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# EFFECT OF THE TRIDECAMER OF COMPOUND 48/80, A $Ca^{2+}$-DEPENDENT HISTAMINE RELEASER, ON PHOSPHOLIPID METABOLISM DURING THE EARLY STAGE OF HISTAMINE RELEASE FROM RAT MAST CELLS

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**Abstract**—When the tridecamer component of compound 48/80 (Fraction D, Fr.D), a $Ca^{2+}$-dependent histamine releaser, was incubated with rat mast cells that had been prelabeled with [$^{32}$P]phosphate, [$^{3}$H]inositol or [$^{3}$H]glycerol, it induced a rapid decrease in [$^{32}$P]phosphatidylinositol-4,5-bisphosphate ($PIP_2$) followed by increases of [$^{3}$H]inositol-1,4,5-trisphosphate ($Ins P_3$) and [$^{3}$H]diacylglycerol during the 10 sec prior to detectable histamine release. Fr.D-induced changes of the metabolism of these compounds occurred even in the absence of $Ca^{2+}$, but to a lesser extent than in the presence of $Ca^{2+}$. In contrast, the accumulation of [$^{3}$H]arachidonic acid into phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidic acid (PA) in [$^{3}$H]arachidonic acid-prelabeled mast cells was $Ca^{2+}$-dependently stimulated by Fr.D with a concomitant decrease in [$^{3}$H]phophatidylethanolamine (PE). These $Ca^{2+}$-dependent changes in PC and PE were not observed in mast cells preloaded with [$^{32}$P]phosphate, while [$^{32}$P]PI and [$^{32}$P]PA increased $Ca^{2+}$ independently. Fr.D also increased $^{45}Ca^{2+}$ uptake by mast cells within 5 sec after the stimulation. These results indicate that Fr.D binding to mast cell $Ca^{2+}$ independently induces rapid changes of PI cycle-related metabolism of plasma membrane components, while it also induces $Ca^{2+}$-dependent accumulation of arachidonic acid into PC, PI and PA in association with the decrease of PE, which may be important during the latent period prior to the $Ca^{2+}$-dependent release of histamine from Fr.D-stimulated mast cells.

We previously reported that compound 48/80 (compd 48/80) comprises several oligomers, which differ in the $Ca^{2+}$ requirement for histamine release from rat mast cells [1] and in the characteristics of binding to the cells [2]. The tridecamer of compd 48/80 (Fraction D, Fr.D), the most potent $Ca^{2+}$-dependent histamine releasing component, binds to mast cell $Ca^{2+}$ independently. Both the binding and subsequent histamine-releasing activities of Fr.D are partially suppressed by the prior treatment of the mast cells with dinitrophenylated (DNP)-ascaris antiserum [1, 2], indicating a partial sharing of binding sites of Fr.D with IgE antiserum. Although the exact mechanism of histamine release from mast cells has not been fully clarified, it has been suggested that changes in plasma membrane phosphatidylinositol metabolism as well as in $Ca^{2+}$ influx are required to trigger the histamine release induced by DNP-ascaris antiserum [3] and also that by compd 48/80 [4]. Since in the absence of $Ca^{2+}$, Fr.D binds to mast cells without causing detectable histamine release, in the present study we utilized Fr.D to investigate phospholipid metabolism during the postreceptor-ligand binding reaction in relation to the $Ca^{2+}$-dependent histamine release from mast cells.

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## MATERIALS AND METHODS

### *Preparation of mast cells and Fr.D of compd* 48/80

Mast cells were collected from peritoneal cavity fluid of male Wistar rats (300–350 g) and concentrated by Ficoll density gradient centrifugation to 90–98% purity as described elsewhere [5]. The cells were suspended in the mast cell medium without (MCM) or with phosphate (MCM-P) [150 mM NaCl, 3.7 mM KCl, 1 mM $CaCl_2$, 5.55 mM glucose, 5 mM sodium phosphate, 1 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) and 1 mg/ml bovine serum albumin (BSA), pH 6.8] [6]. The cell number and cell viability were determined as described previously [7]. The synthesis of compd 48/80 and fractionation of Fr.D from the mixture of various oligomers were performed as described in a previous paper [1].

### *Histamine release assay*

Mast cells ($0.5 \times 10^5$ cells), suspended in 1 ml of MCM-P containing $Ca^{2+}$ or no $Ca^{2+}$, were incubated with Fr.D (0.3 $\mu$g/10 $\mu$l) for up to 1 min at 37°. The reaction was terminated by cooling the mixture in an ice-bath. After centrifugation at 280 *g* for 1 min, histamine released into the supernatant fraction was assayed by the method of Shore *et al.* [8] and expressed as a percentage of the total histamine content of the cells, which was determined after extraction with 2.2% (final concentration) $HClO_4$.

*Calcium flux assay*

Mast cells ($3 \times 10^6$ cells) were suspended at 0° in 0.5 ml of a HEPES medium, which contained 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 137 mM NaCl, 4.15 mM KCl, 0.9 mM $CaCl_2$ and 1.05 mM $MgCl_2$, pH 7.4, and then incubated with $^{45}CaCl_2$ (40 $\mu$Ci) at 37° for 30 min. Then the active oligomer of compd 48/80 (0.15 $\mu$g in 5 $\mu$l) or the vehicle was added, and the reaction mixture was further incubated at 37° for 5 sec. The cell suspension was filtered through HAWP 0.45 $\mu$m Millipore filters, which were immediately rinsed three times with 2 ml of $Ca^{2+}$-free Locke solution (137 mM NaCl + 4.15 mM KCl) containing 2 mM ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA) and 3 mM $LaCl_3$ to remove surface-bound $Ca^{2+}$, for estimation of the intracellular $Ca^{2+}$ uptake as described previously [7].

*Preparation and incubation of* [$^{32}$P]-, [$^3$H]*arachidonic acid-,* [$^3$H]*glycerol- or* [$^3$H]*inositol-prelabeled mast cells*

Mast cells ($0.5–1.5 \times 10^7$ cells) were washed three times with 2 ml of MCM-P or MCM (for [$^{32}$P]-labeled cells), resuspended in the same medium (1 ml) and incubated at 37° with [$^{32}$P]phosphate (200 $\mu$Ci/ml) or [$^3$H]arachidonic acid (20 $\mu$Ci/ml BSA-free MCM-P) for 30 min, or with [$^3$H]glycerol (100 $\mu$Ci/ml) or [$^3$H]myo-inositol (40 $\mu$Ci/ml) for 60 min, and then washed three times with MCM-P (2 ml). