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Analysis of the stimulative effect of thapsigargin, a non-TPA-type tumour promoter, on arachidonic acid metabolism in rat peritoneal macrophages

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1 At concentrations above 10 ng ml^{-1}, the tumour promoter thapsigargin stimulates the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages harvested from rat peritoneal cavity.

2 The release of radioactivity from prelabelled macrophages was augmented more than additively when the cells were incubated in the medium containing both thapsigargin (10 ng ml^{-1}) and other tumour promoters (10 ng ml^{-1}), such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), teleocidin and aplysiatoxin.

3 Thapsigargin required extracellular Ca^{2+} for the stimulation of arachidonic acid release, while TPA did not.

4 Cytoplasmic free calcium level was increased by thapsigargin treatment but not by TPA treatment.

5 An inhibitor of protein kinases, H-7 inhibited the effect of TPA dose-dependently, whereas H-7 did not inhibit that of thapsigargin.

6 These results suggest that thapsigargin stimulates arachidonic acid release by a mechanism different from that of TPA, *viz* by acting as a selective Ca^{2+} mobilizer, but not by activating protein kinase C as TPA does.

Introduction

Thapsigargin, a hexaoxygenated tetraacylated sesquiterpene lactone, is a major skin irritating constituent isolated from the roots of *Thapsia garganica* L. (Apiaceae) (Christensen *et al.*, 1982; Christensen & Norup, 1985) and has potent histamine releasing activity in rat peritoneal mast cells (Rasmussen *et al.*, 1978; Ali *et al.*, 1985; Ohuchi *et al.*, 1986). Recently, Hakii *et al.* (1986) found that thapsigargin is a tumour promoter in a two-stage mouse skin carcinogenesis experiment. The percentage of tumour bearing mice in the group treated with 7,12-dimethylbenz(a)anthracene plus thapsigargin was 53.5 in week 22 but the group treated with thapsigargin alone did not produce any tumours. Since thapsigargin induces the activity of histidine decarboxylase but not ornithine decarboxylase in mouse skin, and does not bind to phorbol ester receptor in a particulate fraction of mouse skin, thapsigargin was classified as a non-TPA (12-*O*-tetradecanoylphorbol-13-acetate)-type tumour promoter (Hakii *et al.*, 1986). In a previous paper (Ohuchi *et al.*, 1985), we showed that palytoxin, another non-TPA-type tumour promoter, and TPA-type tumour promoters such as TPA, teleocidin (Fujiki *et al.*, 1982a) and aplysiatoxin (Fujiki *et al.*, 1982b) stimulate arachidonic acid metabolism in rat peritoneal macrophages at very low concentrations. Recently, we found that thapsigargin, also at very low concentrations, stimulates arachidonic acid metabolism in rat peritoneal macrophages (Ohuchi *et al.*, 1987a),

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and suggested that tumour promoting activity is associated with the stimulation of arachidonic acid metabolism, irrespective of the type of substance under investigation (Ohuchi *et al.*, 1985; 1987a). The aim of the present paper was to compare the mechanism by which thapsigargin stimulates the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages with that of the TPA-type tumour promoters.

Methods

Preparation of peritoneal macrophages

Male rats of the Sprague-Dawley strain, specific pathogen free (Charles River Japan, Inc., Kanagawa, Japan), weighing 300–350 g, were used. The peritoneal macrophages were prepared according to a procedure described previously (Ohuchi *et al.*, 1985). In brief, a solution containing soluble starch (Wako Pure Chemical Ind., Tokyo, Japan) and bacto peptone (Difco Lab., Detroit, MI, U.S.A.), 5% each, was injected into the rats intraperitoneally under light diethyl ether anaesthesia at a dose of 5 ml $100\,g^{-1}$ body weight. The stimulant solution was autoclaved at 120°C for 15 min and cooled to room temperature before injection. Four days after the injection, the rats were killed by cutting the carotid artery under diethyl ether anaesthesia and peritoneal cells were harvested as described previously (Ohuchi *et al.*, 1985).

