Methyl p-hydroxybenzoate (E-218) a preservative for drugs and food is an activator of the ryanodine receptor Ca²⁺ release channel

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1 Haloperidol is a drug used in the management of several psychotic disorders and its use has been linked to Neuroleptic Malignant Syndrome. In the present study we have investigated the effect of a commercial preparation of haloperidol, Serenase, on skeletal muscle sarcoplasmic reticulum.

2 Addition of Serenase to isolated terminal cisternae caused a rapid release of calcium. We tested whether the active Ca²⁺-releasing substance was haloperidol or another compound present in the preparation.

3 Our results show that methyl p-hydroxybenzoate, one of the preservatives and a commonly used anti-microbial agent (E-218) is an activator of Ca²⁺ release (E.C.50 = 2.0 mM), mediated by a ruthenium red-sensitive Ca²⁺ release channel present in skeletal muscle terminal cisternae.
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Introduction

Neuroleptic Malignant Syndrome (NMS) is a rare life-threatening complication of neuroleptic treatment characterized by fever, muscle rigidity, tachycardia and altered consciousness; its appearance has been linked to the use of high potency anti-psychotic drugs such as haloperidol and usually appears within 2 weeks of initiating neuroleptic treatment or increasing their dose (Kaufmann & Wyatt, 1987; Jahan *et al.*, 1992; Levitt *et al.*, 1990; Tanaka *et al.*, 1998; Konikoff *et al.*, 1984). Creatine phosphate kinase (skeletal muscle isoenzyme) is elevated in approximately 40% of the patients (Meltzer *et al.*, 1996) and the use of dantrolene has had a favourable effect on NMS patients (Bismouth *et al.*, 1984; Tanaka *et al.* 1998). This clinical picture closely resembles that of Malignant Hyperthermia (MH), a pharmacogenetic disease characterized by increase in body temperature and muscle contracture which is triggered by volatile inhalational anaesthetics and myorelaxants (Denborough & Lovell, 1960; MacLennan & Phillips, 1992; Michelson & Louis, 1996). In more than 50% of the cases, the latter inherited disorder has been linked to mutations in the ryanodine receptor gene (MacLennan *et al.*, 1990; McCarthy *et al.*, 1990) and to date more than 18 point mutations have been linked to human MH (for a recent review see Jurkat-Rott *et al.*, 2000). The ryanodine receptor (RYR) is a homotetramer of four 565 kDa subunits that mediates Ca²⁺ release from intracellular stores (Smith *et al.*, 1986). The primary sequence of this protein has been determined from a number of species and Northern blot analysis demonstrated that three isoforms exist, one specific for cardiac muscle, one present in the brain and one specific for skeletal muscle (McPherson & Campbell, 1993).

A large number of chemically diverse substances such as caffeine, thymol, halothane, 4-Cl-m-cresol, doxorubicin, ryanodine, bastadin and Ca²⁺ have been reported to activate the RYR leading to Ca²⁺ release (Palade, 1987; Palade *et al.*, 1989; Mack *et al.*, 1994; Zorzato *et al.*, 1986, 1993; Zucchi & Ronchi-Testoni, 1997). One could thus postulate the presence of multiple activator binding sites present on the large hydrophilic amino terminal domain of the molecule. Because of the similarities between some of the clinical features of NMS and MH we hypothesized that the former syndrome may be due, at least in part, to the effect of the drug formulation on the RYR Ca²⁺ release channel. In the present report we show that a commercial preparation of haloperidol, a neuroleptic most frequently implicated in NMS, is capable of releasing Ca²⁺ from a ruthenium red-sensitive Ca²⁺ release channel selectively localized in terminal cisternae (TC). A closer investigation of the chemical substances present in this preparation, revealed that the compound inducing Ca²⁺-release was not haloperidol *per se*, but rather the preservative methyl p-hydroxybenzoate. These results support the view that *in vivo* the RYR could be the target of chemical substances present as preservatives and additives.

Methods

Materials

Serenase was from Lusofarmaco (Ist. Luso Farmaco Ita. Spa, Milano, Italy); 4-Cl-m-cresol, antipyrylazo-III, haloperidol, propyl p-hydroxybenzoate, methyl p-hydroxybenzoate were from Fluka (Buchs, Switzerland); doxorubicin and the Ca²⁺

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ionophore A23187 were from Calbiochem (San Diego, CA, U.S.A.); fura-2/AM, ionomycin, creatine phosphate kinase, creatine phosphate, ATP, caffeine and ruthenium red were from Sigma Chemicals (St. Louis, MO, U.S.A.); phosphatidyl-ethanolamine (PE), phosphatidylcholine (PC) and phosphati-dylserine (PS) were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The EBV-transformed B-lymphoblastoid cell line GS-EBV was cultured in RPMI 1640, 10% FCS and was a generous gift of Prof Giulio Spagnoli, Universität Kantons-spital (Basel, Switzerland). All other chemicals were reagent or highest available grade.

