*Biochemical Pharmacology*, Vol. 36, No. 5, pp. 621–626, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

# SYNERGISM BETWEEN THAPSIGARGIN AND THE PHORBOL ESTER 12-*O*-TETRADECANOYLPHORBOL 13-ACETATE ON THE RELEASE OF [$^{14}$C]ARACHIDONIC ACID AND HISTAMINE FROM RAT PERITONEAL MAST CELLS*

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(*Received* 20 *May* 1986; *accepted* 1 *September* 1986)

**Abstract**—Thapsigargin is a potent skin irritating sesquiterpene lactone isolated from the roots of *Thapsia garganica* L. (Apiaceae). In rat peritoneal mast cells thapsigargin induced a calcium-dependent non-cytotoxic [$^{14}$C]arachidonic acid and histamine release. A minor amount of the released [$^{14}$C]arachidonic acid was converted to [$^{14}$C]prostaglandin $D_2$. A potentiated effect was found between thapsigargin and 12-*O*-tetradecanoylphorbol 13-acetate, as well as between A23187 and the phorbol ester with respect to release of [$^{14}$C]arachidonic acid and histamine.

It is suggested that the synergistic effect is due to activation of protein kinase C by 12-*O*-tetradecanoylphorbol 13-acetate and to increased intracellular calcium concentration mediated by A23187 as well as thapsigargin.

[$^{14}$C]arachidonic acid was released mainly from phosphatidylcholine. It is suggested that this release may be due to an action of phospholipase $A_2$, and that protein kinase C is involved in activation of phospholipase $A_2$.

Numerous stimuli are capable of inducing non-cytotoxic histamine release from rat mast cells, e.g. the calcium-ionophore A23187, compound 48/80, anti-IgE and concanavalin A. Thapsigargin is another potent releaser of histamine from rat peritoneal mast cells [1]. Thapsigargin is a constituent of the root of *Thapsia garganica* L. (Apiaceae) [2]. The skin-irritating effect of the root has been used in the folk medicine in the treatment of rheumatism.

When rat peritoneal mast cells are incubated with thapsigargin in a calcium free medium, the secretory response of histamine starts when $Ca^{2+}$ is added [3]. A similar effect on the release of histamine is seen when $Ca^{2+}$ is added after preincubation with A23187 [4]. However, the effect of thapsigargin differs from that of A23187 in several aspects: thapsigargin does not release histamine from isolated histamine-retaining granules [3]; thapsigargin is unable to transfer $^{45}Ca^{2+}$ from an aqueous phase into an organic phase [5]; thapsigargin does not cause a release of potassium ion from human red blood cells in the presence of 1 mM extracellular $Ca^{2+}$ [5].

A synergistic action between the calcium ionophore A23187 and the tumor promotor 12-*O*-tetradecanoylphorbol 13-acetate (TPA)‡ for histamine release from rat peritoneal mast cells has been shown [6]. TPA activates protein kinase C by increasing its affinity for $Ca^{2+}$ [7]. The synergistic effect is mediated by both an activation of protein kinase C and an increase in the intracellular $Ca^{2+}$ concentration.

Here we report that extracellular $Ca^{2+}$ is required for the release of [$^{14}$C]arachidonic acid by thapsigargin. [$^{14}$C]Arachidonic acid is released mainly from phosphatidylcholine, and [$^{14}$C]prostaglandin $D_2$ ([$^{14}$C]$PGD_2$) is formed as a minor component. The phorbol ester potentiates the release of histamine and [$^{14}$C]arachidonate, when rat peritoneal mast cells are stimulated with thapsigargin.

## MATERIALS AND METHODS

*Materials*. Ficoll 400 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. [1-$^{14}$C]Arachidonic acid (58 mCi/mmol) and [5,6,8,9,12,14,15 (*n*)-$^{3}$H]prostaglandin $D_2$ (160 Ci/mmol) were from Amersham International (Bucks, U.K.). Bovine serum albumin (crystallized and lyophilized) from Sigma was used without further purification. Ionophore A23187 and 12-*O*-tetradecanoylphorbol 13-acetate were from Sigma Chemical Co. (St. Louis, MO). Silicagel 60, high performance thin layer plates 10 × 20 cm and 10 × 10 cm were obtained from E. Merck (Darmstadt, F.R.G.). Polystyrene tubes were from Teknunc (Roskilde, Denmark). LKB Ultrofilm $^{3}$H was from LKB (Bromma, Sweden). X-Omat film was from Kodak (Albertslund, Denmark). Phospholipid standards were from Serdary Research Laboratories (London, Ontario, Canada).