Macrophage culture

The peritoneal cells were suspended in Eagle's minimum essential medium (Nissui, Inc., Tokyo, Japan) supplemented with 10% (v/v) calf serum (Flow Lab., North Rydge, N.S.W., Australia), penicillin G potassium ($30\,mg\,l^{-1}$) and streptomycin sulphate ($100\,mg\,l^{-1}$) (Meiji Seika Co., Tokyo, Japan). The macrophages were seeded at 6×10^6 cells per 60 mm Falcon tissue culture dish (Div. Becton, Dickinson and Co., Cockeysville, MD, U.S.A.) in 4 ml of the medium and incubated for 2 h at 37°C. After the incubation, the dishes were washed three times with the medium to wash-out non-adherent cells. The adherent cells were further incubated for 20 h with 4 ml of medium containing 1 μCi of [^{3}H]-arachidonic acid ($61\,Ci\,mmol^{-1}$, New England Nuclear, Boston, MA, U.S.A.) to label the cellular lipids (Ohuchi *et al.*, 1985). Analysis of the radioactive materials in the cells after extraction with 2:1 chloroform:methanol (v/v) showed that 71% of the radioactivity was associated with phospholipids, 11% with triglycerides, 16.5% with unidentified materials, 1% with prostaglandins, and 0.5% with free arachidonic acid (Ohuchi *et al.*, 1981). More than 95% of the adherent cells were found to engulf or attach to sheep red blood cells when examined 2 h after the incubation in the medium containing sheep red blood cells (Ohuchi *et al.*, 1981).

Measurement of the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages

After the 20 h incubation, the cells were washed five times with 2 ml of medium to remove free [^{3}H]-arachidonic acid. The cells were then incubated with tumour promoters in 4 ml of medium containing bovine albumin (Essential fatty acid-free, Sigma Chemical Co., St Louis, MO, U.S.A.) at $100\,\mu g\,ml^{-1}$ instead of calf serum (Ohuchi *et al.*, 1987a). At appropriate times of incubation, 100 μl of the medium was withdrawn and counted for the released radioactivity.

Measurement of cytoplasmic free calcium level

The method for loading cells with quin 2 was essentially the same as described by Tsien *et al.* (1982). The peritoneal cells were incubated for 30 min at 37°C in HEPES-buffered Hank's solution (HBHS, pH 7.4) containing 20 μM of quin 2/AM (Dojinkagaku Inst., Tokyo, Japan). After 30 min, the medium was diluted 10 times by adding HBHS and further incubated for 30 min at 37°C. Then, the cells were washed twice with phosphate buffered saline (Ca^{2+}, Mg^{2+}-free). Quin 2-loaded cells (3 ml), suspended as 10^7 cells ml^{-1} in a simplified saline solution (Tsien *et al.*, 1982), were equilibrated in a cuvette at 37°C for 5 min. Fluorescence was measured continuously in a Shimadzu RF-540 spectrofluorometer (Shimadzu Co., Tokyo, Japan) at 37°C with monochromator settings of 492 nm emission and 339 nm excitation. The addition of 30 μl dimethylsulphoxide, the vehicle for A23187 (Hoechst Japan Ltd., Tokyo, Japan) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Sigma Chemical Company, St Louis, MO, U.S.A.), or 30 μl ethanol, the vehicle for TPA and thapsigargin, had no effect in the measurement.

