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Induction of calcium release from sarcoplasmic reticulum of skeletal muscle by xanthone and norathyriol

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1 Effects of xanthone and its derivative, 1,3,6,7-tetrahydroxyxanthone (norathyriol), on Ca^{2+} release and ryanodine binding were studied in isolated sarcoplasmic reticulum (SR) vesicles from rabbit skeletal muscle.

2 Both xanthone and norathyriol dose-dependently induced Ca^{2+} release from the actively loaded SR vesicles which was blocked by ruthenium red, a specific Ca^{2+} release inhibitor, and Mg^{2+}.

3 Xanthone and norathyriol also dose-dependently increased apparent [^{3}H]-ryanodine binding. Norathyriol, but not xanthone, produced a synergistic effect on binding activation when added concurrently with caffeine.

4 In the presence of Mg^{2+}, which inhibits ryanodine binding, both caffeine and norathyriol, but not xanthone, could restore the binding to the level observed in the absence of Mg^{2+}.

5 Xanthone activated the Ca^{2+}-ATPase activity of isolated SR vesicles dose-dependently reaching 70% activation at 300 μM.

6 When tested in mouse diaphragm, norathyriol potentiated the muscle contraction followed by twitch depression and contracture in either a Ca^{2+}-free bathing solution or one containing 2.5 mM Ca^{2+}. These norathyriol-induced effects on muscle were inhibited by pretreatment with ruthenium red or ryanodine.

7 These data suggest that xanthone and norathyriol can induce Ca^{2+} release from the SR of skeletal muscle through a direct interaction with the Ca^{2+} release channel, also known as the ryanodine receptor.

Keywords: Skeletal muscle; calcium release; xanthones; ryanodine receptor

Introduction

Sarcoplasmic reticulum (SR) is the major internal Ca^{2+} storage site of the skeletal muscle cell and the uptake and release of Ca^{2+} from and into the myoplasma are controlled by two molecules located on the SR membrane, namely, the Ca^{2+}-ATPase and the Ca^{2+} release channel, also known as ryanodine receptor, respectively. The ryanodine receptor, which binds the plant alkaloid ryanodine specifically (Fleischer *et al.*, 1985; Pessah *et al.*, 1985), is a homotetramer of four 565-kDa subunits that mediates Ca^{2+} release from SR (Lai *et al.*, 1988; Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), and it forms a tetrameric complex whose electron microscopic structure is identical to that of an individual foot (Ferguson *et al.*, 1984; Wagenknecht *et al.*, 1989). The ryanodine receptor 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) and the channel activity was increased by Ca^{2+} and ATP (Morri & Tonomura, 1983) but inhibited by Mg^{2+} (Kirino *et al.*, 1983; Meissner *et al.*, 1986) and ruthenium red (Miyamoto & Racker, 1982), suggesting that the protein responsible for the rapid Ca^{2+} release from SR might be the ryanodine receptor. The Ca^{2+} release properties and channel activities were also affected by several structurally unrelated chemicals (Palade *et al.*, 1989; Zorzato *et al.*, 1993; Abramson *et al.*, 1993; Beeler & Gable, 1993; Kang *et al.*, 1994).

Xanthone derivatives are widely distributed in the plant among the families of *Gentianaceae* and *Guttiferae*. They have been found to show schistosomicidal activity, CNS stimulant, cardiotonic, antidepressant and hydrocholeretic effects (Shankaranarayan *et al.*, 1979) and antiallergic or bronchodilator activity (Lin *et al.*, 1984). Recently, 1,3,6,7-tetrahydroxyxanthone (norathyriol, NT; Figure 1), an aglycon of a xanthone glycoside, mangiferin, isolated from the aerial parts of *Tripterospermum lanceolatum* (Lin *et al.*, 1982), was found to inhibit platelet aggregation (Teng *et al.*, 1991), relax rat thoracic aorta (Ko *et al.*, 1991) and inhibit cutaneous plasma extravasation (Wang *et al.*, 1994).

In this study, we have investigated the effect of xanthone and norathyriol on Ca^{2+} release and muscle contraction. We have found that both xanthone and norathyriol can induce a rapid Ca^{2+} release from SR vesicles isolated from skeletal muscle and activate the ryanodine binding to its receptor on the SR membrane. Norathyriol, but not xanthone, also induces a muscle contracture of isolated mouse diaphragm. These data suggest that xanthone and norathyriol can interact directly with the Ca^{2+} release channel of skeletal muscle.