Thapsigargin ($M_r$ = 650) was kindly provided by Dr S. B. Christensen, Dept. Chemistry BC, Royal

* This paper was presented in part at the International Conference on Leukotrienes and Prostanoids in Health and Disease, 20–25 October 1985, in Tel-Aviv and Rehovot, Israel, and appeared in abstract form.

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‡ Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PG, prostaglandin.

Danish School of Pharmacy, Copenhagen, Denmark. Prostaglandin standards were kindly provided by Dr J. E. Pike, Upjohn Company, Kalamazoo, MI. 5-Lipoxygenase products were kindly provided by Dr J. Rokach, Merck Frost Laboratories, Quebec, Canada.

*Mast cell purification and labelling.* Mast cells were isolated from the peritoneal cavity of male Wistar rats (250–400 g) using a Ficoll density gradient, as described by Uvnäs *et al.* [8]. Peritoneal lavage yielded about $10^6$ mast cells per rat. The purity of the peritoneal mast cell preparations was greater than 95% as assessed by metachromatic staining with toluidine blue dye (1%, w/v). Mast cells from several rats were pooled for an experiment. The cells were washed in buffered salt solution containing 145 mM NaCl, 2.7 mM KCl, 4.7 mM $Na_2HPO_4$, $2H_2O$, 2 mM $KH_2PO_4$, 2 mM glucose, 1 mg/ml bovine serum albumin, pH 7.0. Purified mast cells were incubated with [$^{14}$C]arachidonic acid (1–1.5 $\mu$Ci/6 × $10^6$ cells) at 37° for 30 min. After incubation the mast cells were washed 3 times with buffered salt solution to remove free [$^{14}$C]arachidonic acid and resuspended in the same medium.

*Cell activation and lipid metabolism.* Labelled mast cells (134,000 cells/ml, total volume 5.0 ml) were stimulated by adding thapsigargin, A23187 or TPA solubilized in dimethyl sulfoxide. To controls were added dimethyl sulfoxide only. The final concentration of dimethyl sulfoxide (0.01%) did not affect histamine secretion or release of [$^{14}$C]labelled compounds. Calcium chloride (final concentration 1 mM) was added simultaneously with the stimulator or with the vehicle. Incubation was carried out for 15 min at 37°. Cells were then sedimented by centrifugation (10 min, 125 *g*) and the supernatant was removed. The supernatant was acidified (pH 3) with acetic acid and extracted twice with double volume of ethylacetate.

The combined extracts were evaporated under nitrogen, and the residue was dissolved in 1.00 ml ethylacetate. Aliquots were counted in a liquid scintillation counter (Tri-Carb 2660, Packard). The cell sediments were extracted with 10 vol. chloroform/methanol (2:1, v/v). The phospholipids and neutral lipids were separated by one-dimensional thin layer chromatography by three consecutive developments, i.e. the first solvent, ethylacetate/iso-propanol/water (50:35:15, by vol.) to a distance of 12 cm above the application line; the second solvent, chloroform/methanol/acetic acid/water (100:30:35:3, by vol.) to a distance of 8.5 cm; the final solvent, butanol/acetone/acetic acid/water (5:5:1:1, by vol.) was allowed to rise 8.5 cm above the application line. The [$^{14}$C]labelled compounds were visualized by autoradiography, and the standards were visualized with 3% (w/v) copper(II)acetate in 8% (w/v) aqueous phosphoric acid and charred at 180° for 10 min. Spots were compared to standards, scraped into vials and the radioactivity was determined in a liquid scintillation counter.

*Identification of prostaglandin $D_2$.* Extracts from the supernatant were applicated on 10 × 10 cm silicagel 60 thin layer chromatography plates and developed by two dimensional thin layer chromatography with solvent A: ethylacetate/iso-octane/acetic acid/water (55:25:10:50, by vol.); and solvent B: chloroform/methanol/acetic acid/water (90:9:1:0.65, by vol.). Authentic standards were prostaglandin $D_2$ ($PGD_2$), prostaglandin $E_2$ ($PGE_2$), thromboxane $B_2$ ($TxB_2$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and leukotriene $B_4$ ($LTB_4$).

