid cell damage and death. The regulation of intracellular free [Ca^{2+}] in skeletal muscle depends on the activities of several membrane molecules, such as the voltage-dependent Ca^{2+} channel of sarcolemma, also known as dihydropyridine receptor (DHPR), and the Ca^{2+}-ATPase and Ca^{2+} release channel, namely the ryanodine receptor, of the sarcoplasmic reticulum (SR). The voltage-dependent mechanism of rapid Ca^{2+} release from SR, commonly referred to as excitation-contraction coupling, is believed to be mediated by a physical interaction between the transverse (T)-tubule voltage-sensing DHPR and the SR ryanodine receptor (McPherson & Campbell, 1993). The ryanodine receptor forms a tetrameric complex, the electron microscopic structure of which is identical to that of an individual foot (Ferguson *et al*., 1984; Wagenknecht *et al*., 1989), and behaves like a Ca^{2+} release channel when incorporated into lipid bilayers (Smith *et al*., 1985; Imagawa *et al*., 1987; Lai *et al*., 1988; Hymel *et al*., 1988) suggesting that the protein responsible for the rapid Ca^{2+} release from SR might be the ryanodine receptor. A large number of chemically diverse substances have been shown to release Ca^{2+} from SR via the ryanodine receptor Ca^{2+} release channel, including Ag^+, halothane, caffeine, Ca^{2+} itself, ryanodine (Fleischer & Inui, 1989; Palade *et al*., 1989) and sulphhydryl reactive compounds, such as heavy metals (Brunder *et al*., 1988) and anthraquinones (Abramson *et al*., 1988).

The anthraquinone derivative, emodin (1,3,8-trihydroxy-6-methyl-anthraquinone), is one of the major compounds in many traditional Chinese medical herbs especially among the families of *Rhizoma* and *Radix*. It has been shown to inhibit protein tyrosine kinase (Jayasuriya *et al*., 1992), modulate the immune response (Huang *et al*., 1992), have an anti-inflammatory effect (Chang *et al*., 1996) and an anti-viral effect (Kawai *et al*., 1984).

In this study, we have investigated the effect of emodin on skeletal muscle by using the mouse diaphragm preparation and isolated SR membrane vesicles. We found that emodin induced muscle contracture by promoting the influx of extracellular Ca^{2+} and inducing of Ca^{2+} release from the internal Ca^{2+} pool, namely the SR.

Methods

Mouse diaphragm preparation

Mice (ICR strain) of either sex, weighing 15–20 g, were used. The diaphragm was isolated according to the methods of Bülbring (1946). The diaphragm was placed in Krebs solution of the following composition (mM): NaCl 118.5, KCl 4.8,

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$MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.5, glucose 11.1 and $CaCl_2$ 2.5; pH 7.4 and constantly gassed with 95% O_2 + 5% CO_2 at 37 ± 0.5°C. For Ca^{2+} free experiments, the diaphragm preparations were first washed three times in Krebs solution with the $CaCl_2$ replaced by 2.5 mM EGTA and then immersed in a Ca^{2+} free medium without EGTA. Twitches of the diaphragm were elicited by direct stimulation of the muscle with a pulse of 0.5 ms at 0.1 Hz. The muscle was loaded with a resting tension of 1 g, and the changes of tension were recorded via an isometric transducer (Grass FT.03) on a Gould RS3200 polygraph (Gould Instrument Co.).

Preparation of sarcoplasmic reticulum fraction

The triad enriched heavy fraction of SR was prepared from back muscles of either rat or rabbit by a differential centrifugation as previously described (Kang *et al.*, 1994). Briefly, the muscles were homogenized and centrifuged at 10,000 *g* for 5 min in a JA-14 rotor (Beckman) and the supernatant fraction filtered through eight layers of cheese cloth and then centrifuged at 17,000 *g* for 50 min. The sediment fraction was homogenized in a solution containing 0.3 M sucrose, 150 mM KCl, 0.2 mM phenyl methyl sulphonyl floride (PMSF), and 20 mM 3-(N-morpholino) propane sulphonic acid (MOPS) (pH 6.8), and centrifuged at 17,000 *g* for 40 min in a JA-20 rotor (Beckman). The final 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 after protein determination (Lowry *et al.*, 1951) and stored at −70°C.