Methods

Preparation of sarcoplasmic reticulum fraction

The triad enriched heavy fraction of sarcoplasmic reticulum (SR) was prepared from rabbit leg and back muscles by differential centrifugation as described by Ikemoto *et al.* (1984) with modifications. Briefly, the muscle was homogenized in three times its volume of ice cold 20 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), 0.1 mM EDTA, 0.1 mM EGTA, and 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 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 sediment fraction was homogenized in a solution containing 0.3 M sucrose, 150 mM KCl, 0.2 mM PMSF, and 20 mM MOPS (pH 6.8), and centrifuged at 17,000 *g* for 40 min in a JA-20 rotor (Beckman). The sediment fraction was homogenized in

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the above solution at a final protein concentration of 20–30 mg ml^{-1}. The Ca^{2+} content of the isolated SR was determined by EGTA titration in the medium containing 100 μM antipyrylazo III (AP) and calculated as outlined in the calcium release assay. The preparation was quickly frozen in liquid nitrogen and stored at −70°C.

Mouse diaphragm preparation

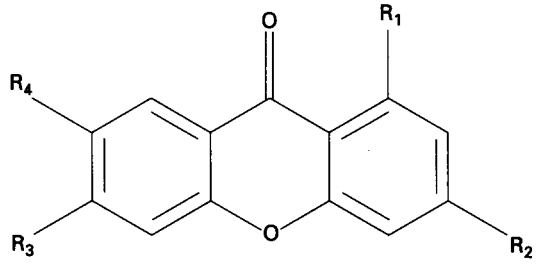
Mice (ICR strain) of either sex, weighing 15–20 g, were used. The diaphragm preparation was isolated according to the method of Bülbring (1946). A modified Krebs solution of the following composition (mM) was used: NaCl 130.6, KCl 4.8, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1, pH 7.4, with (2.5 mM) or without (0 mM + 2.5 mM EGTA) the addition of $CaCl_2$. The diaphragm preparation 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 by direct stimulation of the muscle with a pulse of 0.5 ms at 0.2 Hz. Caffeine contracture was elicited by addition of 10 mM caffeine to the unstimulated muscle. 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.)

Ca^{2+} release assay

The time course of Ca^{2+} release from SR vesicles was investigated with a Ca^{2+} sensitive probe, the antipyrylazo III (AP III), in a dual wavelength spectrophotometer (SLM, Amico DW-2000) with no addition of precipitating agent modified from Palade (1987a). SR vesicles (0.5 mg ml^{-1}) were actively loaded with 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 the saturation of SR vesicles. Release inducers, such as polylysine (2 μg ml^{-1}, Mol. wt. = 3,800 Da, Sigma) or 2 mM caffeine, were added to induce Ca^{2+} release from SR. The amount of total Ca^{2+}, that released by A23187 or drug were calculated according to the absorbance-concentration curve derived from the titration of buffer containing 100 μM AP III with the addition of a known concentration of Ca^{2+}.

[^{3}H]-ryanodine binding

Ryanodine binding was measured according to Pessah *et al.* (1987) with modification. Five hundred μg ml^{-1} of SR were incubated at 37°C for 2 hr in a medium containing 250 mM KCl, 15 mM NaCl, 50 μM $CaCl_2$ 5–50 nM [^{3}H]-ryanodine (NEM, 87.0 Ci $mmol^{-1}$), 20 mM Tris, pH 7.1 with the test compounds at concentrations indicated in each experiment. Non-specific binding was measured in the presence of 1 μM cold ryanodine (Calbiochem). At the end of the incubation period, 200 μl of each reaction mixture was withdrawn, added to 5 ml of ice cold buffer to quench the reaction, rapidly filtered through the Whatman GF/B glass filter, rinsed once with 5 ml ice cold buffer. Drug effect on apparent (equilibrium) ryanodine binding was determined as above at 10 nM [^{3}H]-ryanodine. The data shown are the average of triplicate determinations of at least three different preparations.



$R_1 = R_2 = R_3 = R_4 = H$: Xanthone
$R_1 = R_2 = R_3 = R_4 = OH$: Norathyriol

Figure 1 Chemical structures of norathyriol and xanthone.

Cation-dependent ATPase

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 20 mM MOPS, pH 6.8, 0.3 mg ml^{-1} NADH, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.45 mM phospho(enol) pyruvate (PEP), 5 units ml^{-1} pyruvate kinase and 10 units ml^{-1} lactate dehydrogenase (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; 1 mM ATP was then added to start the reaction. Ca^{2+}-ATPase was measured with the addition of 0.2 mM $CaCl_2$ and 4 μM calcium ionophore, A23187. When tested with norathyriol, which has a strong absorbance at 340 nm, ATPase activity was determined by measuring the phosphate concentration, as described by Chen *et al.* (1956).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951)) with bovine serum albumin as standard.