The standards were visualized after spraying with 10% phosphomolybdic acid in ethanol and heating at 110° for 10 min. The [$^{14}$C]labelled spots were visualized by autoradiography and compared to the standards.

The radioactivity which migrated as the standard $PGD_2$ was scraped from the thin layer plate and extracted twice with ethylacetate after acidification with acetic acid (pH 3). The pooled extracts were mixed with [$^3$H]$PGD_2$ (20,000 cpm) and 50 $\mu$g $PGD_2$. The solution was evaporated to dryness and dissolved in a 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM dithioerythreitol. This mixture was incubated at 37° with 4 mM NADPH and a 100,000 *g* supernatant from rabbit liver homogenate, which contains a $PGD_2$-11-ketoreductase [9]. Formation of $PGF_{2\alpha}$ was followed by taking aliquots after 0, 15, 30, 45, 60 min of incubation. $PGF_{2\alpha}$ was separated from $PGD_2$ by thin layer chromatography using solvent A. The labelled spots were scraped into vials and the radioactivity was determined in a liquid scintillation counter.

*Histamine release assay.* Histamine release experiments were carried out with the same preparations of cells as used for the measurement of [$^{14}$C]arachidonic acid metabolism. The release was terminated by transferring 250 $\mu$l of the cell suspension to 2 ml ice-cold, buffered salt solution, followed by centrifugation at 250 *g* for 10 min. Histamine in the pellet and in the supernatant was measured fluorometrically as described by Shore *et al.* [10].

*Statistics.* Statistical evaluation of results was carried out using Student's *t*-test for the difference between independent experiments. Values of $P < 0.05$ were considered indicating a statistically significant difference.

## RESULTS

### [$^{14}$C]*Arachidonic acid incorporation and release from mast cells*

When 6 × $10^6$ cells were incubated for 30 min with 1.6 × $10^6$ cpm [$^{14}$C]arachidonic acid, 11.6 ± 2.2% (N = 6) of the label was found in the washed cells. The label was mainly incorporated into phospholipids (Fig. 1). Figure 1 also shows the changes in distribution of radioactivity, when the mast cells were stimulated with A23187 (1 $\mu$g/ml), or thapsigargin (1 $\mu$g/ml) for 15 min. A decrease of approximately 20% in the [$^{14}$C]radioactivity was seen in the phosphatidylcholine fractions when the cells were stimulated with either A23187 or thapsigargin. A similar percentage of free [$^{14}$C]arachidonic acid was released to the supernatant during this period. Studies of response-time indicated that the release of [$^{14}$C]arachidonic acid was maximal after 5–10 min of activation with either stimulant (data not shown).

Histamine was released linearly for the first 2 minutes reaching a plateau of approximately 70%

