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Effects of methyl *p*-hydroxybenzoate (methyl paraben) on Ca^{2+} concentration and histamine release in rat peritoneal mast cells

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1 Mechanisms of methyl *p*-hydroxybenzoate (methyl paraben) action in allergic reactions were investigated by measuring the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and histamine release in rat peritoneal mast cells (RPMCs).

2 In the presence or absence of extracellular Ca^{2+}, methyl paraben (0.1 – 10 mM) increased $[Ca^{2+}]_i$, in a concentration-dependent manner. Under both the conditions, methyl paraben alone did not evoke histamine release.

3 In RPMCs pretreated with a protein kinase C (PKC) activator (phorbol 12-myristate 13-acetate (PMA) 3 and 10 nM), methyl paraben (0.3 – 3 mM) induced histamine release. However, a high concentration (10 mM) of the agent did not increase the histamine release.

4 U73122 (0.1 and 0.5 μM), an inhibitor of phospholipase C (PLC), significantly inhibited the methyl paraben-induced histamine release in PMA-pretreated RPMCs. U73343 (0.5 μM), an inactive analogue of U73122, did not inhibit the histamine release caused by methyl paraben.

5 In Ca^{2+}-free solution, PLC inhibitors (U73122 0.1 and 0.5 μM, D609 1 – 10 μM) inhibited the methyl paraben-induced increase in $[Ca^{2+}]_i$, whereas U73343 (0.5 μM) did not.

6 Xestospongin C (2 – 20 μM) and 2 aminoethoxydiphenyl borate (30 and 100 μM), blockers of the inositol 1,4,5-trisphosphate (IP_3) receptor, inhibited the methyl paraben-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution.

7 In conclusion, methyl paraben causes an increase in $[Ca^{2+}]_i$, which may be due to release of Ca^{2+} from storage sites by IP_3 via activation of PLC in RPMCs. In addition, methyl paraben possibly has some inhibitory effects on histamine release via unknown mechanisms.
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Abbreviations: 2APB, 2 aminoethoxydiphenyl borate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMSO, dimethyl sulphoxide; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PSS, physiological salt solution; RPMCs, rat peritoneal mast cells

Introduction

There have been numerous reports on cases of anaphylactic reactions caused by various drugs (Fisher & More, 1981; Mertes & Laxenaire, 2002). Anaphylactic shock is the type I allergy reaction mediated by IgE antibodies and mast cells. The symptom similar to anaphylactic shock is called an anaphylactoid reaction (Fisher & Pennington, 1982), where the mechanism involves activation of mast cells without IgE antibodies (Stellato & Marone, 1995). A preservative, methyl *p*-hydroxybenzoate (methyl paraben), may be responsible for some cases of anaphylactic shock and anaphylactoid reactions caused by various commercially available medicines (Nagel *et al.*, 1977; Wildsmith *et al.*, 1998). Methyl paraben is nonstimulating and nontoxic, and has a broad antibiotic spectrum. The compound is widely used as a preservative for foods, cosmetics and medicines. Those methyl paraben-containing products caused contact dermatitis and drug hypersensitivity (Larson, 1977; Mowad, 2000), but there has been no fundamental study on allergic reactions related to methyl paraben.

In an immunological mechanism, degranulation of mast cells is triggered off by the aggregation of high-affinity receptor for the Fc region of IgE (FcεRI) caused by crosslinking of IgE by polyvalent antigens. However, specific IgE antibodies for methyl paraben have not been identified (Kokubu *et al.*, 1989). Simple chemicals such as methyl paraben are incapable of producing sensitization and induction of immediate or delayed hypersensitivity without prior conjugation to carrier molecules, usually proteins. The bound methyl paraben is then considered a hapten, whereas its chemical properties are not clear (Soni *et al.*, 2002).

It was reported that methyl paraben activated the ryanodine receptor Ca^{2+} release channel in skeletal muscle terminal cisternae (Cavagna *et al.*, 2000). On the other hand, Teraoka *et al.* (1997) reported that caffeine, an activator of the ryanodine receptor, did not increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat peritoneal mast cells, and Soboloff & Berger (2002) described that ryanodine did not

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significantly increase $[Ca^{2+}]_i$ in bone marrow-derived mast cells. However, the lack of stimulatory effects of caffeine and ryanodine on Ca^{2+} release does not accordingly indicate the absence of ryanodine receptor in many types of nonexcitable cells (Hosoi *et al.*, 2001). The existence of ryanodine receptor is still under controversy, raising the question as to how methyl paraben affects the intracellular events during the allergic reactions.