Results

Effects of xanthone and norathyriol on Ca^{2+} release

The effect of xanthones on the Ca^{2+} release was studied in the sarcoplasmic reticulum (SR) membrane enriched microsomes isolated from rabbit skeletal muscle by use of the metallochromic Ca^{2+} indicator dye, antipyrylazo III, to monitor the Ca^{2+} uptake and release. As shown in Figure 2, Ca^{2+} in the reaction medium, originating from the SR vesicles, was rapidly translocated back into the SR vesicles upon addition of MgATP by the Ca^{2+}-ATPase located on the SR membrane as indicated by the decrease of optical absorbance. A small amount of Ca^{2+} (5–10 nmol) was then added sequentially to ensure the saturation of the SR vesicles and the drug-induced

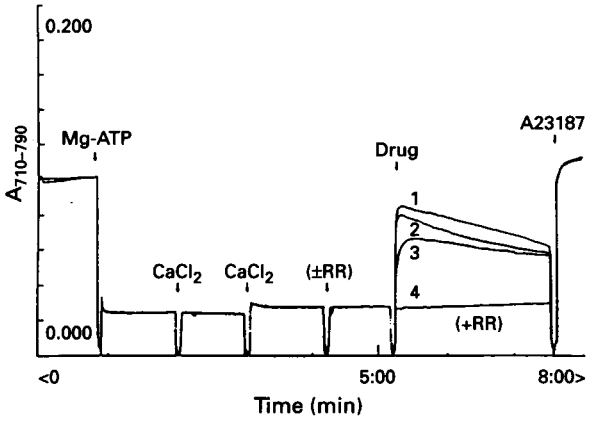


Figure 2 Effects of xanthone and norathyriol on calcium release from sarcoplasmic reticulum (SR). Five hundred micrograms of SR vesicles were loaded with calcium as described in the text and the calcium concentration was monitored by the absorbance difference at 710 nm and 790 nm according to the procedure described in Methods. Trace 1, calcium release induced by 2 μg ml^{-1} polylysine; trace 2, calcium release induced by 100 μM xanthone; trace 3, calcium release induced by 100 μM norathyriol; trace 4, calcium release induced by 100 μM norathyriol with prior addition of 2 μM ruthenium red.

Ca^{2+} release experiments were performed after the SR was loaded with a near maximal amount of Ca^{2+}. Addition of 2 μg ml^{-1} polylysine (trace 1), a Ca^{2+} release inducer (Cifuentes *et al.*, 1989; Kang *et al.*, 1992), induced a rapid release of the loaded Ca^{2+} as indicated by the sharp increase in absorbance, and the released Ca^{2+} was slowly taken up by the SR vesicles. Four micromolar of Ca^{2+} ionophore, A23187, was then added to induce an irreversible release of the resequestered Ca^{2+}. Both xanthone (trace 2) and norathyriol (trace 3), at 100 μM, induced a rapid Ca^{2+} release from SR vesicles and the Ca^{2+} released by xanthone but not by norathyriol was quickly resequestered by the SR vesicles. Ca^{2+} release induced by norathyriol (trace 4) and xanthone (not shown) was inhibited by pretreatment with 2 μM ruthenium red, a Ca^{2+} release inhibitor, suggesting that both compounds induce Ca^{2+} release through the Ca^{2+} release channel, also known as the ryanodine receptor, of the SR. Ca^{2+} release induced by xanthone and norathyriol was dose-dependent with EC_{50} values of 25 μM and 50 μM and maximal release occurred at concentrations greater than 100 μM and 200 μM, respectively (Figure 3). Ca^{2+} release induced by 100 μM xanthone and norathyriol as well as by 2 μg ml^{-1} and 2 mM caffeine were inhibited by 1 mM Mg^{2+} (data not shown).

Effects of xanthone and norathyriol on Ca^{2+}-ATPase activity

Several chemicals, such as cyclopiazonic acid (Goeger & Riley, 1989), have been shown to increase the Ca^{2+} permeability of SR vesicles by inhibiting the Ca^{2+}-ATPase activity of the SR. We tested the effect of xanthone and norathyriol on the Ca^{2+}-ATPase activity of SR and found that xanthone dose-dependently stimulated the ATPase activity (Figure 4), reaching nearly 70% activation at 300 μM. Norathyriol, in contrast to xanthone, inhibited the ATPase activity at a high concentration with an IC_{50} value of 350 μM (data not shown).

Effects of norathyriol and xanthone on [^{3}H]-ryanodine binding

The plant alkaloid, ryanodine, has been shown to bind specifically to the Ca^{2+} release channel of SR (Fleischer *et al.*, 1985) and the ligand binding was used as a probe for the channel activity (Chu *et al.*, 1990). Xanthone and norathyriol dose-dependently increased [^{3}H]-ryanodine binding with the EC_{50} values of 35 μM and 100 μM respectively (Figure 5). Caffeine, a known Ca^{2+} release channel activator stimulates ryanodine binding (Pessah *et al.*, 1987); it induced a 3 fold stimulation of ryanodine binding at 30 mM and norathyriol, but not xanthone, potentiated this stimulant effect (Figure 6).