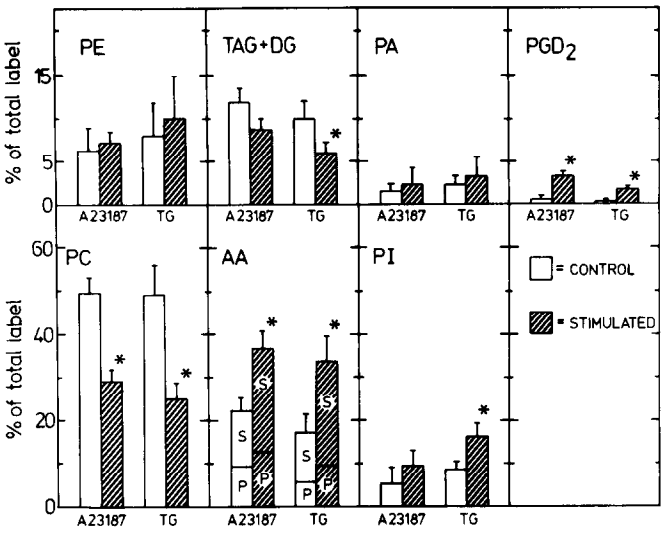


Fig. 1. Distribution of [$^{14}$C]labelling in [$^{14}$C]arachidonic acid prelabelled mast cells after stimulation with the ionophore A23187 (1 μg/ml) or thapsigargin (TG) (1 μg/ml). Control values (open columns) and stimulated values (hatched columns), N = 4 or 6. The results are mean ± SD. Asterisks indicate significant differences from control values * $P < 0.05$. Rat peritoneal mast cells, prelabelled with [$^{14}$C]arachidonic acid for 30 min at 37°, were stimulated with the ionophore A23187 (1 μg/ml), thapsigargin (1 μg/ml) or the vehicle dimethyl sulfoxide (0.01%) for 15 min at 37°. $Ca^{2+}$ (final concentration 1 mM) was added simultaneously with the stimulator or the vehicle. The phospholipids and neutral lipids were separated by thin layer chromatography and radioactive spots were located by autoradiography. 100% = 3650 ± 226 cpm. PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; TAG, triacylglycerol; DG, diacylglycerol; AA, arachidonic acid; p, pellet; s, supernatant; $PGD_2$, prostaglandin $D_2$ in the supernatant. About 5% of the radioactivity was found in phosphatidylserine and other non-identified compounds.

of total histamine content at approximately 5 min (data not shown) with either A23187 or thapsigargin as stimulants. $Ca^{2+}$ (final concentration 1 mM) was added to the mast cells simultaneously with either stimulant or the vehicle. Earlier experiments (unpublished results) have shown that the presence of $Ca^{2+}$ in the buffer used in the lavage of the peritoneum of the rat and in the purification of the cells resulted in a reduced histamine release compared to the histamine release obtained, when $Ca^{2+}$ and thapsigargin were added simultaneously to the cells.

### [$^{14}$C]*Arachidonic acid and* [$^{14}$C]$PGD_2$ *release*

Thin layer plates with standards were developed in two dimensions, and autoradiography was used to locate the released [$^{14}$C]labelled compounds. In the supernatant 98% of the recovered radioactivity comigrated with standards of arachidonic acid and $PGD_2$ (Fig. 1). A minor amount (<2%) comigrated with 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) in the stimulated extracts, but the amount was not significantly different from that of the control.

To characterize further the metabolite comigrating with $PGD_2$ in the thin layer system, a crude extract of rabbit liver $PGD_2$ 11-ketoreductase was incubated with the [$^{14}$C]-labelled metabolite, [$^{3}$H]$PGD_2$ and $PGD_2$ standard. After 60 min both the [$^{14}$C] and the [$^{3}$H]labelled compounds were converted to metabolites comigrating with $PGF_{2\alpha}$ (Fig. 2). These

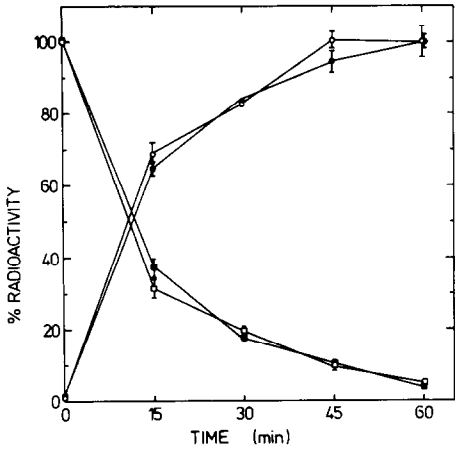


Fig. 2. Identification of [$^{14}$C]$PGD_2$ by its conversion to [$^{14}$C]$PGF_{2\alpha}$. The [$^{14}$C]labelled radioactivity, which migrated as $PGD_2$ in the thin layer system was mixed with [$^{3}$H]$PGD_2$ (20,000 cpm) and 50 μg $PGD_2$. This mixture was incubated with 4 mM NADPH and a 100,000 *g* supernatant from rabbit liver homogenate. Formation of labelled $PGF_{2\alpha}$ was followed by thin layer chromatography using solvent A, which separated $PGD_2$ and $PGF_{2\alpha}$ spots. The spots were scraped into vials and counted. 100% [$^{14}$C] = 457 cpm labelled compound pooled from six experiments. 100% [$^{3}$H] = 10 412 cpm. 100% of both [$^{14}$C] and [$^{3}$H]radioactivity is the radioactivity recovered from the thin layer plate. Results are mean and range of two experiments, each in duplicate: ■—■, [$^{14}$C]$PGD_2$; ●—●, [$^{14}$C]$PGF_{2\alpha}$; □—□, [$^{3}$H]$PGD_2$; ○—○, [$^{3}$H]$PGF_{2\alpha}$.