In the present study, in order to clarify the mechanism of allergic reactions caused by methyl paraben, we investigated the effects of the agent on the changes in $[Ca^{2+}]_i$ and histamine release in RPMCs.

Methods

Mast cell isolation and purification

Male Sprague – Dawley rats weighing 400 – 800 g were anaesthetized with diethyl ether, and then killed by bleeding from the carotid arteries. Rat peritoneal mast cells were isolated and purified over Percoll density gradient as previously described (Chan *et al.*, 2000). The peritoneal cavity was injected with the physiological salt solution (PSS) containing BSA (0.3 mg ml^{-1}). After gentle massage of the rat abdominal region, mixed peritoneal cells (30 – 35 ml of fluid) were obtained by peritoneal lavage. The mixed peritoneal cells were then washed twice by centrifugation (1100 r.p.m., 5 min, 4°C) and were resuspended in 1 ml of PSS. The cell suspension was then mixed with 2 ml of 33% Percoll. BSA-supplemented PSS (1 ml) was then carefully layered over the Percoll-cell mixture. Purification was then performed by centrifugation (3000 r.p.m., 20 min, 4°C), which allowed cell separation and gradient formation. Harvesting of the mast cells posed no problem since these cells gathered in a layer at the bottom of the tube, whereas other cells formed a rather compact layer on top of the gradient and could easily be removed by aspiration. The cell fraction was then washed twice in PSS by centrifugation and finally resuspended to the desired cell density in PSS.

Intracellular calcium measurements

Intracellular Ca^{2+} images were obtained with the confocal laser scanning microscope (IX70, Olympus). Cells were incubated in PSS containing the acetoxymethyl ester of fluo-3 (fluo-3 AM 5 μM) for 30 min at room temperature (22 – 25°C). Cells attached to a glass coverslip were mounted on the bottom of a chamber of 500 μl capacity and placed in the microscope for fluorescence measurements. Cells were excited at 488 nm with an argon laser beam through the objective lens (UplanApo40X, Olympus). Fluo-3 fluorescent images (emission 530 nm) were collected with the scan unit (FVX-SU, Olympus) every 0.42 s. To estimate $[Ca^{2+}]_i$, the mean intensity of cell area except nucleus was calculated with the analysing software (FLUOVIEW FV500, Olympus). The data were expressed as ratios of fluorescence intensity changes (F) relative to control values before stimulation (F_0), namely $(F\text{-}F_0)\ F_0^{-1}$.

Histamine measurements

Drugs were applied to cell suspensions (10^6 cells ml^{-1}), making a total of 1 ml solution. The histamine-releasing reactions were terminated by placing the test tube in ice-cold water for 10 min. Cell suspensions were then centrifuged and divided into fractions of supernatant (0.5 ml) and supernatant plus pellets (0.5 ml), both of which were acidified with perchloric acid (50 μl) to abolish histamine breakdown. After both fractions were centrifuged at 1100 r.p.m. for 10 min at 4°C to remove proteins, secreted histamine was determined by the fluorometric method. Histamine release was expressed as a percentage of the total cell contents. All samples were stored at −25°C until the histamine level was measured using the high-performance liquid chromatography (HPLC) postlabel system as previously described (Yamatodani *et al.*, 1985; Horinouchi *et al.*, 1993). The system was composed of an intelligent pump (Hitachi, L-6200), a reaction pump (Hitachi, 655-A-13), a fluorescence spectrophotometer (Hitachi, F-1150), an autosampler (Hitachi, AS-4000), a Chromato-integrator (Hitachi, D-2500) and a 6 ∅, 15 cm column (Catecholepak, Toyosoda, Tokyo, Japan) warmed at 50°C by a column oven (Hitachi, L-5020). Each 100 μl of supernatant was injected into the HPLC for each sample. The excitation wavelength used was 340 nm and the emission was 450 nm.

Chemicals

The drugs used were BSA, EGTA, methyl paraben, HEPES, phorbol 12-myristate 13-acetate (PMA), compound 48/80, U73343 and U73122 (Sigma Chemicals, St Louis, U.S.A.), dimethyl sulphoxide (DMSO) (Merck, Darmstadt, Germany), A23187, 2APB, xestospongin C and D609 (Calbiochem, San Diego, CA, U.S.A.), Fluo-3 AM (Dojindo Laboratories, Kumamoto, Japan) and Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden).