Magnesium has been shown to inhibit Ca^{2+} release (Meissner *et al.*, 1986) and suppress ryanodine binding (Pessah *et al.*, 1987). We tested the effect of Mg^{2+} on ryanodine binding in the presence of xanthone, norathyriol and caffeine and the data are summarized in Table 1. Xanthone, norathyriol and caffeine stimulated ryanodine binding and Mg^{2+} inhibited ryanodine binding. In the presence of Mg^{2+}, norathyriol and caffeine, but not xanthone could restore binding to the level observed in the absence of Mg^{2+}.

Effects of xanthone and norathyriol on diaphragm isolated from mouse

The mouse isolated diaphragm was used to test the effects of xanthone and norathyriol on intact muscle. As shown in Figure 7a, 50 μM norathyriol induced a muscle contracture and twitch depression with a lag period in a bathing solution containing either 2.5 mM Ca^{2+} (upper trace) or no Ca^{2+} (not shown). The lag period shortened and contracture force increased as the concentration of norathyriol increased (middle trace). These norathyriol-induced effects on muscle were inhibited by pretreatment with 50 μM ruthenium red (lower trace). In contrast to norathyriol, xanthone potentiated the muscle contraction without causing contracture (Figure 7b) at concentrations up to 300 μM. The effect was dose-dependent, was not blocked by pretreatment with ruthenium red or nifedipine, and persisted in a Ca^{2+} free bathing solution (data not shown).

Caffeine induced a phasic contraction followed by a slow developing tonic contraction (Figure 8a) of the resting muscle by inducing Ca^{2+} efflux from the internal storage site, SR, and influx of extracellular Ca^{2+}, respectively (Kang *et al.*, 1994). Pretreatment of norathyriol selectively eliminated the phasic

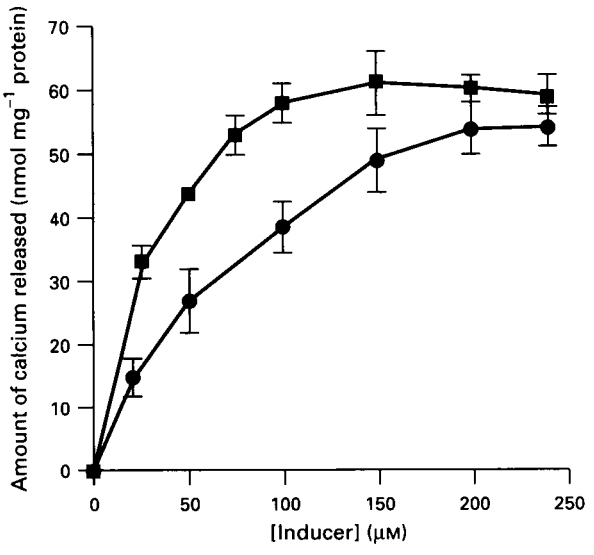


Figure 3 Dose-response curves for the effects of xanthone and norathyriol on calcium release from sarcoplasmic reticulum (SR). Calcium release induced by norathyriol (●) and xanthone (■) were measured as described in Figure 2. The amount of calcium released was calculated by measuring the absorbance at maximal release and compared to the standard curve derived from the known concentration of Ca^{2+} as described in the Methods. Points show means and vertical lines represent s.e.mean.

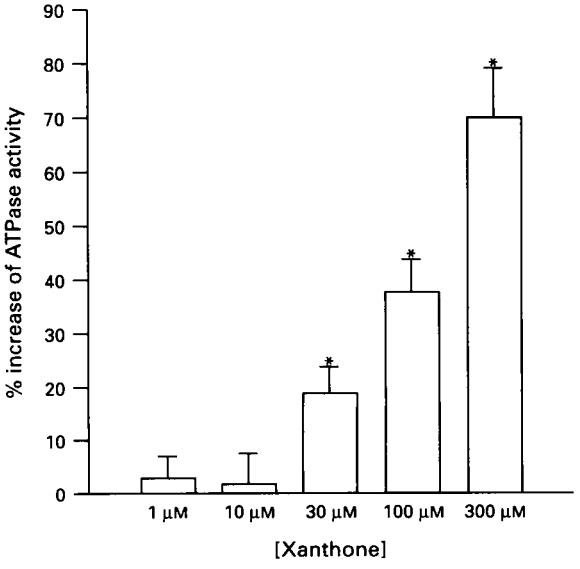


Figure 4 The effect of xanthone on Ca^{2+}-ATPase activity. Xanthone, at the concentrations indicated, was incubated with sarcoplasmic reticulum (SR) for 5 min in the presence of 2 μM A23187 and the ATPase activity was measured according to the procedure outlined in Methods. Data are presented as the mean ± s.e.mean. *$P<0.01$ as compared with the respective control and was considered to be statistically significant by Student's t test ($n=8$).