Subcellular fractionation

Sarcoplasmic reticulum (SR) was isolated from the white muscles of New Zealand White rabbits and was fractionated into longitudinal tubules (LSR) and terminal cisternae (TC) in the presence of antiproteolytic agents as described by Saito *et al.* (1984). The SR fractions were resupended in 0.3 M sucrose, 5 mM imidazole pH 7.4, 100 μM PMSF, 1 μg ml^{-1} leupeptin and were stored in liquid nitrogen until used. Protein concentration was determined according to the method of Bradford, using bovine serum albumin as standard (Bradford, 1976).

Ca^{2+} release measurements

Ca^{2+} release from isolated SR fractions (TC and LSR) was measured in a Beckman DU7400 diode spectrophotometer by monitoring the $A_{710}-A_{790}$ value of the Ca^{2+} indicator antipyrylazo-III as described by Palade (1987). The assay was carried out at 37°C in a medium containing, in a final volume of 1.5 ml, 1 mM MgATP, 10 mM creatine phosphate, 10 μg ml^{-1} creatine phosphate kinase, 200 μg protein, 7.5 mM K-pyrophosphate pH 7.0, 18.5 mM MOPS pH 7.0, 100 mM KCl, 250 μM antipyrylazo-III and 0.1 mM CaCl$_2$. Pulses of 25 nmol calcium were administered to load the SR fractions with approximately 2.3 μM Ca^{2+} mg protein^{-1}. When steady state was reached Ca^{2+} release was triggered by the addition of different compounds. At the end of the experiment the signals were calibrated by the addition of 25 nmol Ca^{2+}. Measure-ments of the initial rate of calcium release were performed with the 'kinetics time program' software package of the Beckman DU7400 spectrophotometer, as previously described (Zorzato *et al.*, 1993).

Intracellular Ca^{2+} measurements

Changes in the intracellular Ca^{2+} concentration of the B-lymphocyte cell line were monitored with the fluorescent calcium indicator fura-2/AM (final concentration 5 μM) as described (Grynkiewwicz *et al.*, 1985; Zorzato *et al.*, 1993). Fluorescent changes (ratio 340/380 nm) were measured in an LS50 Perkin Elmer spectrofluorimeter equipped with a magnetic stirrer and thermostated at 37°C. All measurements were made in Ca^{2+}-free Krebs-Ringer containing 0.5 mM EGTA.

Planar lipid bilayer measurements

TC vesicles were incorporated into planar lipid bilayers of the Mueller/Rudin type (Mueller *et al.*, 1963). Bilayers were formed across the aperture in a 12 μm Teflon septum, using a mixture at a 5:2:3 ratio of PE, PS and PC phospholipids (30 mg ml^{-1} in n-decane). For a full description of the conditions please refer to Buratti *et al.* (1995). Currents were

recorded at 0 mV holding potential and are shown as upwards deflections. Single channel analysis was carried out according to Colquhoun & Sigworth (1983). Channel open probability (Po values) was determined from the areas of Gaussian distributions which best fit to peaks of amplitude histograms from recordings of 60–80 s duration.

Data analysis and curve fitting

Statistical analysis was performed using the student's *t*-test for paired samples. Dose-response curves were fit with the Sigmoidal, Boltzman and Gaussian fit functions using the Origin Microcal 4.1 software program.