Solutions

The PSS contained (in mM): NaCl 138.6, KCl 5.0, $MgCl_2$ 1.5, $CaCl_2$ 2.0, HEPES 10, glucose 5.6, KH_2PO_4 1.0, pH 7.2. Ca^{2+}-free solution was made by substituting an equimolar concentration of $MgCl_2$ instead of $CaCl_2$ and adding 0.5 mM EGTA.

Statistics

Data were expressed as mean ± s.e.m. and statistical significance was determined using the paired or unpaired Student's t-test. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Methyl paraben-induced increase in $[Ca^{2+}]_i$ in the presence or absence of external Ca^{2+}

Figure 1a and b shows representative traces of the effects of methyl paraben (3 mM) on $[Ca^{2+}]_i$ in rat peritoneal mast cells (RPMCs). Methyl paraben was applied for 75 s. The agent produced a transient increase in $[Ca^{2+}]_i$ both in Ca^{2+}-containing solution (PSS, 1a) and in Ca^{2+}-free solution (1b). Figure 1c shows the average of the peak values of the changes in $[Ca^{2+}]_i$ induced by methyl paraben (0.1 – 10 mM). The $[Ca^{2+}]_i$ was increased concentration-dependently in the pre-

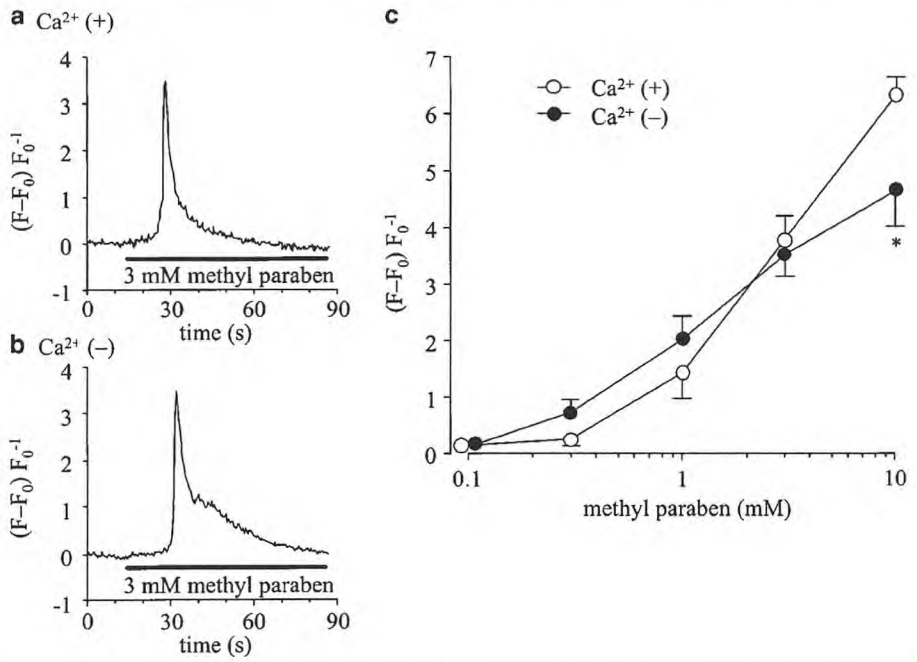

Figure 1 Effects of methyl paraben on $[Ca^{2+}]_i$ in RPMCs. The fluo-3 AM-loaded RPMCs were stimulated with methyl paraben for 75 s at room temperature (22 – 25°C). (a) Methyl paraben (3 mM) increased the $[Ca^{2+}]_i$ in an RPMC incubated in PSS containing 2 mM Ca^{2+}. (b) Methyl paraben was applied in Ca^{2+}-free solution containing 0.5 mM EGTA after a 5 min removal of Ca^{2+}. Also in Ca^{2+}-free condition, methyl paraben increased the $[Ca^{2+}]_i$ in an RPMC. (c) The Concentration – response relation of methyl paraben-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-containing (open circles) and Ca^{2+}-free (filled circles) mediums. The ordinate shows the net maximum $[Ca^{2+}]_i$ of the response with baseline subtracted. The data points indicate mean ± s.e.m. of 20 experiments. The asterisk shows a significant difference in Ca^{2+}-containing and Ca^{2+}-free solutions.

sence of extracellular Ca^{2+}. A similar result was obtained also in the Ca^{2+}-free solution. At a high concentration (10 mM), however, the increase was significantly greater in the Ca^{2+}-containing solution.