Ca^{2+} release assay

The time course of Ca^{2+} release from SR vesicles was investigated with the Ca^{2+} sensitive probe, antipyrylazo III (AP III), in a dual wavelength spectrophotometer (SLM, Aminco DW-2000) modified from Palade (1987). SR vesicles (0.5 mg ml^{-1}), were actively loaded with the addition of 1 mM MgATP in a reaction mixture containing 150 mM KCl, 100 μM AP III, 20 mM MOPS (pH 6.8). Aliquots of 5–10 nmol Ca^{2+} were added sequentially until no more Ca^{2+} could be taken up into the SR vesicles. Release inducers, such as polylysine (2 μg ml^{-1}, M.W. = 3,800 Da, Sigma Co. St. Louis, U.S.A.) or emodin (Sigma) at the concentration indicated, were added to induce Ca^{2+} release from SR.

[^{3}H]-ryanodine binding

Ryanodine binding was measured according to Pessah *et al.* (1987) with modifications. SR, 500 μg ml^{-1}, was incubated at 37°C for 2 h in a medium containing 250 mM KCl, 15 mM NaCl, 50 mM $CaCl_2$, 10 nM [^{3}H]-ryanodine (68.3 Ci $mmol^{-1}$; NEM, U.S.A.), 20 mM Tris, pH 7.1 in the absence or presence of emodin at the concentrations indicated in each experiment. Non-specific binding was measured in the presence of 1 μM cold ryanodine (Latoxan, France). At the end of the incubation, 200 μl of each reaction mixture was withdrawn and added to 5 ml of ice-cold buffer to quench the reaction, rapidly filtered through the Whatman GF/B glass filter, and rinsed once with 5 ml ice-cold buffer. The data shown are the average of triplicate determinations in at least three different preparations.

Ca^{2+}-ATPase measurement

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 with Beckman DU-650 spectrophotometer. SR protein (10 μg) in the absence or presence of emodin at different concentrations was incubated in a 1 ml assay mixture containing 20 mM MOPS (pH 6.8), 0.3 mg ml^{-1} NADH, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.45 mM phosphoenopyruvate, 5 u ml^{-1} pyruvate kinase, 10 u ml^{-1} lactate dehydrogenase and 4 μM Ca^{2+} ionophore, A23187, at 37°C for 5 min, and the reaction was started by the addition of 100 μM ATP. Ca^{2+}-ATPase activity was calculated as the difference of activities measured with and without the addition of 0.2 mM $CaCl_2$.

Statistical analysis

The statistical significance of difference between control and drug effects was evaluated by Student's *t* test. A *P* value of 0.05 or less was considered statistically significant.

Results

Emodin dose-dependently induced a contraction in mouse diaphragm

The effect of emodin on intact skeletal muscle was investigated by using the mouse isolated diaphragm. As shown in Figure 1, emodin induced a marked contraction in mouse diaphragm at concentrations of 10 μM and 30 μM. The emodin-induced contraction was produced rapidly and dose-dependently (Figure 2a) and the time to peak tension was also reduced as the concentration of emodin increased (Figure 2b). Neither tubocurarine (5 μM) nor tetrodotoxin (5 μM) blocked the contraction induced by emodin, suggesting that the emodin-induced contraction is caused myogenically (data not shown).

Emodin induced muscle contraction by induction of Ca^{2+} release from an internal Ca^{2+} store and promoting the influx of extracellular Ca^{2+}

The effect of emodin on the role of the intracellular Ca^{2+} pool (the SR) was examined by placing the diaphragm in the Ca^{2+} free medium. Typical traces of response are shown in Figure 3 and the data are summarized in Table 1. The contraction induced by emodin (Figure 3a) persisted in the Ca^{2+} free medium (Figure 3b). However, the maximal force of contraction was reduced by 35% (Table 1). The contraction

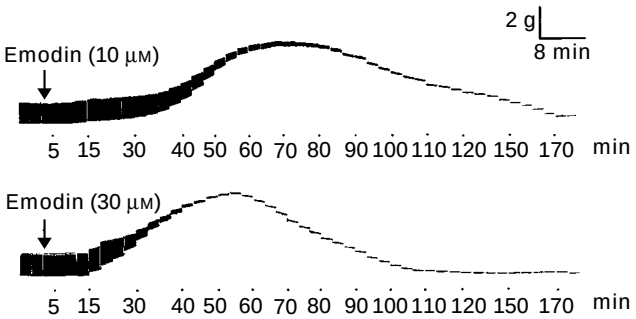

Figure 1 Emodin-induced muscle contraction and twitch depression of mouse diaphragm. The effects of 10 μM and 30 μM emodin were examined in the normal Krebs medium. The traces shown represent a typical reaction. The experiments were repeated at least three times with three different preparations.