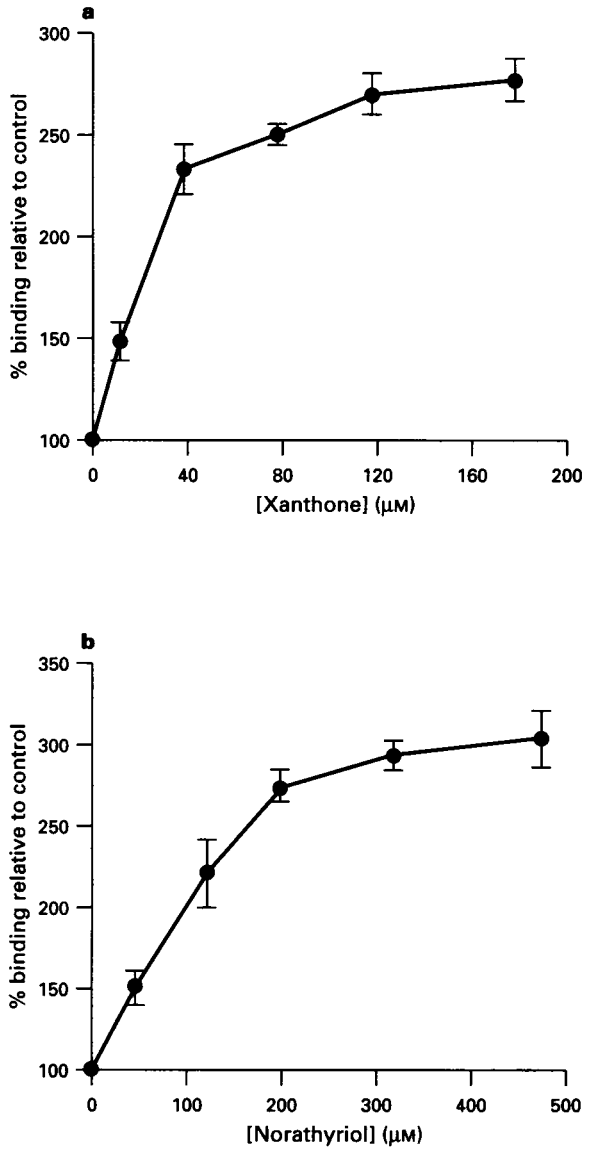


Figure 5 Dose-response curves for the effects of xanthone and norathyriol on [^{3}H]-ryanodine binding. Different concentrations of xanthone (a) and norathyriol (b) as indicated were added to the assay medium and the apparent binding of [^{3}H]-ryanodine measured according to the procedure outlined in the Methods. Each data point represents a triplicate measurement of three different preparations and vertical lines show s.e.mean.

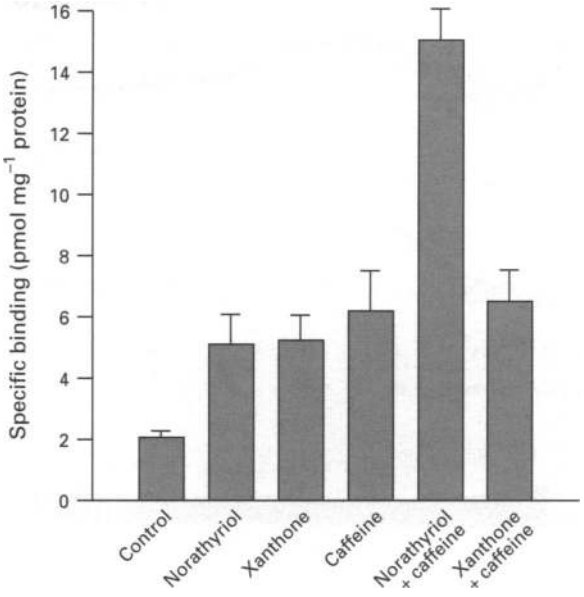


Figure 6 Effect of xanthone, norathyriol and caffeine on [^{3}H]-ryanodine binding. Xanthone (120 μM), norathyriol (300 μM) and caffeine (30 mM) were added alone or in combination to the assay medium and the apparent [^{3}H]-ryanodine binding measured according to the procedure outlined in the Methods. Each data point represents a triplicate measurement of three different preparations and vertical lines show s.e.mean.