Methyl paraben did not evoke histamine release from RPMCs

The amount of histamine release was measured as an index of the degranulation of RPMCs and illustrated in Figure 2. The basal spontaneous release was around 10% of the total contents in RPMCs (control). Compound 48/80 (0.3 μg ml^{-1}) potentiated the histamine release both in PSS and in Ca^{2+}-free solution, increasing it up to 63.9 ± 5.2% ($n=4$) and 34.6 ± 1.9% ($n=4$), respectively. A combination of a Ca^{2+} ionophore (A23187 0.1 μM) and the phorbol ester (PMA 10 nM) caused the release of histamine in PSS (40.9 ± 1.2%, $n=5$). In contrast, methyl paraben did not increase histamine release in PSS (4.32 ± 0.58%, $n=4$, by 3 mM and 9.84 ± 1.70%, $n=4$, by 10 mM). It was also the case in Ca^{2+}-free solution (6.49 ± 0.91%, $n=4$, by 3 mM and 8.92 ± 1.18%, $n=4$, by 10 mM).

Methyl paraben-evoked histamine release from PMA-pretreated RPMCs

Incapability of methyl paraben to release histamine in the present study may be due to insufficient activation of protein kinase C (PKC), since it has been reported that both increase in $[Ca^{2+}]_i$ and activation of PKC are needed for the release (Beaven & Cunha-Melo, 1988). We, therefore, pretreated RPMCs with PMA for 5 min to activate PKC. The spontaneous histamine release was normalized as a relative histamine release of 1.0. Application of methyl paraben alone at 0.3 – 10 mM did not induce histamine release (open circles). In the absence of methyl paraben (control), PMA at 3 nM (closed circles) and 10 nM (closed squares) increased the release to 1.17 ± 0.30 ($n=4$) and 2.05 ± 0.54 ($n=4$) fold, respectively. Pretreatment with PMA for 5 min greatly enhanced the histamine release evoked by methyl paraben (0.3 – 10 mM). The peak increase was 2.23 ± 0.11 times the control increase in the presence of 3 nM PMA ($n=4$, 1 mM methyl paraben) and 1.53 ± 0.53 times the control increase in the presence of 10 nM PMA ($n=4$, 3 mM methyl paraben). A high concentration (10 mM) of methyl paraben, however, did not augment or even suppressed the histamine release from the PMA-pretreated RPMCs. The 10 nM PMA-induced increase in histamine release was suppressed to 0.79 ± 0.18 ($n=4$) of the control increase (Figure 3).

Effects of U73122 and U73343 on histamine release induced by combination of PMA and methyl paraben

To assess the involvement of phospholipase C (PLC) in the histamine release reaction by methyl paraben in the PMA-pretreated RPMCs, effects of a PLC inhibitor U73122 and its inactive analogue U73343 were examined. The spontaneous histamine release in PSS containing 2 mM Ca^{2+} was normalized as a relative histamine release of 1.0. A volume of 10 nM of PMA increased the release to 1.80 ± 0.17 fold ($n=12$), and U73122 (0.5 μM) did not significantly inhibit the increase ($n=12$). The histamine release caused by methyl paraben (3 mM) applied 5 min after application of PMA (10 nM) was

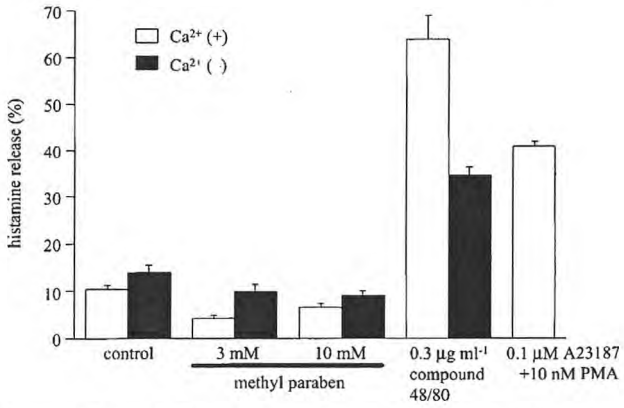

Figure 2 Effects of methyl paraben (3 and 10 mM) and various stimuli on histamine release from RPMCs. Cells were preincubated for 5 min at 37°C and subsequently incubated with methyl paraben (3 and 10 mM), compound 48/80 (0.3 μg ml^{-1}) or a combination of A23187 (0.1 μM) and PMA (10 nM) for 30 min in the external solution with (open columns) or without (closed columns) Ca^{2+}. The released histamine in the 30 min period was calculated as a percentage of the total histamine content of the cells. Results are expressed as mean ± s.e.m. of eight to 10 experiments.