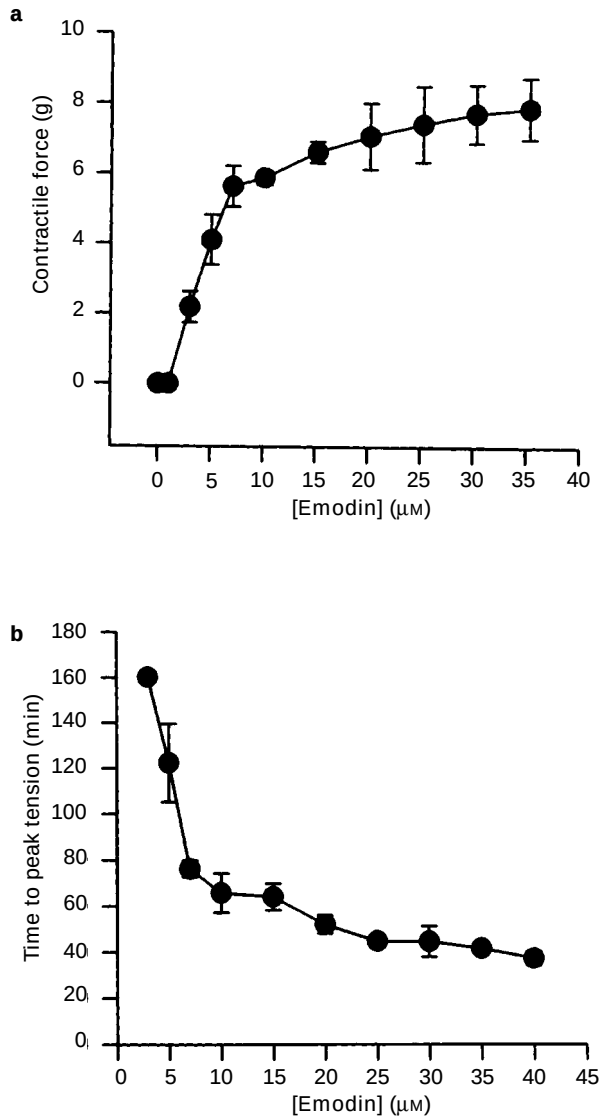

Figure 2 Dose-response effects for emodin on muscle contraction in mouse diaphragm. Mouse diaphragm was prepared as described in Methods and the effects of different doses of emodin on contractile force (a) and time to peak tension (b) were measured. Data are expressed as mean and vertical lines show s.e.mean ($n=6$).

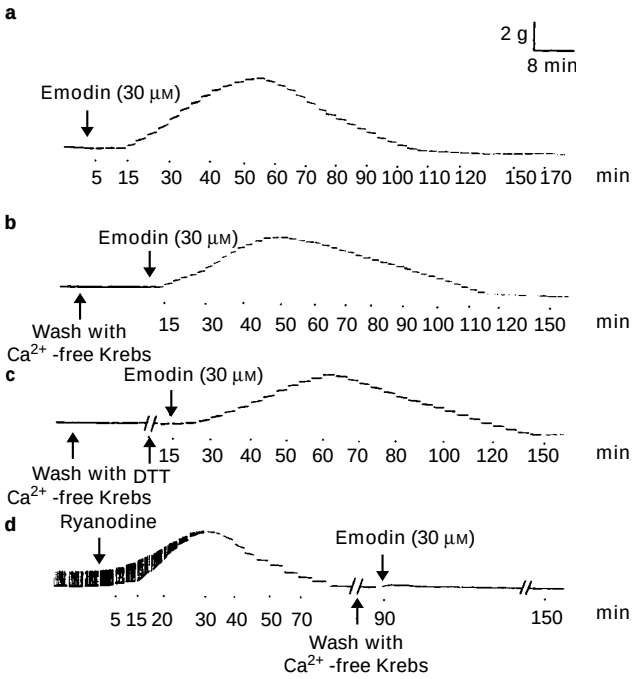

Figure 3 Emodin-induced contraction in the Ca^{2+} free medium. The muscle contraction induced by emodin was examined in either normal (a) or Ca^{2+} free (b) Krebs and after the addition of 2 mM DTT (c). In (d), the muscle was precontracted with 2 μM ryanodine in normal Krebs and then switched to a Ca^{2+} free medium followed by the addition of emodin.