Tumour promoters

TPA was purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Teleocidin was isolated from *Streptomyces mediocidicus* (Fujiki *et al.*, 1982a). Aplysiatoxin was isolated from marine blue-green alga, *Lyngbya majuscula* (Fujiki *et al.*, 1982b). Thapsigargin was isolated from an ethanolic extract of *Thapsia garganica* L. (yield 0.1% of fresh material) (Rasmussen *et al.*, 1978). The systematic name of thapsigargin is as follows: 6-(acetoxy)-2,3,3a,4,5,6,6a,

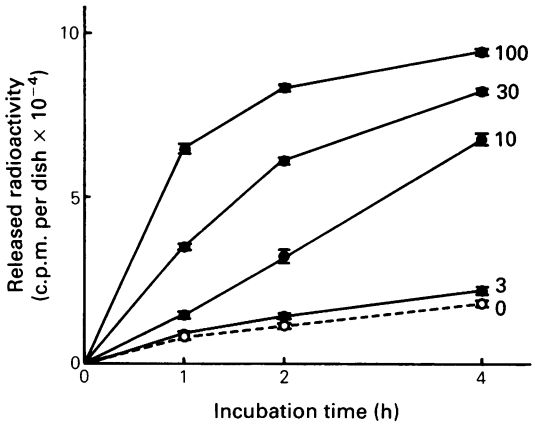

Figure 1 Effects of varying concentrations of thapsigargin on the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages. [^{3}H]-arachidonic acid-labelled macrophages (6×10^6 cells) were incubated at 37°C in 4 ml of the medium containing indicated concentrations of thapsigargin (ng ml^{-1}). Concentrations of thapsigargin at 3, 10, 30 and 100 ng ml^{-1} are 4.6, 15.4, 46.2 and 153.8 nM, respectively. Each point represents the mean from 4 dishes; vertical lines indicate s.e.mean. The results were confirmed by two additional experiments.

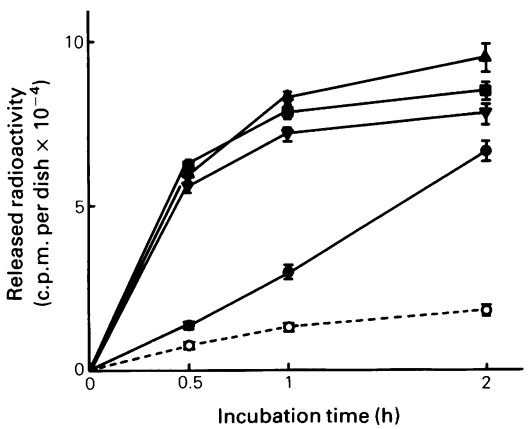

Figure 2 Effects of thapsigargin, TPA, teleocidin and aplysiatoxin on the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages. [^{3}H]-arachidonic acid-labelled macrophages (6×10^6 cells) were incubated for 2 h at 37°C in 4 ml of the medium containing 10 ng ml^{-1} of thapsigargin (●), TPA (▼), teleocidin (■) and aplysiatoxin (▲). The broken line represents control. Concentrations of 10 ng ml^{-1} of thapsigargin, TPA, teleocidin and aplysiatoxin are 15.4, 16.2, 22.5 and 14.9 nM, respectively. Values are the means from 4 dishes; vertical lines indicate s.e.mean. The results were confirmed by a separate experiment.