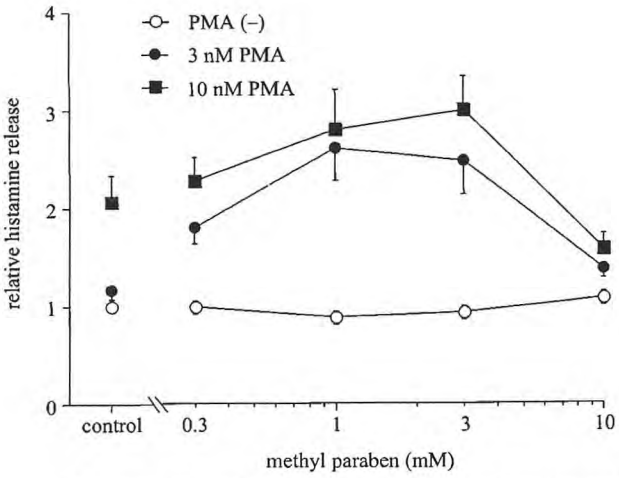

Figure 3 Effects of combined application of methyl paraben and PMA on histamine release in PSS. Cells were preincubated for 5 min at 37°C in the external solution with Ca^{2+} and subsequently incubated without (open circles) or with PMA (3 nM, closed circles or 10 nM, closed squares) for 5 min. The cells were then stimulated with methyl paraben (3 mM) for 30 min in the continuous presence or absence of PMA. The spontaneous histamine release was used for normalization as a relative histamine release of 1.0. The symbols refer to the mean of four to 12 experiments and the error bars represent s.e.m.

3.86 ± 0.28 times the control ($n = 5$). U73122 significantly reduced this augmented histamine release by the combination of PMA and methyl paraben. The normalized inhibitory value by U73122 on the combination of PMA and methyl paraben-induced augmentation was 0.68 ± 0.04 ($n = 5$) and 0.10 ± 0.02 ($n = 5$) at 0.1 and 0.5 μM, respectively. In contrast, U73343 (0.5 μM) did not inhibit the histamine release caused by the combined application of PMA and methyl paraben (Figure 4).

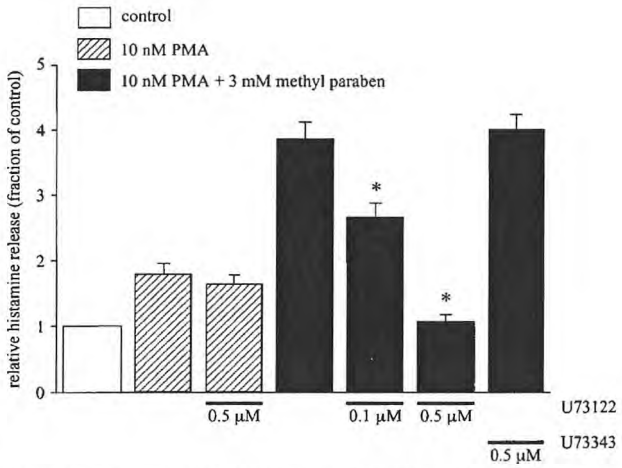

Figure 4 Effects of U73122 and U73343 on histamine release induced by a combination of PMA and methyl paraben. After preincubation for 5 min at 37°C in PSS, cells were pretreated with PMA (10 nM) or a combination of PMA and U73122 (0.1 and 0.5 μM) or U73343 (0.5 μM) for 5 min. The cells were then stimulated with methyl paraben (closed columns) or not (hatched columns) and incubated for 30 min in PSS with continued treatment of PMA in the continuous presence or absence of U73122 or U73343. The spontaneous histamine release was used for normalization as a relative histamine release of 1.0 (open column). Results are expressed as mean ± s.e.m. of five to 12 experiments. Asterisks indicate a significant difference from the combined application of PMA and methyl paraben in the absence of U73122 or U73343 with $P < 0.05$.