Results

We tested the effect of the commercial preparation of haloperidol, Serenase on the initial rate of Ca^{2+} release from actively loaded TC vesicles, by following the Ca^{2+} fluxes spectrophotometrically with the indicator antipyrylazo-III. Addition of 1:10 dil of Serenase caused a rapid release of Ca^{2+} from the TC fraction (Figure 1). We subsequently wanted to establish: (i) what compound in the commercial drug preparation was responsible for this effect and (ii) the mechanism through which calcium was being released. Figure 2 shows the chemical structures of the compounds present in Serenase; each vial contains 2.66 mM haloperidol, 11.1 mM lactic acid, 3 mM propyl p-hydroxybenzoate and 3 mM methyl p-hydroxybenzoate. Each one of these substances was individually tested for its capacity to release Ca^{2+} from actively loaded TC vesicles. Figure 3 shows that neither haloperidol (trace a), nor lactic acid (trace b) nor propyl p-hydroxybenzoate (trace c) significantly affected
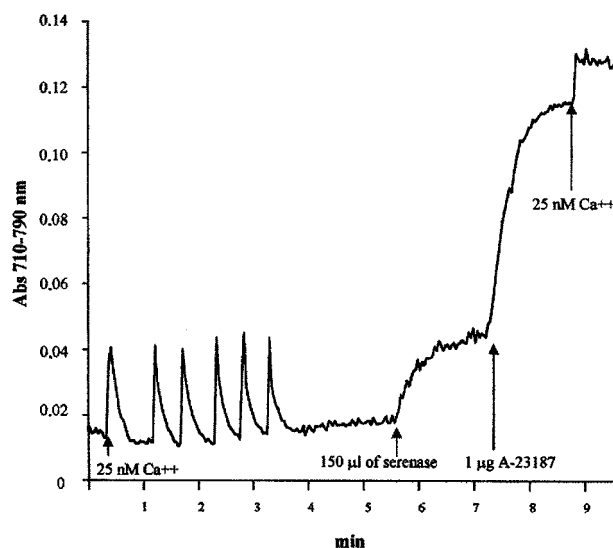
Figure 1 Serenase, a commercial preparation of haloperidol, stimulates Ca^{2+} release from isolated rabbit skeletal muscle terminal cisternae. The experiments were carried out as described in the Methods section, using the Ca^{2+} indicator antipyrylazo-III. Where indicated six consecutive additions of 25 nmol of CaCl$_2$ were administered; after Ca^{2+} loading, 150 μl Serenase (1:10 dilution) were added. At the end of the experiment the Ca^{2+} ionophore A23187 was added to release remaining Ca^{2+}, followed by the addition of 25 nmol CaCl$_2$ used to calibrate the dye response. Upward deflections indicate Ca^{2+} release and downward deflections indicate Ca^{2+} accumulation.
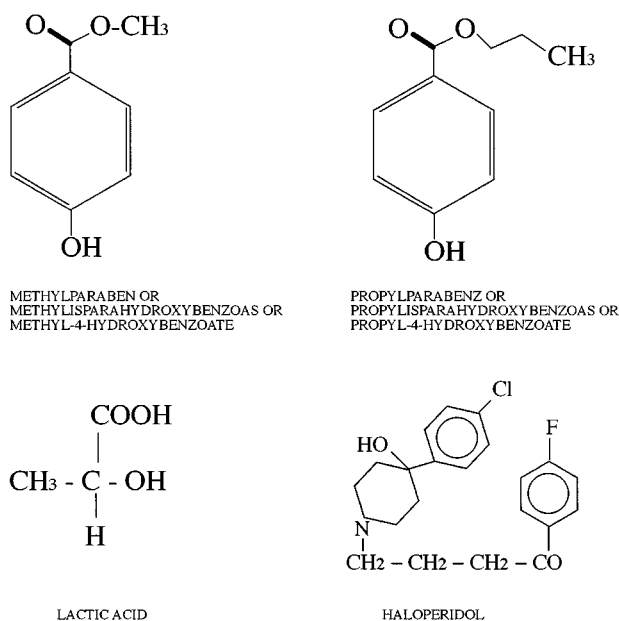
METHYLPARABEN OR
METHYLISPARAHYDROXYBENZOAS OR
METHYL-4-HYDROXYBENZOATE

PROPYLPARABENZ OR
PROPYLISPARAHYDROXYBENZOAS OR
PROPYL-4-HYDROXYBENZOATE

LACTIC ACID   HALOPERIDOL

Figure 2 Chemical structure of the components present in a vial of Serenase.

Ca^{2+} release. The small upwards and downward deflections seen after the addition of haloperidol and lactic acid are due to the presence of small amounts of contaminating Ca^{2+} in these preparations. On the other hand methyl p-hydroxybenzoate (trace d) had a small Ca^{2+} releasing effect. We then tested in greater detail each of these substances for its Ca^{2+} releasing ability; higher concentrations of haloperidol (up to 3 mM), lactic acid (up to 10 mM) and propyl p-hydroxybenzoate (up to 5 mM) had no discernible effect on Ca^{2+} release from TC (not shown). At higher concentrations methyl p-hydroxybenzoate was capable of releasing Ca^{2+}

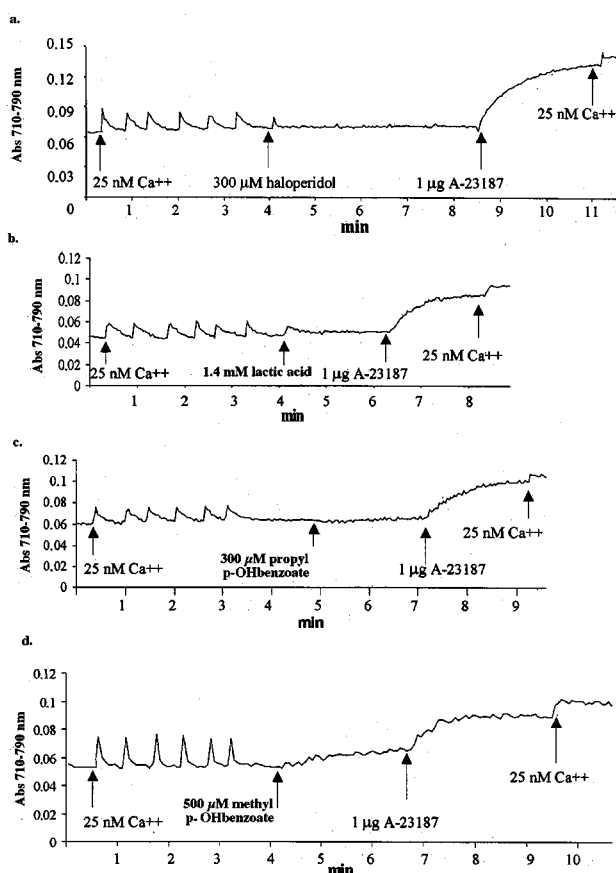**Figure 3** Ca^{2+}-releasing properties of the single components present in Serenase. Conditions were as those described in Figure 1; where indicated the following compounds were added. Trace a, 300 μM haloperidol; trace b, 1.4 mM lactic acid; trace c, 300 μM propyl p-hydroxybenzoate and trace d, 500 μM methyl p-hydroxybenzoate. Traces are representative of experiments carried out at least four times.