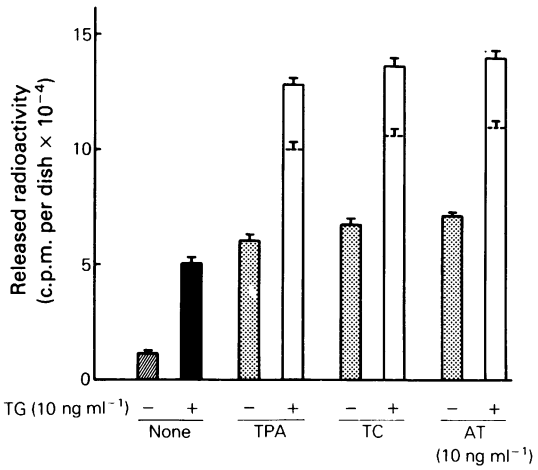

Figure 3 Effects of a combined treatment with thapsigargin plus TPA, thapsigargin plus teleocidin, and thapsigargin plus aplysiatoxin on the release of radioactivity from prelabelled macrophages. [^{3}H]-arachidonic acid-labelled macrophages (6×10^6 cells) were incubated for 2 h at 37°C in 4 ml of the medium containing indicated concentrations of the drug. Radioactivity was measured 2 h after the incubation. Values are the means from 4 dishes; vertical lines indicate s.e.mean. Broken lines in the open columns show the additive values for each net increase in the release of radioactivity induced by thapsigargin (TG) and TPA, TG and teleocidin (TC) or TG and aplysiatoxin (AT). These were significantly different ($P < 0.001$) from measured values. Concentrations of 10 ng ml^{-1} of TG, TPA, TC and AT are 15.4, 16.2, 22.5 and 14.9 nM, respectively. The results were confirmed by two additional experiments.

7,8,9b-decahydro-3,3a-dihydroxy-3,6,9-trimethyl-8-[(2-methyl-1-oxo-2-butenyl)oxy]-2-oxo-4-(1-oxobutoxy)azuleno[4,5-*b*]furan-7-yl octanoate. Each tumour promoter was dissolved in ethanol and added to the incubation medium. The final concentration of ethanol was adjusted to 0.1%. The control medium contained the same amount of the vehicle.

Other chemicals

An inhibitor of protein kinases, H-7 (Hidaka *et al.*, 1984; Kawamoto & Hidaka, 1984) (1-(5-isoquinoline-sulphonyl)-2-methylpiperazine dihydrochloride) was purchased from Seikagaku Kogyo Ltd, Tokyo, Japan.

Statistical analysis

Results were analysed for statistical significance by Student's *t* test for paired observations.

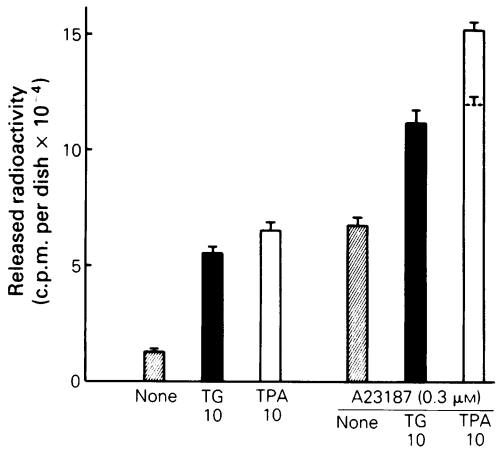

Figure 4 Effects of A23187 on thapsigargin- or TPA-stimulated release of radioactivity from prelabelled macrophages. [^{3}H]-arachidonic acid-labelled macrophages (6×10^6 cells) were incubated for 2h in the medium containing thapsigargin (TG, 10 ng ml^{-1}) or TPA (10 ng ml^{-1}) with or without A23187 (0.3 μM). Broken line in the open column shows the additive value for each net increase in the release of radioactivity induced by TPA and A23187. This was significantly different ($P < 0.001$) from the measured value. Concentrations of 10 ng ml^{-1} of TG and TPA are 15.4 and 16.2 nM, respectively. Values are the means from 4 dishes; vertical lines indicate s.e.mean. The results were confirmed by two additional experiments.

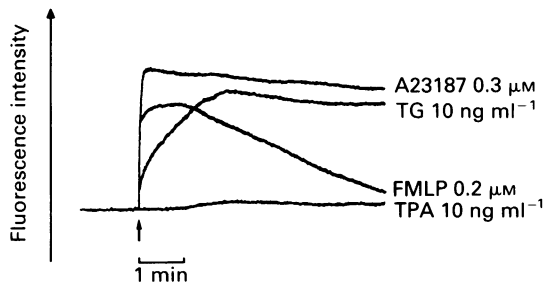

Figure 5 Changes in cytoplasmic free calcium level after treatment with thapsigargin, TPA, A23187 and FMLP. Additions of thapsigargin (TG, 10 ng ml^{-1}), TPA (10 ng ml^{-1}), A23187 (0.3 μM) and FMLP (0.2 μM) are shown by the arrow. The peritoneal cells were loaded with quin 2 and measurements of fluorescence changes were made in a simplified saline solution (Tsien *et al.*, 1982). Concentrations of 10 ng ml^{-1} of TG and TPA are 15.4 and 16.2 nM, respectively.