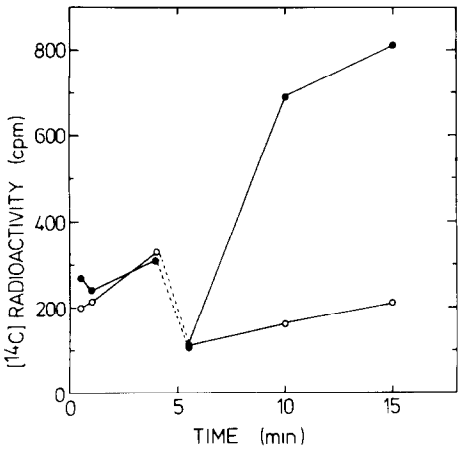


Fig. 3. The $Ca^{2+}$-dependence of [$^{14}$C]radioactivity release induced by thapsigargin. Rat peritoneal mast cells, prelabelled with [$^{14}$C]arachidonic acid, were incubated with 1 μg/ml thapsigargin in the absence of $Ca^{2+}$ for 4 min. After two washing procedures with buffer without $Ca^{2+}$, the cells were resuspended in a buffer containing 1 mM $Ca^{2+}$ (●); control (○) (mean of two) at 5.5 min. Aliquots of the cells (225,000 cells) were stopped at the indicated times. The cells were sedimented, and the supernatant was extracted and counted.

results indicate that A23187 and thapsigargin both stimulate the release of [$^{14}$C]arachidonic acid, and that [$^{14}$C]$PGD_2$ is formed as a minor component (about 5% of the released [$^{14}$C]arachidonic acid).

### *$Ca^{2+}$-dependence of [$^{14}$C]radioactivity release induced by thapsigargin*

Rat peritoneal mast cells stimulated with thapsigargin in buffered salt solution, showed no release

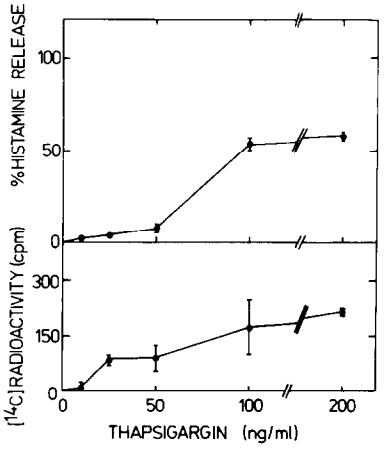


Fig. 4. Dose response curves for thapsigargin (TG)-induced release of histamine and [$^{14}$C]radioactivity from rat peritoneal mast cells. The number of mast cells were $1.3 \times 10^5$ cells/ml. The prelabelled mast cells were incubated for 15 min. Aliquots of the cells were taken for histamine release. The cell suspensions were centrifugated, and the supernatant was extracted and counted in a liquid scintillation counter. The values for both the histamine release and [$^{14}$C]arachidonic acid were corrected for the control values, which were 4.3% and 419 cpm respectively. Results are mean and range of two experiments, each in duplicate.

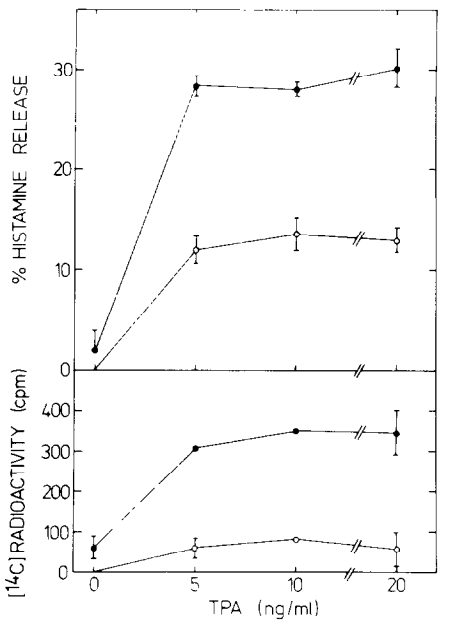


Fig. 5. Synergism between thapsigargin and TPA on histamine release and [$^{14}$C]arachidonic acid release in rat peritoneal mast cells. Mast cells ($1.34 \times 10^5$ cells/ml) were incubated for 15 min at 37° with various concentrations of TPA in the absence of (○) or in the presence of thapsigargin (●) (10 ng/ml). The values for both the histamine release and [$^{14}$C]arachidonic acid were corrected for the control values, which were 2.6% and 644 cpm, respectively. Results are mean and range of two experiments, each in duplicate.