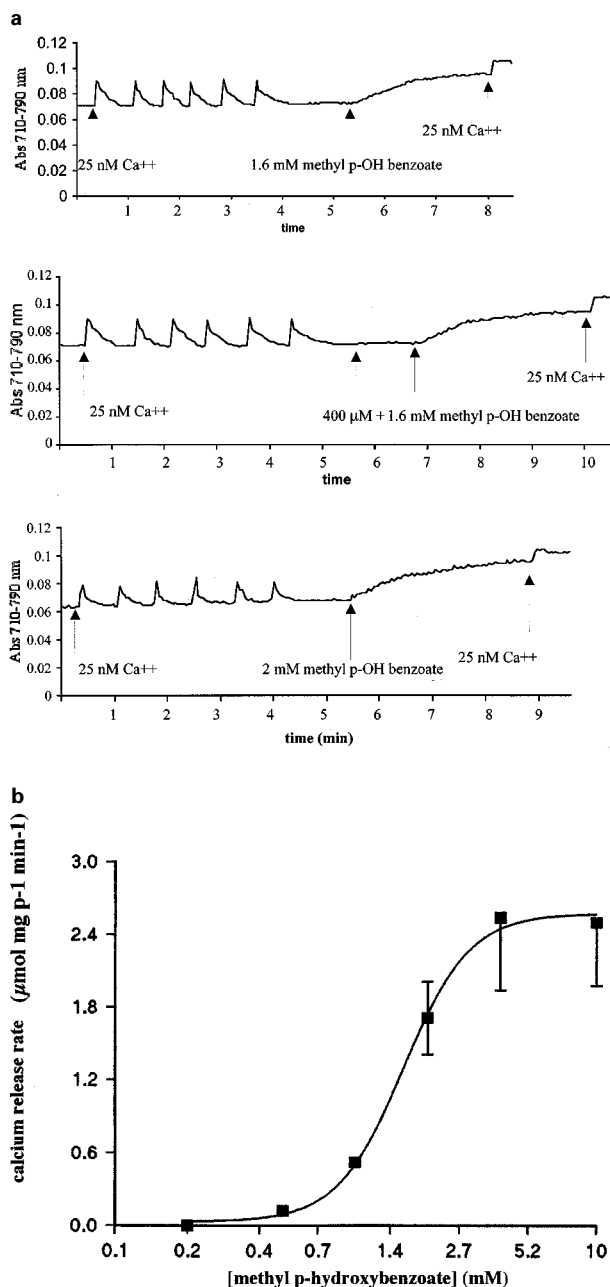**Figure 4** Dose dependence of methyl p-hydroxybenzoate-induced net Ca^{2+} release from rabbit skeletal muscle terminal cisternae. Experiments were carried out as described in Figure 1. (a): Ca^{2+} release was stimulated by the addition of the indicated concentrations of 4 mM methyl p-hydroxybenzoate. The traces are representative of experiments carried out six separate times. (b) Increasing concentrations of methyl p-hydroxybenzoate were added to terminal cisterane in order to obtain the initial rate of Ca^{2+} release. Results are the mean ± standard deviation of four experiments.