Results

Thapsigargin stimulated the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages in a dose-dependent manner as shown in Figure 1. At 3 ng ml^{-1} thapsigargin, no significant increase in the release of radioactivity was observed. At 10 ng ml^{-1} thapsigargin, the release of radioactivity continued to increase linearly until 4h of incubation. In contrast to thapsigargin, treatment with TPA, teleocidin or aplysiatoxin each at 10 ng ml^{-1}, induced a rapid release of radioactivity within 30 min of incubation (Figure 2). When the labelled macrophages were incubated for 2h in the medium containing both thapsigargin and TPA, each 10 ng ml^{-1}, the release of radioactivity was stimulated more than additively ($P < 0.001$) (Figure 3). Augmentation ($P < 0.001$) was also observed when the cells were incubated for 2h in the medium containing both thapsigargin

Table 1 Effects of extracellular Ca^{2+} on the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages

Treatment	*$CaCl_2$ (0.9 mM)*	*Released radioactivity* (c.p.m. per dish × 10^{-3})
None	−	5.37 ± 0.27
	+	5.11 ± 0.24
Thapsigargin (10 ng ml^{-1})	−	4.89 ± 0.31
	+	8.06 ± 0.23*,a
Thapsigargin (30 ng ml^{-1})	−	4.92 ± 0.15
	+	12.50 ± 0.39*,a
TPA (10 ng ml^{-1})	−	9.04 ± 0.24a
	+	11.34 ± 0.22*,a

[^{3}H]-arachidonic acid-labelled macrophages (6×10^6 cells) were incubated for 1h at 37°C in 4 ml of a phosphate buffered saline solution (composition in mM: NaCl 137, KCl 2.7, $Na_2HPO_4 \cdot 12H_2O$ 8.1, KH_2PO_4 1.5 and $MgCl_2 \cdot 6H_2O$ 0.5; pH 7.4) with or without 0.9 mM $CaCl_2$. Concentrations of thapsigargin 10, 30 ng ml^{-1} and TPA 10 ng ml^{-1} are 15.4, 46.2 and 16.2 nM, respectively. Values are the means ± s.e.mean from 4 dishes. Similar results were obtained in two additional experiments. Statistical significance: *$P < 0.001$ vs corresponding Ca^{2+}-free control; a$P < 0.001$ vs no treatment (None).