Table 1 Effect of Mg^{2+} on [^{3}H]-ryanodine binding

Addition	*[^{3}H]-ryanodine binding (pmol mg^{-1} protein)*	
	$-Mg^{2+}$	$+Mg^{2+}$
None	2.02 ± 0.3	1.0 ± 0.2
Norathyriol, 300 μM	5.13 ± 1.0	2.3 ± 0.5
Xanthone, 120 μM	5.20 ± 0.9	0.9 ± 0.35
Caffeine, 30 mM	6.20 ± 1.3	2.5 ± 0.3

The apparent ryanodine bindings were measured in the absence of Mg^{2+} ($-Mg^{2+}$) or in the presence of 1 mM Mg^{2+} ($+Mg^{2+}$) according to the procedure stated in Methods. Data are presented as mean ± s.e.mean of triplicate measurements from three different preparations.

contraction (Figure 8b) with little effect on tonic contraction suggesting a depletion of the caffeine sensitive internal Ca^{2+} pool.

Norathyriol induced a contracture of resting muscle (Figure 8c) as seen with electrically stimulated muscle (Figure 7a), and this norathyriol induced contraction was inhibited when the muscle was precontracted with 2 μM ryanodine (Figure 8d), suggesting that norathyriol and ryanodine were acting on the same Ca^{2+} pool. Neither norathyriol nor ryanodine inhibited muscle contraction induced by high K^+ (depolarization-induced contraction; Figure 8c and 8d); this contraction could be blocked by pretreatment with 1 μM nifedipine (data not shown).

Discussion

In this study, we have presented evidence showing that both xanthone and its derivative, norathyriol, induce Ca^{2+} release from SR vesicles by a direct interaction with the Ca^{2+} release channel of skeletal muscle, namely, the ryanodine receptor. Xanthone is used in the preparation of xanthydrol and as an ovicide for codling moth eggs while xanthone derivatives are widely distributed in plants among the families of *Gentianaceae* and *Guttiferae*, such as *Tripterospermum lanceolatum* (Hayata) Hara ex Satake (*Gentianaceae*) and *Garcinia mangostana* Linn. (*Guttiferae*), with variable pharmacological actions (Shankaranarayan *et al.*, 1979; Wang *et al.*, 1994). From the study with isolated SR vesicles, we have demonstrated that both xanthone and norathyriol induce Ca^{2+} release from actively loaded SR vesicles and this effect was blocked by the Ca^{2+} release channel blocker, ruthenium red, suggesting that the effect of xanthone and norathyriol is specific for the ryanodine sensitive Ca^{2+} release channel. Although both xanthone and norathyriol induced rapid release of Ca^{2+} from SR vesicles, the uptake of the released Ca^{2+} showed a different kinetic pattern. In the case of xanthone, the released Ca^{2+} was quickly resequestered by the SR. However, when the Ca^{2+} release was induced by norathyriol, the released Ca^{2+} was resequestered at a very slow rate. It has been shown that both Ca^{2+} release (Kwok & Best, 1991) and channel activity (Morii & Tonomura, 1983) can be activated by low concentrations of Ca^{2+} but inhibited by high concentrations of Ca^{2+}. Several chemicals, like caffeine, induce

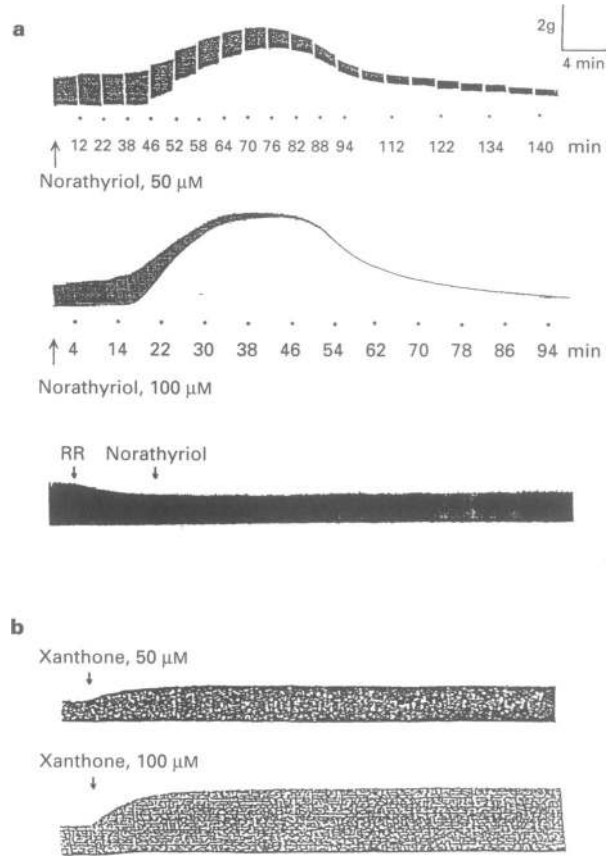


Figure 7 Effect of xanthone and norathyriol on muscle contraction of mouse diaphragm. The effect of 50 μM and 100 μM norathyriol (a) and xanthone (b) on muscle contraction were tested in the isolated mouse diaphragm as outlined in the Methods. For the inhibitory experiment, 50 μM ruthenium red was added at least 30 min before the addition of test compounds.