from actively loaded TC vesicles (Figure 4a); the addition of successive increasing amounts of this compound did not result in a synergistic stimulation of Ca^{2+} release (Figure 4a). In fact addition of 2 mM or 0.4 plus 1.6 mM methyl p-hydroxybenzoate resulted in a net Ca^{2+}-release rate of 0.59 (± 0.12; $n=6$) and 0.63 (± 0.12; $n=6$) μmoles of Ca^{2+} mg protein^{-1} min^{-1} ($P=0.587$). A similar additive effect was seen at higher concentrations of methyl p-hydroxybenzoate (not shown). Figure 4b shows the dose-dependent effect of methyl p-hydroxybenzoate on the kinetics of Ca^{2+} release from TC. The half-maximal stimulatory dose of Ca^{2+} release was 2 mM whereas at 4 mM this compound released approximately 2.4 μmoles of Ca^{2+} mg protein^{-1} min^{-1}. Such a rate is in the same order of magnitude as that seen with 5 mM caffeine (Palade, 1987) and 1 mM 4-Cl-m-cresol (Zorzato *et al.* 1993). This Ca^{2+}-releasing effect could be due to inhibition of the Ca^{2+}ATPase, to a damage of the vesicles or to a direct effect of the compound on the RYR Ca^{2+} release channel. In order to address this problem LSR vesicles which are enriched in the Ca^{2+}ATPase were tested. In this case, methyl p-hydroxybenzoate failed to elicit any Ca^{2+} release (Figure 5a) indicating that the above mentioned effect (i) could not be due to a damage inflicted on the vesicles and (ii) was not the consequence of an inhibitory action on the Ca^{2+} pump but most likely due to
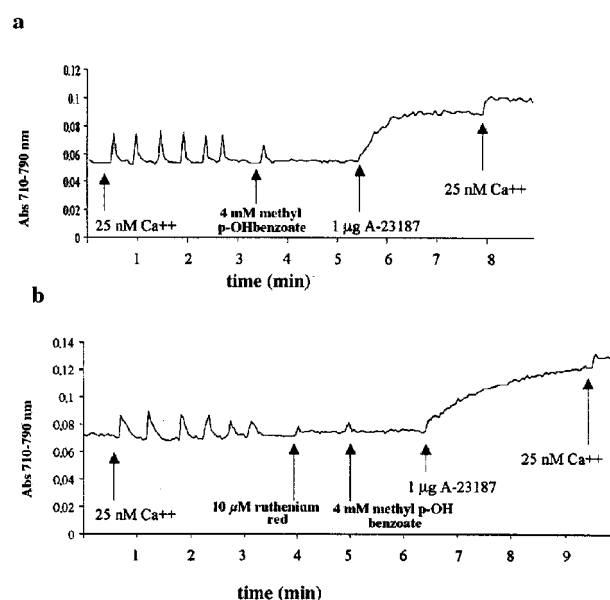
Figure 5 Characteristics of methyl p-hydroxybenzoate-induced Ca^{2+} release. No Ca^{2+} is released from isolated rabbit longitudinal sarcoplasmic reticulum (LSR, trace a) while its effect in terminal cisternae (TC) is blocked by pre-treatment with 10 μM ruthenium red (trace b). Conditions as described in Figure 1.
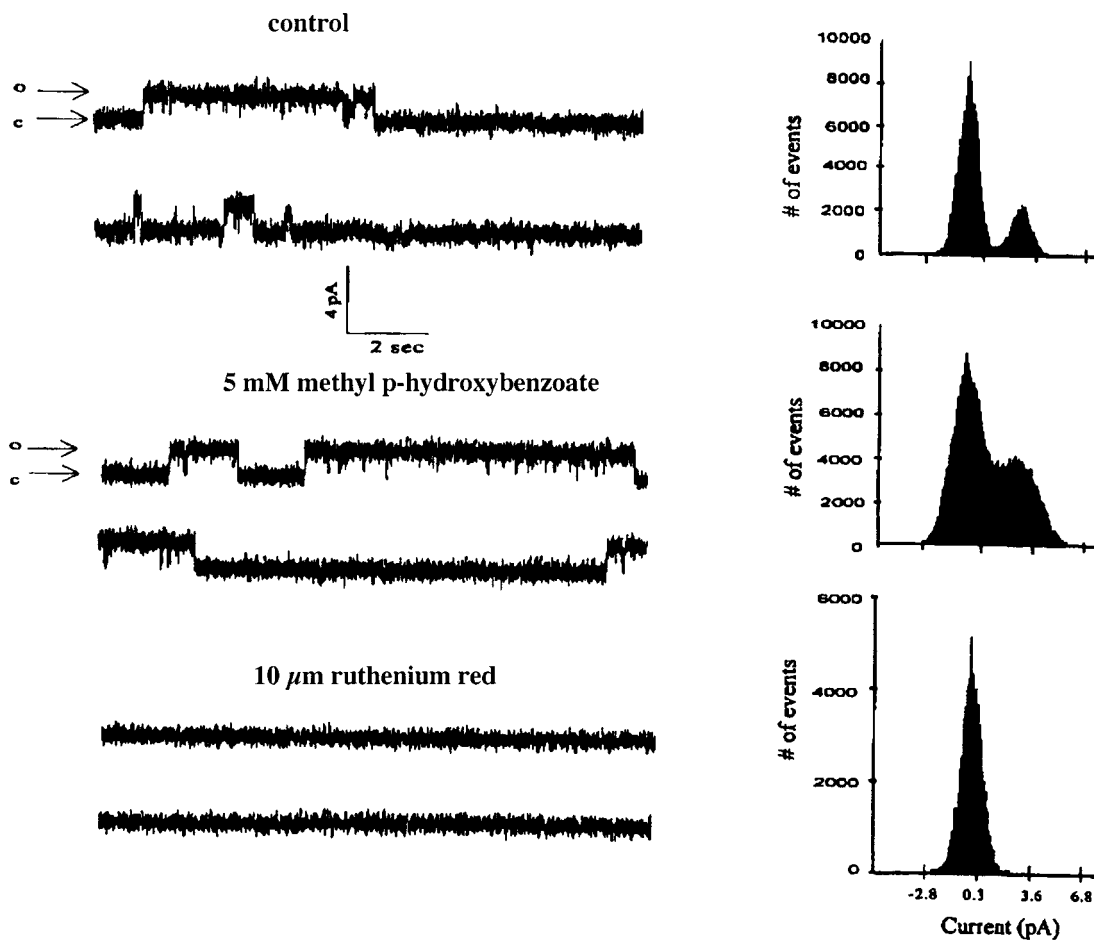
Figure 6 Effect of methyl p-hydroxybenzoate on the open probability of single channels incorporated into lipid bilayers. TC vesicles were fused with lipid bilayers and single channel currents and amplitude histograms were measured as described in the Methods section. Single channel activity, recorded at 0 mV holding potential, exhibited an open level having a conductance of 75 pS and an open probability (Po value) of 0.221 in the controls (top trace) and 0.457 in the presence of 5 mM methyl p-hydroxybenzoate (middle trace). The subsequent addition of 10 μM ruthenium red fully inhibited the current fluctuations (bottom trace). The charge carrier was Na$^+$ in a gradient concentration in the trans/cis compartment of 50/250 mM NaCl; c = closed channel, o = open channel.