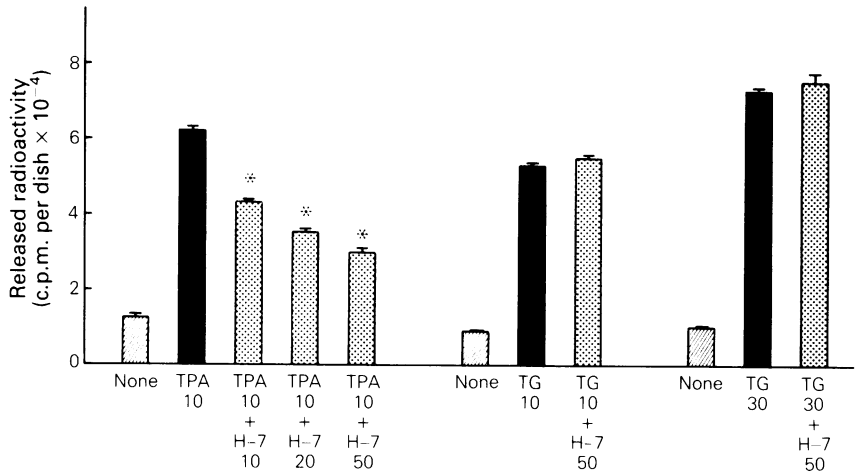

Figure 6 Effects of H-7 on TPA- and thapsigargin-induced release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages. [^{3}H]-arachidonic acid-labelled macrophages (6×10^6 cells) were incubated for 4 h at 37°C in 4 ml of the medium containing indicated concentrations of TPA (10 ng ml^{-1}), thapsigargin (TG, 10 ng ml^{-1}) and H-7 (10, 20 and 50 μM). Concentrations of 10 ng ml^{-1} of TPA and TG are 16.2 and 15.4 nM, respectively. The results were confirmed by two additional experiments. Values are the means from 4 dishes; vertical lines indicate s.e.mean. Effects of H-7 were statistically significant ($P < 0.001$) where indicated by an asterisk.

(10 ng ml^{-1}) and other TPA-type tumour promoters such as teleocidin (10 ng ml^{-1}) or aplysiatoxin (10 ng ml^{-1}) (Figure 3). However, as shown in Figure 4, a combination of thapsigargin (10 ng ml^{-1}) and calcium ionophore A23187 (0.3 μM) stimulated the release only additively, but a combination of TPA (10 ng ml^{-1}) and A23187 (0.3 μM) stimulated the release more than additively ($P < 0.001$).

Table 1 shows the effects of extracellular Ca^{2+} on the release of radioactivity. When cells were incubated in medium without Ca^{2+}, thapsigargin at concentrations of 10 and 30 ng ml^{-1} failed to exert its effect. In the presence of Ca^{2+}, both concentrations of thapsigargin stimulated the release of radioactivity dose-dependently. In contrast to thapsigargin, TPA (10 ng ml^{-1}) stimulated the release of radioactivity in the medium with or without Ca^{2+}. In the case of TPA, a slightly higher release of radioactivity was induced in the presence of Ca^{2+} than in the absence of Ca^{2+} (Table 1).

When the peritoneal cells were incubated in a simplified saline solution containing thapsigargin (10 ng ml^{-1}), the cytoplasmic free calcium level ($[Ca^{2+}]_i$) was increased, as shown in Figure 5. The maximal $[Ca^{2+}]_i$ was reached about 2 min after the addition of thapsigargin and was maintained at a high steady-state level. In TPA (10 ng ml^{-1})-treated peritoneal cells, very little increase in $[Ca^{2+}]_i$ was observed. Teleocidin or aplysiatoxin, at 10 ng ml^{-1}, also provoked very little increase in $[Ca^{2+}]_i$ (not shown). A23187 (0.3 μM) and FMLP (0.2 μM) showed a rapid increase in $[Ca^{2+}]_i$. The high level of $[Ca^{2+}]_i$ induced by A23187 was maintained but that induced by FMLP was not.

TPA-induced release of radioactivity was inhibited dose-dependently by the protein kinase inhibitor, H-7 (10–50 μM) (Figure 6). The inhibitory effect was apparent after 30 min incubation with H-7 (data not shown). However, H-7 at a concentration of 50 μM failed to inhibit the release of radioactivity induced by two concentrations of thapsigargin, 10 and 30 ng ml^{-1} (Figure 6). Even after 6 h incubation with H-7, thapsigargin-induced release of radioactivity was not inhibited (data not shown). The spontaneous release of radioactivity was not affected by H-7 at 50 μM, released radioactivity after 4 h incubation being 1.01 ± 0.06 and 1.09 ± 0.04 c.p.m. per dish $\times 10^{-4}$ (mean $\pm$ s.e.mean from 4 dishes) for control and H-7-treated groups, respectively.

In the incubation conditions employed in the present work, no cytotoxicity was observed as judged by a Trypan blue exclusion test.