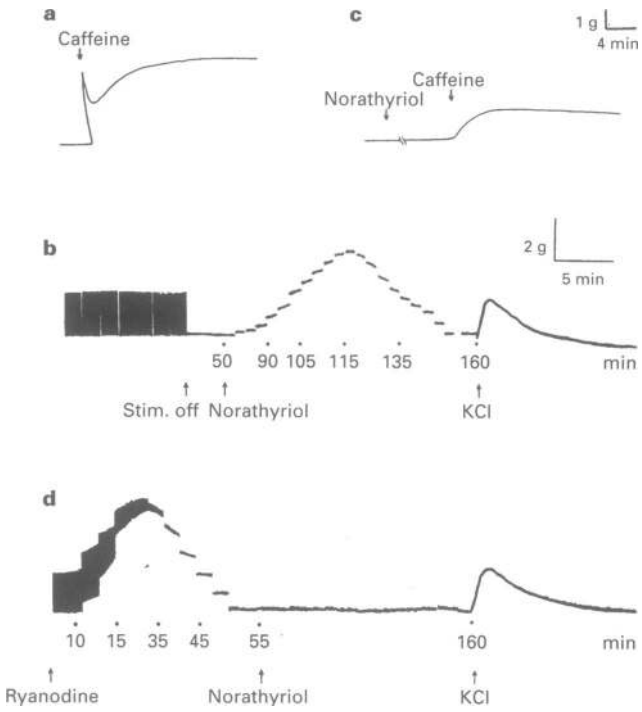


Figure 8 Effect of norathyriol, ryanodine, caffeine and KCl singly and in combination on muscle contraction of mouse diaphragm. The effect of 10 mM caffeine on muscle without electrical stimulation (a) or pretreatment with 100 μM norathyriol (b), and the effect of 100 μM norathyriol on muscle without electrical stimulation (c) or pretreatment with 2 μM ryanodine (d) were tested in the isolated mouse diaphragm and tension measured as outlined in Methods. In traces (c) and (d), the muscles were depolarized with Krebs solution containing 80 mM K^+ after treatment with norathyriol and ryanodine.

Ca^{2+} release by lowering the threshold of Ca^{2+} sensitivity (Endo, 1975). Other drugs, such as ryanodine, induce Ca^{2+} release by locking the channel in an open state (Meissner, 1986). It is possible that xanthone mimics the action of caffeine in activation of the channel which then becomes inactivated as the concentration of Ca^{2+} increases and norathyriol, possibly by acting through a different mechanism, might lock the channel in an open state like ryanodine.

Ryanodine binding has been shown to be a useful probe for release channel activity (Chu *et al.*, 1990) and several Ca^{2+} release inducers have been shown to increase the apparent ryanodine binding (Pessah *et al.*, 1987). We have shown that xanthone and norathyriol, like caffeine, the known calcium release inducer, can dose-dependently increase the apparent ryanodine binding with maximum activation at 120 μM and 300 μM, respectively. Although both xanthone and norathyriol greatly increased the apparent ryanodine binding, they showed different effects when added in combination with caffeine and Mg^{2+} (Figure 6 and Table 1). Concurrent addition of xanthone and caffeine did not produce a further increase in ryanodine binding; however, the ryanodine binding showed a synergistic increase when norathyriol and caffeine were added together. While in the presence of Mg^{2+}, which inhibits ryanodine binding, both caffeine and norathyriol, but not xanthone, restored the binding to the level observed in the absence of Mg^{2+}. These data suggest that xanthone binds to the caffeine site but its binding or actions are blocked by Mg^{2+}; whereas, although norathyriol might behave like caffeine, it additionally binds at some other site.

Norathyriol, at 50 μM, induced a contracture of mouse diaphragm in bathing solution with or without Ca^{2+} under direct electrical stimulation which was blocked by pretreatment with the Ca^{2+} release channel blocker, ruthenium red (Figure 7a). Twitches evoked by direct electrical stimulation were eventually blocked and the muscle tone returned to the resting state, suggested depletion of the internal Ca^{2+} store. These data and the ability of norathyriol to deplete the caffeine sensitive internal Ca^{2+} pool (Figure 8b) suggest that norathyriol can induce Ca^{2+} release from the SR of intact muscle, in agreement with the study on isolated SR vesicles. This is further supported by the fact that norathyriol could not induce a contraction in muscle precontracted and internal Ca^{2+} store depleted with ryanodine (Figure 8d).