Table 1 Factors affecting emodin-induced muscle contraction

	Contractile force (g)
Normal Krebs	5.67 ± 0.24
+DTT	3.78 ± 0.32^{a}
Ca^{2+} free medium	3.57 ± 0.36^{a}
+DTT	3.32 ± 0.32^{a}
Ryanodine-treated	1.60 ± 0.11^{a}
+DTT	0.23 ± 0.12^{b}
+nifedipine	0.64 ± 0.10^{c}

The contraction of mouse isolated diaphragm was induced by 30 μM emodin in various conditions after the addition of either 2 mM DTT or 1 μM nifedipine, and tension measured according to procedure outlined in Methods. In Ca^{2+} free medium, no $CaCl_2$ was added in the Krebs medium. For depletion of the internal Ca^{2+} pool, the diaphragm was precontracted with 2 μM ryanodine in normal Krebs solution. Data are expressed as mean $\pm$ s.e.mean ($n=10$). $^{a}P<0.001$, compared to normal Krebs. $^{b}P<0.05$, $^{c}P<0.01$, compared to ryanodine pretreated (no addition).

induced by emodin in normal Krebs was inhibited to the same degree by prior addition of 2 mM dithiothreitol (DTT), a membrane impermeable thiol reducing agent (Table 1). In addition, the contraction induced by emodin in the Ca^{2+} free medium was not affected by DTT (Figure 3c; Table 1). The contraction induced by emodin in the Ca^{2+} free medium was completely blocked when the internal Ca^{2+} pool of the muscle was depleted with 2 μM ryanodine (Figure 3d). These data suggest that the contraction induced by emodin in the Ca^{2+} free medium might be due to the release of Ca^{2+} from the intracellular ryanodine-sensitive pool.

The effect of emodin on muscle was further studied with the ryanodine-treated diaphragm in normal Krebs solution. As shown in Table 1, emodin caused a small contraction when the internal Ca^{2+} was depleted by 2 μM ryanodine. In contrast to the contraction-induced in Ca^{2+} free medium, the contraction seen in ryanodine-treated muscle was inhibited by DTT. We also found that the contraction-induced by emodin in the ryanodine-treated muscle could be inhibited by nifedipine, a Ca^{2+} channel blocker.

Emodine induced Ca^{2+} release from sarcoplasmic reticulum

Figure 4 shows a typical experiment in which the metallochromic Ca^{2+} indicator dye, antipyrylazo III, was used to monitor the Ca^{2+} uptake and release of isolated sarcoplasmic reticulum (SR) membrane vesicles. As shown in Figure 4, Ca^{2+} in the reaction medium were rapidly translocated into the SR upon addition of Mg ATP as indicated by the decrease of optical absorbance difference at 710 nm and 790 nm. The drug-induced Ca^{2+} release experiments were performed after the SR vesicles had been loaded with a near maximal amount of Ca^{2+}. To obtain the near maximal loading, 5–10 nmol of

$CaCl_2$ was added sequentially into the reaction medium after the addition of Mg ATP until no more Ca^{2+} could be taken up by the SR (usually 80–100 nmol Ca^{2+} mg^{-1} protein). Addition of 2 μg ml^{-1} polylysine (trace 1), a Ca^{2+} release inducer (Cifuentes *et al*., 1989), induced a rapid release of the loaded Ca^{2+} and total release can be obtained upon addition of 4 μM A23187, a Ca^{2+} ionophore. The Ca^{2+} release induced by polylysine was blocked by the Ca^{2+} release channel blocker, ruthenium red (RR) (trace 5). Emodin does-dependently induced Ca^{2+} release from SR (traces 2–4) and the Ca^{2+} release induced by 30 μM of emodin was blocked by prior addition of 2 μM RR (trace 6) or 500 μM DTT (trace 7). These data suggest that emodin triggers Ca^{2+} release via an oxidation reaction with the Ca^{2+} release channel, also known as ryanodine receptor, of the SR.

Previously Ag^{+}, a thiol reactive metal ion, was shown to induce Ca^{2+} release from SR by inhibition of the Ca^{2+}-ATPase (Gould *et al*., 1987) possibly through the oxidation of a crucial SH moiety on the Ca^{2+}-ATPase. By measuring the Ca^{2+}-ATPase of the isolated SR vesicles, we found that emodin, at concentrations up to 100 μM, did not affect the Ca^{2+}-ATPase activity of SR (data not shown).