activation of the RYR Ca^{2+} channel. Figure 5b shows that pretreatment of TC vesicles with ruthenium red (final concentration 10 μM) a known inhibitor of the RYR, completely abolished the Ca^{2+}-releasing action of methyl p-hydroxybenzoate confirming that its effect is modulated by the RYR intracellular calcium channel.

This effect of methyl p-hydroxybenzoate on the skeletal muscle RYR Ca^{2+} channel was also confirmed by testing the preservative on single channels incorporated into planar lipid bilayers. As shown in Figure 6 addition of 5 mM of the compound to the cis chamber increased the Po from 0.221 (controls) to 0.457 (5 mM methyl p-hydroxybenzoate), corresponding to an increment of $103\pm14\%$ ($n=4$) of the RYR channel probability being in the open state. This effect was reversible and could be inhibited by the addition of 10 μM ruthenium red (bottom trace, Figure 6).

Because methyl p-hydroxybenzoate is present in a variety of formulations, we also tested whether its presence affected the calcium release rate of two other well known RYR activators, doxorubicin and caffeine. The curves depicted in Figure 7a show that the presence or absence of 2 mM methyl p-hydroxybenzoate did not significantly affect the calcium release dose response curve of isolated TC to caffeine. On the other hand, the presence of the preservatives had a small but significant effect on doxorubicin-induced calcium release from isolated TC fractions: in fact it shifted the EC_{50} of doxorubicin from 40 μM to about 25 μM, though the maximal effect was not altered (Figure 7b).

In a recent report Sei *et al*. (1999) show that human B-lymphocytes express the skeletal muscle type 1 ryanodine receptor. We thus tested whether methyl p-hydroxybenzoate affects the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, of a human B-lymphocyte cell line. The addition of 4 mM methyl p-hydroxybenzoate caused a rapid increase in the $[Ca^{2+}]_i$ of fura-2 loaded cells, which slowly (8–10 min) declined back to resting levels. The subsequent addition of 4-Cl-m-cresol, a specific activator of Ca^{2+} release *via* the skeletal muscle RYR Ca^{2+} channel, had no effect on the $[Ca^{2+}]_i$. On the other hand ionomycin was still capable of releasing Ca^{2+} (Figure 8a). Thus the lack of effect of 4-Cl-m-cresol was not due to depletion of all the intracellular Ca^{2+} stores but rather these results suggest that methyl p-hydroxybenzoate and 4-Cl-m-cresol induce Ca^{2+} release by activating the same channel. Figure 8b shows that addition of 4-Cl-m-cresol to the B-cell line caused a large and sustained Ca^{2+} transient, completely abolished the effect of methyl p-hydroxybenzoate and greatly reduced the effect of ionomycin. These experiments demonstrate (i) that the intracellular Ca^{2+} pool that is sensitive to methyl p-hydroxybenzoate is the same as that which is sensitive to 4-Cl-m-cresol and (ii) that the latter compound is more potent at activating Ca^{2+} release. Pre-treatment of the B-cell line with ionomycin completely blocked the Ca^{2+}-releasing effect of methyl p-hydroxybenzoate (Figure 8c); a result consistent with the observation that the methyl p-hydroxybenzoate-sensitive pool is contained within the ionomycin-sensitive one.