Discussion

The mechanism by which thapsigargin stimulated the release of [^{3}H]-arachidonic acid from prelabelled macrophages was different from that stimulated by TPA. Firstly, release stimulated by thapsigargin (10 ng ml^{-1}) was slow and sustained whereas release stimulated by TPA, teleocidin or aplysiatoxin

($10\,ng\,ml^{-1}$) was rapid (Figures 1 and 2). The rate of release induced by thapsigargin $100\,ng\,ml^{-1}$ was similar to that induced by TPA $10\,ng\,ml^{-1}$ (Figures 1 and 2). It might suggest a difference in potency between thapsigargin and TPA. However, when examined at the same concentration, $10\,ng\,ml^{-1}$, TPA was slightly less effective with respect to prostaglandin E_2 production when measured 6 h after the incubation (Ohuchi *et al.*, 1987b). Secondly, the release of radioactivity was stimulated more than additively by a combination of thapsigargin and each TPA-type tumour promoter (Figure 3). A similar synergism between non-TPA-type tumour promoters and TPA-type tumour promoters was also observed with histamine release from purified peritoneal mast cells of the rat (Ohuchi *et al.*, 1986), with human platelet activation (Thastrup *et al.*, 1987a), and with superoxide anion formation by porcine and human neutrophils (Kano *et al.*, 1987). Thirdly, in Ca^{2+}-free medium thapsigargin failed to stimulate [^{3}H]-arachidonic acid release but TPA did stimuate the release of radioactivity (Table 1), suggesting that Ca^{2+} influx into cells is a prerequisite for stimulation of release by thapsigargin. The combined treatment with TPA and the calcium ionophore A23187 also increased the release of radioactivity more than additively, but only an additive increase in release was induced by combined treatment with thapsigargin and A23187 (Figure 4). These results suggest that thapsigargin may act as a calcium ionophore like A23187. In fact, as shown in Figure 5, TPA ($10\,ng\,ml^{-1}$) caused very little increase in cytoplasmic free Ca^{2+} level in quin 2-loaded peritoneal cells, but thapsigargin ($10\,ng\,ml^{-1}$) increased fluorescence intensity almost to the same level as observed in the cells treated with $0.3\,\mu M$ A23187. However, in platelets, thapsigargin has been demonstrated not to be a Ca^{2+} ionophore, because removal of extracellular calcium by EGTA had no significant effect on the thapsigargin-induced rise in cytoplasmic free calcium (Thastrup *et al.*, 1987b). It was suggested that thapsigargin stimulates the efflux of calcium from intracellular stores rather than influx through the plasma membranes of platelets. We did not examine whether $[Ca^{2+}]_i$ was increased more than additively by the combined treatment with thapsigargin and TPA. However, since TPA has been shown to decrease A23187- or concanavalin A-induced Ca^{2+} influx in pig neutrophils (Rickard & Sheterline, 1985), the combined effect by thapsigargin and TPA on arachidonic acid release cannot be explained solely by stimulation of Ca^{2+} influx. Fourthly, an inhibitor of protein kinases H-7 (Hidaka *et al.*, 1984; Kawamoto & Hidaka, 1984) failed to suppress thapsigargin-stimulated release of radioactivity (Figure 6). This is consistent with the finding that thapsigargin did not displace the phorbol ester receptor binding, presumably to protein kinase C, in a particulate fraction of mouse skin (Hakii *et al.*, 1986). Conversely, H-7 inhibited TPA-stimulated release of radioactivity in a dose-dependent manner (Figure 6), consistent with the release of radioactivity by TPA being mediated through activation of protein kinase C, which possesses a receptor for TPA-type tumour promoters (Blumberg *et al.*, 1984; Fujiki *et al.*, 1984).

In conclusion, thapsigargin acts as a Ca^{2+} mobilizer and stimulates the release of radioactivity from [^{3}H]-arachidonic acid-labelled macrophages. This release of radioactivity is more than additively augmented by the TPA-type tumour promoters which activate protein kinase C. An activity common to both TPA-type and non-TPA-type tumour promoters is the stimulation of [^{3}H]-arachidonic acid release. Although both types of tumour promoter appear to have different mechanisms of action, the stimulation of arachidonic acid metabolism might play a role in the tumour promotion and inflammatory reactions induced by these agents (Ohuchi *et al.*, 1987b).

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