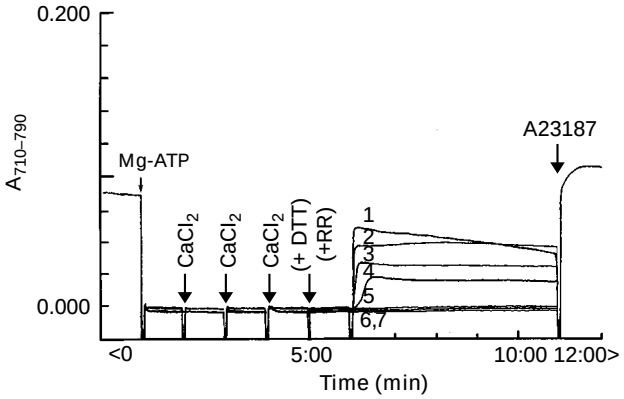

Figure 4 Induction of Ca^{2+} release by emodin from SR membrane vesicles. SR vesicles (0.5 mg ml^{-1}) were loaded with Ca^{2+} and the Ca^{2+} concentration was monitored by the absorbance difference at 710 nm and 790 nm according to the procedure outlined in Methods. Typical traces are shown. Trace 1, Ca^{2+} release induced by 2 μg ml^{-1} polylysine; traces 2–4, Ca^{2+} release induced by 30, 10 and 5 μM emodin, respectively; trace 5, Ca^{2+} release induced by polylysine with prior addition of 2 μM ruthenium red (RR). Traces 6 and 7: Ca^{2+} release induced by 30 μM emodin after prior addition of 2 μM RR and 1 mM DTT, respectively.

Table 2 Effect of emodin on [^{3}H]-ryanodine binding to isolated SR vesicles

Addition		*[^{3}H]-ryanodine binding* (pmol mg^{-1} protein)
None		1.35±0.38
Emodin	0.3 μM	1.99±0.01
	1.0 μM	3.28±0.58[a]
	10 μM	5.86±0.51[b]
	30 μM	3.42±0.19[a]
	50 μM	2.92±0.89
	100 μM	1.46±0.63

The ryanodine binding was measured according to the procedure stated in Methods in the presence of various concentration of emodin (0.3–100 μM). Control binding was measured after the addition of 2 μM DMSO instead of emodin. Data are presented as mean±s.e.mean of triplicate measurements from three different preparations. The binding in the presence of emodin was compared to the control and analysed by Student's *t* test. [a]$P<0.05$; [b]$P<0.01$ ($n=3$).

Effect of emodin on [^{3}H]-ryanodine binding

The plant alkaloid, ryanodine, has been shown to bind specifically to the Ca^{2+} release channel of the SR (Fleischer *et al*., 1985) and the ligand binding could be used as a probe for the channel activity (Chu *et al*., 1990). Emodin, at lower concentrations (0.3–10 μM), dose-dependently potentiated [^{3}H]-ryanodine binding (Table 2). However, the degree of potentiation was gradually decreased as the concentration of emodin increased (30–100 μM). The effect of emodin on ryanodine binding, as in Ca^{2+} release induction, was also prevented in the presence of DTT (data not shown).

Discussion

In skeletal muscle, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) plays an important role in contraction. The sources of Ca^{2+} are Ca^{2+} entry through voltage-dependent Ca^{2+} channels in the plasma membrane, as in the case of KCl-induced contractions (Frank, 1990), and Ca^{2+} release through Ca^{2+} release channels of SR, as in the case of ryanodine-induced contractions (Lai *et al*., 1988; Takeshima *et al*., 1989; Zorzato *et al*., 1990) or both as in the case of caffeine-induced contractions (Røed, 1991). SH-reactive agents have been shown to produce contraction of intact skeletal muscle through the oxidation of a critical SH group of the Ca^{2+} release channel, also known as ryanodine receptor, of SR and/or DHPR of plasma membrane (Oba & Yamaguchi, 1990; Oba *et al*., 1996).