Discussion

The initial aim of our study was to investigate whether the pharmacological basis of Neuroleptic Malignant Syndrome, which has been associated with the use of substances such as haloperidol, could be explained by a direct effect of the drug on the RYR. This is the basis of the pharmacogenetic disease MH, which is triggered by volatile anaesthetics and succinylcholine in patients who have been shown to have
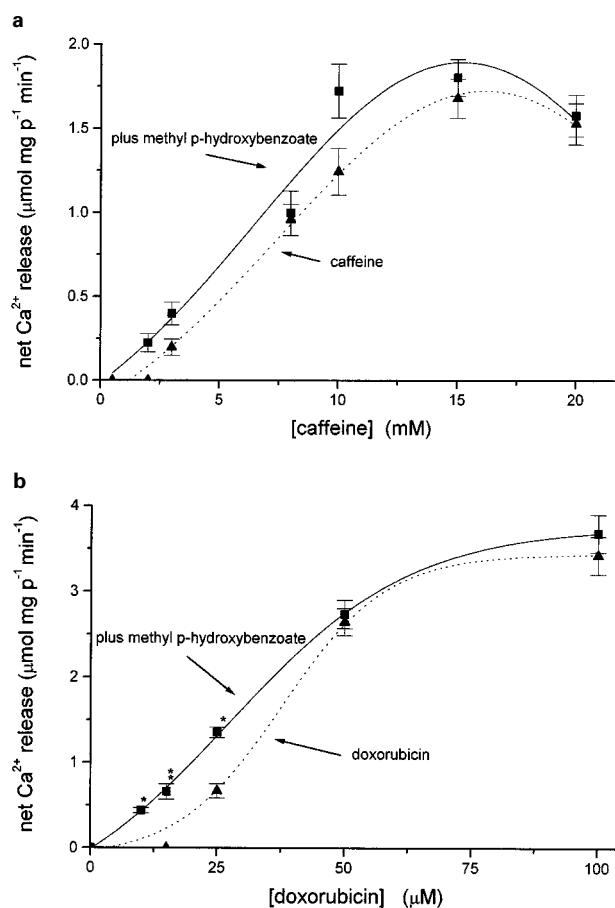
Figure 7 Methyl p-hydroxybenzoate decreases the EC_{50} of doxorubicin-induced Ca_{2+} release but does not affect caffeine-induced calcium release from isolated terminal cisternae. (a) Initial rates of calcium release from terminal cisternae induced by increasing concentrations of caffeine alone or caffeine plus 200 μM methyl p-hydroxybenzoate or (b) doxorubicin alone or doxorubicin plus 200 μM methyl p-hydroxybenzoate. Conditions as in Figure 4b. Results are the mean ($\pm$s.e.mean) of at least eight experiments. *$P<0.0006$; **$P=0.026$.

functional alterations in their RYR Ca^{2+} release channel or other proteins involved in SR calcium homeostasis (MacLennan & Phillips, 1992; Michelson & Louis, 1996). Under our experimental conditions, we could not observe any effect of haloperidol on the RYR Ca^{2+} channel. We report however, that methyl p-hydroxybenzoate is capable of releasing Ca^{2+} from isolated terminal cisternae by directly activating the RYR Ca^{2+} release channel at a concentration similar to that of caffeine a well known RYR activator. The effect is specific since (i) pretreatment of TC vesicles with ruthenium red, an inhibitor of this Ca^{2+} release channel, completely blocks the effect of methyl p-hydroxybenzoate; (ii) the preservative has no effect on LSR vesicles and thus it does not interfere with the Ca^{2+}ATPase and (iii) it does not damage the vesicles. Thus this compound can be added to the growing number of unrelated drugs and chemical substances which have been shown to activate the RYR and induce SR Ca^{2+} release.

Though methyl p-hydroxybenzoate can directly activate Ca^{2+} release, its recommended concentration in drug formulations is in the low millimolar range and thus at this concentration its use is unlikely to trigger activation of the RYR in individuals with a wild type Ca^{2+} channel. However, the association of drug formulations containing other activators of the RYR such as doxorubicin (Zorzato *et al*.,