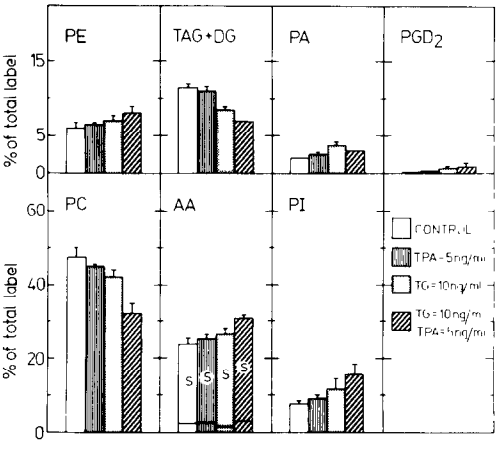


Fig. 6. Distribution of [$^{14}$C]labelling in [$^{14}$C]arachidonic acid prelabelled mast cells after stimulation with TPA (5 ng/ml) (▥); thapsigargin (TG) (10 ng/ml) (□); thapsigargin (TG) (10 ng/ml) and TPA (5 ng/ml) (▨); or the vehicle dimethyl sulfoxide (0.01%). After the reactions were stopped on ice-bath, the cells were sedimented, and the pellet as well as the supernatant were extracted. The phospholipids and neutral lipids were separated by thin layer chromatography. 100% = 3260 cpm. PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; TAG, triacylglycerol; DG, diacylglycerol; AA, arachidonic acid; s, supernatant, (the arachidonic acid in the pellet: the columns under s); $PGD_2$, prostaglandin $D_2$ in the supernatant. About 5% of the radioactivity was found in phosphatidylserine and other non-identified compounds. Results are mean and range of two experiments, each in duplicate.

of either histamine (data not shown) or [$^{14}$C]-radioactivity. When these cells were washed twice and suspended in buffered salt solution containing 1 mM $Ca^{2+}$ without thapsigargin [$^{14}$C]arachidonic acid release was induced (Fig. 3). The release of histamine showed the same pattern (data not shown).

*Potentiation of the effect of thapsigargin by TPA*

Figure 4 shows the dose response curve for the release of histamine and [$^{14}$C]labelled compounds by thapsigargin. Ten ng/ml thapsigargin was chosen as a concentration, which was sub-optimal with respect to the release of histamine and [$^{14}$C]radioactivity. If thapsigargin (10 ng/ml) and TPA (5 ng/ml) were added simultaneously to the cells, the histamine release was in two experiments, each performed in duplicate, 1.9 and 2.1 times greater than the sum of the individual responses (Fig. 5). The [$^{14}$C]radioactivity was 2.5 and 2.7 times greater than the sum of the individual responses (Fig. 5).

The increased release of [$^{14}$C]labelled compounds resulting from the simultaneous addition of thapsigargin (10 ng/ml) and TPA (5 ng/ml) seemed to be released from phosphatidylcholine. Thapsigargin (10 ng/ml) or TPA (5 ng/ml) added separately did not cause changes of [$^{14}$C]labelling of phosphatidylcholine (Fig. 6).

## DISCUSSION

The results showed that the histamine liberating substance thapsigargin released [$^{14}$C]arachidonic acid and histamine from [$^{14}$C]arachidonic acid prelabelled rat peritoneal mast cells in a $Ca^{2+}$-dependent manner. In agreement with results of others the calcium ionophore A23187 was also shown to act in a calcium-dependent manner with respect to the release of histamine and labelled arachidonic acid [11–13].

Phosphatidylcholine apparently was the main source of released [$^{14}$C]radioactivity with thapsigargin or A23187 as stimulators. Recently, Nakamura and Ui [12] have reported similar results in [$^{14}$C]arachidonic acid prelabelled rat peritoneal mast cells, when the cells were stimulated with A23187 (1 $\mu$M) for 10 min. Rat peritoneal mast cells prelabelled with [$^{3}$H]arachidonic acid mainly liberate [$^{3}$H]arachidonic acid from phosphatidylcholine and triacylglycerol, when the cells are stimulated with A23187 for 5 min [11, 14]. This indicates, that an action of phospholipase $A_2$ could be the main pathway for the release of arachidonic acid in rat peritoneal mast cells, when the cells were stimulated with A23187 or thapsigargin. Furthermore, the synergistic effect of TPA and A23187 on [$^{14}$C]arachidonic acid release may indicate that protein kinase C is involved in activation of phospholipase $A_2$.