Effects of U73122, U73343 and D609 on increase in $[Ca^{2+}]_i$ induced by methyl paraben

We investigated the effects of U73122, U73343 and another PLC inhibitor D609 to confirm that the increase in $[Ca^{2+}]_i$ caused by methyl paraben was induced through activation of PLC in RPMCs. The change in $[Ca^{2+}]_i$ caused by 3 mM of methyl paraben in the Ca^{2+}-free solution containing 0.5 mM EGTA was used as a control (Figure 5a). Pretreatment with U73122 (0.5 μM) for 5 min markedly inhibited the $[Ca^{2+}]_i$ increase, while U73343 (0.5 μM) did not (Figure 5b). D609 at 10 μM completely suppressed the increase (Figure 5c). Figure 5d illustrates the effects of U73312 (0.1 and 0.5 μM), U73343 (0.5 μM) and D609 (1 – 10 μM) on the peak increase in $[Ca^{2+}]_i$ caused by 3 mM of methyl paraben. U73122 (0.1 and 0.5 μM) and D609 (1 – 10 μM) inhibited the increases in $[Ca^{2+}]_i$ in a concentration-dependent manner, whereas the inactive analogue (U73343 0.5 μM) did not.

Effects of xestospongin C and 2APB on increase in $[Ca^{2+}]_i$ induced by methyl paraben

PLC increases the cytosolic levels of inositol 1,4,5-trisphosphate (IP_3) and DAG in mast cells (White *et al.*, 1985). IP_3 stimulates release of Ca^{2+} from internal stores (Meyer *et al.*, 1988; Berridge, 1993) and DAG is known to activate PKC (Nishizuka, 1984). We investigated the effects of xestospongin C and 2 aminoethoxydiphenyl borate (2APB), inhibitors of IP_3-induced Ca^{2+} release, on the methyl paraben-induced increase in $[Ca^{2+}]_i$ in RPMCs. Figure 6a depicts a control increase in the $[Ca^{2+}]_i$ induced by methyl paraben (3 mM) in the Ca^{2+}-free solution containing 0.5 mM EGTA. Pretreat-