a

b

c
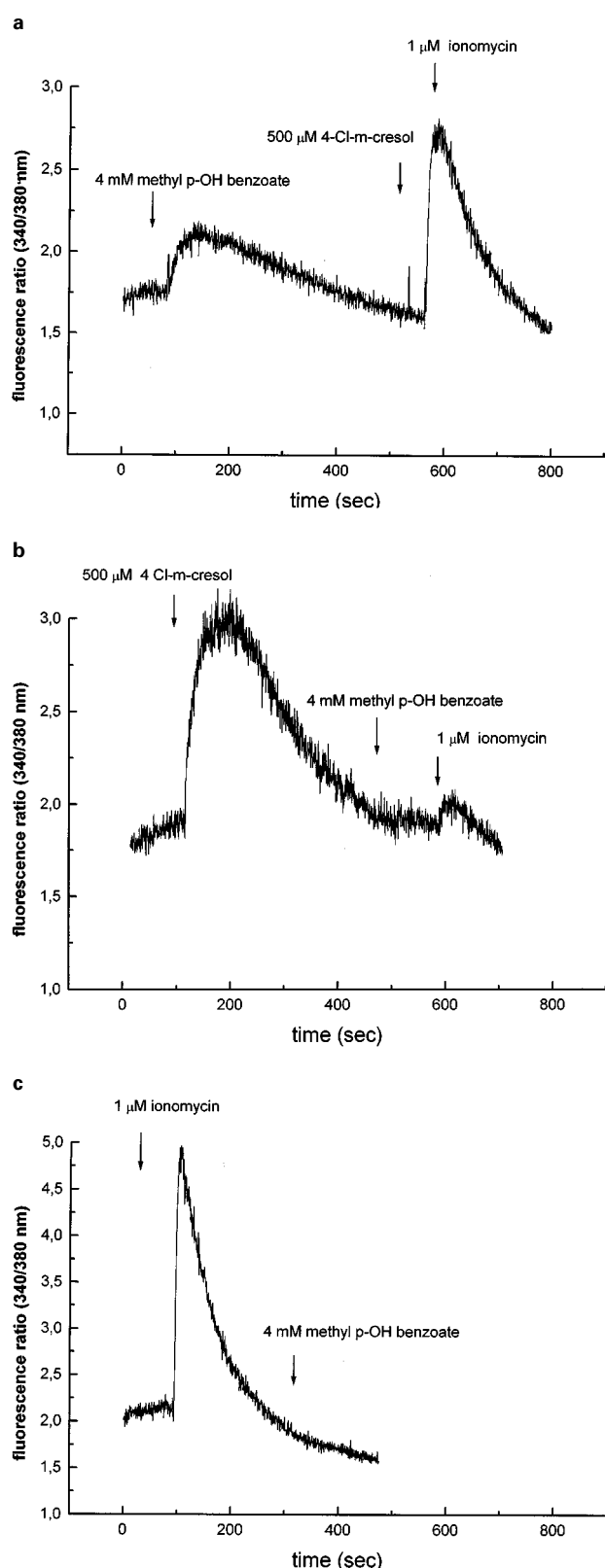
Figure 8 Effect of methyl p-hydroxybenzoate on the $[Ca^{2+}]_i$ of the human B-lymphoblastoid cell line GC-BB. In each experiment 0.5×10^6 cells ml^{-1} loaded with 5 μM fura-2/AM were added to the thermostated, magnetically stirred cuvette and the fluorescence ratio recorded. Once a steady resting $[Ca^{2+}]_i$ was reached, the effect of various agonists on calcium homeostasis was determined. (a) Where indicated, 4 mM methyl p-hydroxybenzoate were added. When $[Ca^{2+}]_i$ returned back to the resting level, 4-chloro-m-cresol and ionomycin were added. (b) Same as (a) except that 4-Cl-m-cresol was added prior to methyl p-hydroxybenzoate. Ionomycin was added at the end to determine the status of the intracellular stores. (c) Same as (a), but ionomycin was added first to deplete all intracellular calcium

1986) and 4-Cl-m-cresol (Zorzato *et al.* 1993), could potentially cause activation of the Ca^{2+} release channel in certain individuals. This conclusion is supported by our observation of the potentiating effect of methyl p-hydroxybenzoate on doxorubicin-induced calcium release. As to its effect, our data suggest that even at such low subthreshold concentrations, the latter compound has a small but significant effect on the EC_{50} for doxorubicin-induced calcium release.

Methyl p-hydroxybenzoate is largely used as an antimicrobial agent in the food industry, where it is short handed E218. According to the Food and Drug Administration methyl p-hydroxybenzoate can be added to food up to a final concentration of 0.1%. Thus, ingestion of 200–300 g of food containing 0.1% E218 would result in the intake of 2–3 mmol of methyl p-hydroxybenzoate. If such an amount reaches the plasma volume, the concentration that would be achieved is quite close to that required for the half maximal-activation of the RYR *in vitro*.

Of interest, our results also show that addition of millimolar concentrations of methyl p-hydroxybenzoate to a human B-lymphocyte-derived cell line causes a rapid and transient increase in the $[Ca^{2+}]_i$. We think that this effect is most likely mediated by direct activation of their RYR1 type calcium channel since (i) in isolated skeletal muscle terminal cisternae and in reconstituted channels incorporated into lipid bilayers, the compound was shown to activate the RYR; (ii) its effect was blocked by ruthenium red, a well unknown inhibitor of the RYR and B-lymphocytes are endowed with type 1 RYR (Sei *et al.*, 1999). The activation of B-lymphocytes could occur in the circulation or more importantly at the level of the mucosa and/ or epithelium lining the gastrointestinal tract. Thus the adsorption of methyl p-hydroxybenzoate may have the undesired side effect of activating B-lymphocytes present in mucosa associated lymphoid tissues. In B-lymphocytes Ca^{2+} signalling has been implicated in various physiological responses including gene expression, cell proliferation and secretion of antigen-specific antibodies (Yamada *et al.*, 1993; Dolmetsch *et al.*, 1997). Sei *et al.* (1999) showed that the skeletal muscle ryanodine receptor present in human B-lymphocytes appears to be involved in calcium signalling *via* the B-cell receptor. Thus it may well be that in some patients who show intolerance or allergy to food, one triggering signal could be represented by the preservative added to the food. Since Ca^{2+} signalling determines the ultimate response of B-cells and particularly secretion of antibodies, the presence of a food additive which can directly cause Ca^{2+} release needs to be carefully reconsidered.

stores. Experiments were performed in Ca^{2+}-free medium containing 0.5 mM EGTA. The traces are representative of at least five separate experiments.

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