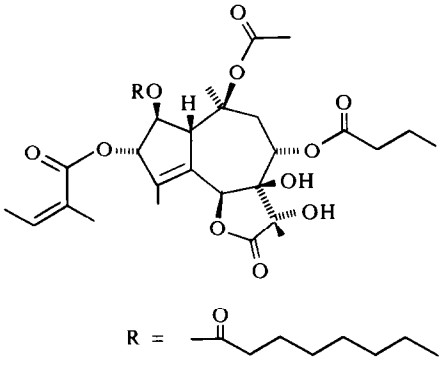


Fig. 7. The structure of thapsigargin [2].

In the present experiments the dose response curves for the release of histamine and of [$^{14}$C]arachidonic acid induced by thapsigargin were alike. Similar dose response curves were obtained by A23187. Nakamura and Ui [12] have also found that the dose response curves for [$^{14}$C]arachidonic acid and histamine release in A23187 stimulated mast cells are very similar. These results might suggest that histamine as well as [$^{14}$C]arachidonic acid were released, when the intracellular $Ca^{2+}$ concentration was increased by A23187. When rat mast cells are stimulated with A23187, the cells produce $PGD_2$ as the major arachidonic acid metabolite [15]. Our results showed that a minor amount of the released [$^{14}$C]arachidonic acid was converted to [$^{14}$C]$PGD_2$ (about 5%), and that this was the major [$^{14}$C]arachidonate metabolite, when the cells were stimulated with thapsigargin or A23187. 5-HETE has been reported to be synthesized in rat mast cells [16]. We found a minor amount of labelled compound comigrating with 5-HETE in the thin layer system, but there was no difference in the amounts in the control and in the stimulated cells. As shown in Fig. 3 thapsigargin did not stimulate [$^{14}$C]arachidonic acid release from prelabelled cells, when the cells were suspended in buffered salt solution without $Ca^{2+}$. After removing free thapsigargin from the cells by two washing procedures, and resuspending the cells in a buffered salt solution with 1 mM $Ca^{2+}$, [$^{14}$C]arachidonic acid and histamine release were induced. This could indicate that thapsigargin was attached to the cell membrane, and that only free thapsigargin in the buffer was removed by the washing procedures. When the buffer containing 1 mM $Ca^{2+}$ was added to the cells, then thapsigargin conceivably acted by transporting $Ca^{2+}$ across the cell membrane. Thus, a proposed increased intracellular $Ca^{2+}$ concentration then would cause the release reactions.

A synergistic action was found between thapsigargin and TPA regarding the release of [$^{14}$C]arachidonic acid and of histamine. TPA has been shown to potentiate A23187 stimulated release of histamine and of [$^{14}$C]arachidonic acid in rat mast cells prelabelled with [$^{14}$C]arachidonic acid [17]. This is in agreement with our results obtained with A23187. Heiman and Crews [17] have also found that stimulation of histamine release in rat mast cell by secretagogues which require extracellular $Ca^{2+}$ is enhanced by phorbolesters, whereas release by agents that do not require extracellular $Ca^{2+}$ is not enhanced by phorbolesters. This may suggest that protein kinase C is involved in the synergistic action of thapsigargin and TPA, and that thapsigargin in mast cells increased the intracellular concentration of $Ca^{2+}$. In human platelets thapsigargin has been shown to increase intracellular $Ca^{2+}$ concentration

[5]. A recent paper [18] confirms the synergistic effect of thapsigargin and TPA on histamine release from rat mast cells.

When the mast cells were stimulated with TPA and thapsigargin, [$^{14}$C]arachidonic acid was released mainly from phosphatidylcholine (Fig. 6). A similar effect was observed with thapsigargin only, but in a higher concentration (Fig. 1). This suggests that phospholipase $A_2$ was activated by high intracellular $Ca^{2+}$ concentration as well as by the combination of a protein kinase C activation and a small increase in intracellular $Ca^{2+}$ concentration. This could indicate that a concerted activation of protein kinase C and an increase in the intracellular $Ca^{2+}$ concentration were necessary for the release of arachidonic acid and histamine in rat peritoneal mast cells.

*Acknowledgements*—Professor E. Aaes-Jørgensen is thanked for critical reading of the manuscript. Jytte Christiansen is thanked for technical assistance. Elsebeth Norup is thanked for performing some of the histamine measurements. This work was supported by the Danish Medical Research Council (J. No. 12-4449 and 12-4790).

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