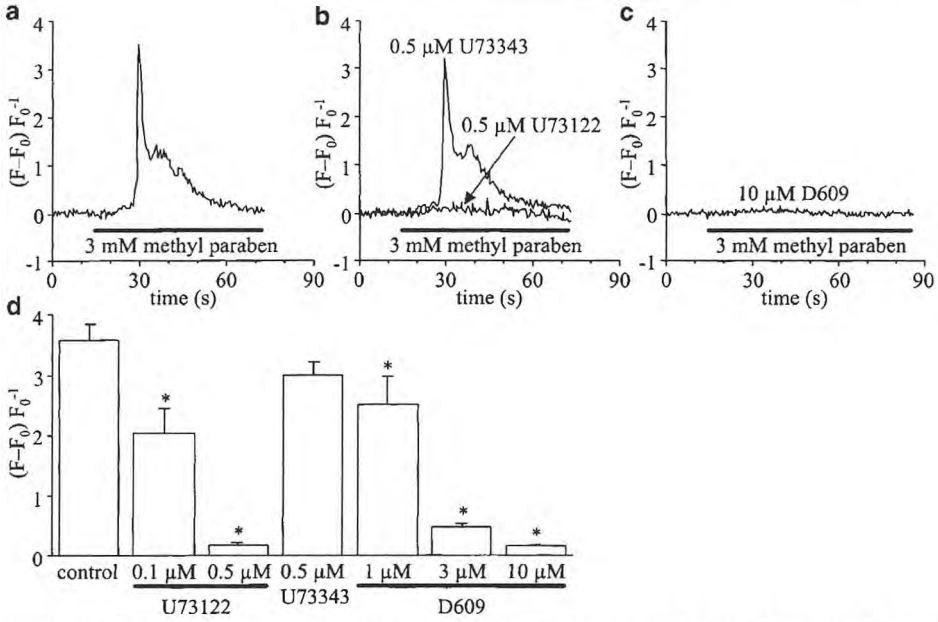

Figure 5 Effects of U73122, U73343 and D609 on the increase in $[Ca^{2+}]_i$ induced by methyl paraben in Ca^{2+}-free solution containing 0.5 mM EGTA. Methyl paraben (3 mM) was applied for 60 – 75 s following a 5 min incubation in the Ca^{2+}-free solution. (a) Methyl paraben increased the $[Ca^{2+}]_i$ in a fluo-3 AM-loaded RPMC. (b) Pretreatment good for 5 min with U73122 (0.5 μM) markedly suppressed the increase, while U73343 (0.5 μM) had no effects. (c) Pretreatment for 5 min with D609 (10 μM) completely blocked the effect of methyl paraben. (d) Each column indicates the average of the peak increase above the base line ($n = 8 - 10$). U73122 and D609 suppressed the $[Ca^{2+}]_i$ increase dose-dependently. The reduction by U73343 was not significant. Bars indicate s.e.m. Asterisks indicate significant difference from the control with $P < 0.05$.

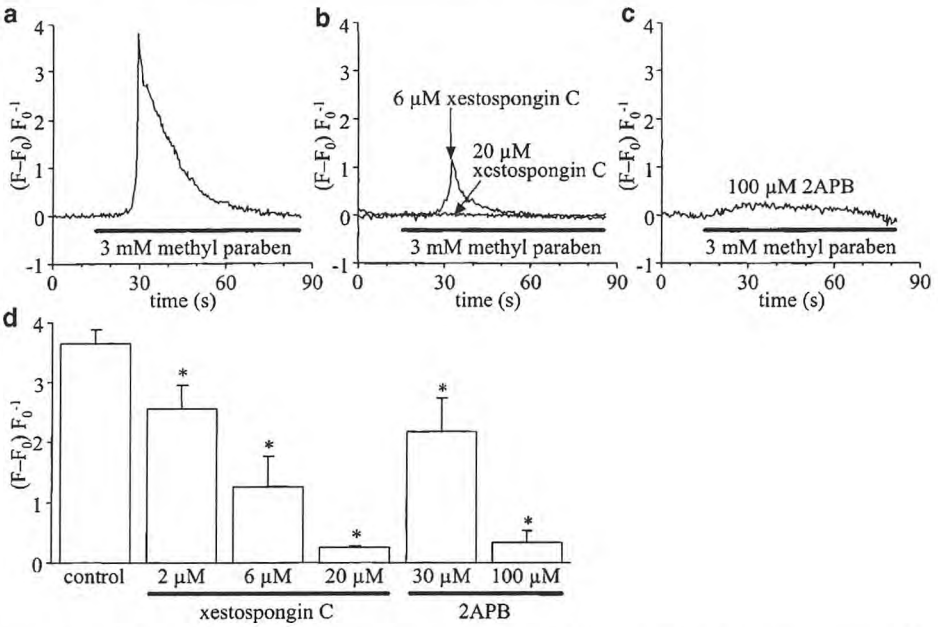

Figure 6 Effects of xestospongin C and 2APB on the increase in $[Ca^{2+}]_i$ induced by methyl paraben in Ca^{2+}-free solution containing 0.5 mM EGTA. Methyl paraben (3 mM) was applied for 70 – 75 s in Ca^{2+}-free solution after a 5 min removal of Ca^{2+}. (a) Methyl paraben increased the $[Ca^{2+}]_i$ in a fluo-3 AM-loaded RPMC. (b) Pretreatment for 5 min with xestospongin C (6 or 20 μM) markedly suppressed the $[Ca^{2+}]_i$ increase. (c) Pretreatment for 5 min with 2APB (100 μM) greatly decreased the $[Ca^{2+}]_i$ increase. (d) Each column indicates the average of the peak increase above the base line ($n = 7 - 10$). Xestospongin C and 2APB dose-dependently suppressed the $[Ca^{2+}]_i$ increase produced by methyl paraben. Bars indicate s.e.m. Asterisks show significant suppression from the control with $P < 0.05$.

ment with xestospongin C (6 and 20 μM; Figure 6b) or 2APB (100 μM; Figure 6c) markedly inhibited the increase in $[Ca^{2+}]_i$. Effects of the IP_3 inhibitors on the peak increase in $[Ca^{2+}]_i$ are shown in Figure 6d. Xestospongin C (2 – 20 μM) and 2APB (30 and 100 μM) suppressed the increases in $[Ca^{2+}]_i$ in a concentration-dependent manner.

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