It was shown that high K^+-induced contractures could be eliminated by removing Ca^{2+} from the extracellular spaces (Frank, 1958, 1960) or blocked by dihydropyridine calcium channel antagonist, such as nitrendipine and nifedipine (Frank, 1990). Such results suggest that the entrance of external Ca^{2+} via the voltage-sensitive, slow calcium channels in the T-tubules is required for excitation-contraction coupling in skeletal muscle during high K^+- or depolarization-induced contractures but not for twitches. We have shown, in this study, that pretreatment with either norathyriol (Figure 8c) or ryanodine and norathyriol (Figure 8d) does not inhibit the contraction induced by high K^+, suggesting that the voltage-sensitive calcium channel in the T-tubule of skeletal muscle is not blocked or inactivated by norathyriol. On the other hand, this high K^+-induced contraction could be eliminated by nifedipine. These results together with the finding that norathyriol induces contracture in the absence of external Ca^{2+} suggest that the contracture induced by norathyriol might not be due to membrane depolarization.

Although xanthone could induce Ca^{2+} release and activate ryanodine binding in isolated SR vesicles, it showed a different

effect on intact muscle. Xanthone dose-dependently potentiated the twitch without causing contracture and this effect was not inhibited by pretreatment with ruthenium red, suggesting that xanthone may not be as potent a Ca^{2+} release inducer as norathyriol in intact muscle under physiological conditions. The results in Table 1 show that, in the presence of Mg^{2+}, norathyriol and caffeine can restore ryanodine binding but not the effect of xanthone, suggesting that the effect of xanthone on the Ca^{2+} release channel is fully antagonized by Mg^{2+}. Therefore, it is reasonable to speculate that xanthone might not be able to induce Ca^{2+} release in intact muscle under physiological conditions (mM Mg^{2+}). This is supported by the finding that Ca^{2+} release induced from the membrane vesicles by xanthone can be blocked by the addition of 1 mM free Mg^{2+} (data not shown). We have also observed that pretreatment of muscle with a Ca^{2+} channel blocker (nifedipine) or the removal of Ca^{2+} from the organ bath did not affect the twitch potentiation seen with xanthone suggesting that the twitch potentiation was not due to an influx of extracellular Ca^{2+}. Physiological second messengers, such as adenosine 3':5'-cyotic monophosphate (cyclic AMP; Su & Malencik, 1982), and chemicals, such as (S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone (gingerol; Kobayashi *et al.*, 1988), have been shown to exert their positive inotropic effects through the activation of Ca^{2+}-ATPase of SR, ultimately making more calcium available for the contractile proteins. We have shown that xanthone activated the Ca^{2+}-ATPase of the isolated SR vesicles in a dose-dependent manner, 70% activation of the ATPase activity being reached at 300 μM. However, we are unable to conclude, at this point, whether xanthone can activate Ca^{2+}-ATPase in intact muscle resulting in twitch potentiation. Sensitization of the calcium release channel of skeletal muscle by protein kinase A-dependent phosphorylation (Hain *et al.*, 1994) has also been suggested. However, we could not detect any effect on muscle twitch after treatment with the cyclic AMP inducer, forskolin, and phosphodiesterase inhibitor, isobutyl methyl xanthine (data not shown). The exact mechanism by which xanthone exerts its effect on twitch potentiation requires further study.

Both ryanodine and caffeine have been used widely to study the internal Ca^{2+} pool of intact skeletal muscle due to their specificity for the ryanodine sensitive Ca^{2+} release channel; however, their use is still limited to some extent. For example, besides a direct interaction with the ryanodine sensitive Ca^{2+} release channels located on the terminal cisternae of the SR (Kumbarachi & Nastuk, 1982), caffeine can also act on several other sites (Endo, 1977; Røed, 1991) resulting in a complex biphasic contraction. Although specific for its binding site, the effect of ryanodine is largely dependent on the free Ca^{2+} concentration and the activity of muscle (Jenden & Fairhurst, 1969) making it difficult to study the effect of ryanodine in resting muscle. Xanthone and norathyriol represent novel chemicals with structures different from known Ca^{2+} release inducers (Palade, 1987b; Abramson *et al.*, 1988; Valdivia *et al.*, 1992; Beeler & Gable, 1993; Furukawa *et al.*, 1994). The data from this study suggest that norathyriol may be a useful tool for studying the internal Ca^{2+} pool of skeletal muscle in either isolated membrane vesicles or intact muscle.

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