In this study, we have shown that emodin, a SH reactive anthraquinone, could trigger skeletal muscle contraction in mouse isolated diaphragm. The contraction induced by emodin persisted in a Ca^{2+} free medium and was not affected by DTT. However, the contraction induced in the Ca^{2+} free medium was completely inhibited by prior treatment with ryanodine. These data suggest that emodin could induce Ca^{2+} release from the internal Ca^{2+} pool and cause the muscle to contract. The direct interaction of emodin with the Ca^{2+} release channel of SR was further indicated by the finding that emodin could induce Ca^{2+} release from isolated SR vesicles. The release was inhibited by the release blocker, ruthenium red, and the thiol reducing agent DTT, suggesting that emodin induced Ca^{2+} release through oxidation of the critical SH of ryanodine receptor, as in the case with SH-reactive heavy metals (Salama & Abramson, 1984; Brunder *et al*., 1988; Nagura *et al*., 1988; Zaidai *et al*., 1989).

The activity of Ca^{2+}-ATPase was shown to be inhibited by SH reactive agents (Murphy, 1976; Scherer & Deamer, 1986) and Ag^{+} was shown to induce Ca^{2+} release from SR through the oxidation of a crucial SH moiety on the Ca^{2+}-ATPase (Gould *et al*., 1987). However, we found that the activity of Ca^{2+}-ATPase was not affected by emodin at concentrations up to 100 μM. In addition, by using a sucrose gradient (Mitchell *et al*., 1988), we isolated the light fraction of SR membrane vesicles which contained only the Ca^{2+}-ATPase and found that neither polylysine nor emodin induce Ca^{2+} release from these SR vesicles (data not shown). These data further suggest that the Ca^{2+} release induced by emodin is due to its interaction with the ryanodine receptor of SR.

Both anthraquinones (Abramson *et al*., 1988) and Ag^{+} (Salama & Abramson, 1984; Brunder *et al*., 1988) induced

Ca^{2+} release from the SR vesicles. However, in contrast to the potentiation of [^{3}H]-ryanodine binding by anthraquinones, Ag^+ causes a rapid displacement of bound ryanodine from its receptor (Pessah *et al*., 1987). It was suggested that anthraquinones and Ag^+ might react with different SH groups of ryanodine receptor complex (Abramson & Salama, 1989). We also found that emodin could dose-dependently potentiate [^{3}H]-ryanodine binding in a biphasic manner. For comparison, we examined the effect of Ag^+ on ryanodine binding and found that Ag^+ at concentrations of 0.01–0.1 μM dose-dependently potentiated the binding and the degree of potentiation was gradually decreased, as seen with emodin, when the concentration of Ag^+ increased to 1 μM. However, the apparent binding decreased and no binding was detected at concentrations higher than 10 μM (unpublished results). In contrast to Ag^+, the binding of ryanodine in the presence of emodin never fell below the control value. These data suggest that the SH groups reacting with emodin might be different from that which reacts with doxorubicin and Ag^+, and that three different SHs, might exist as suggested by Abramson & Salama (1989). The first SH, which reacts with doxorubicin or lower concentrations of Ag^+ (0.01–0.1 μM) and emodin (0.3–10 μM), when oxidized would result in the increase of [^{3}H]-ryanodine binding. The second SH, which reacts with medium range of Ag^+ (0.1–1 μM) and higher concentrations of emodin (10–100 μM), when oxidized would result in the reduction of binding potentiation. The third SH, which reacts only with higher concentrations of Ag^+ (>1 μM), would cause a decrease in apparent binding possibly due to the rapid displacement of bound ryanodine from its receptor (Pessah *et al*., 1987).

Although emodin induced muscle contraction in the Ca^{2+} free medium, the maximal contractile force was reduced by roughly 35%, suggesting that the influx of extracellular Ca^{2+} might also be involved in this contraction induced by emodin. This was further supported by the finding that emodin induced a small but consisted contraction when the internal Ca^{2+} pool was depleted by precontraction with ryanodine. The contraction induced in the ryanodine pretreated muscle was inhibited by prior addition of DTT and nifedipine, suggesting the involvement of SH on the external site of DHPR, as seen in the case with Ag^+ (Oba *et al*., 1992) and H_2O_2 (Oba *et al*., 1996). In addition, we have also found that the contraction induced by emodin in normal Krebs was inhibited to a similar extent by either removing the extracellular Ca^{2+} or by addition of the membrane impermeable thiol reducing agent, DTT.

Results obtained from this study have shown that the muscle contraction induced by emodin was mainly caused by the release of internal Ca^{2+}, which might be due to oxidation of the ryanodine receptor. Emodin also appeared to promote influx of extracellular Ca^{2+} through the voltage-dependent Ca^{2+} channel of the plasma membrane.

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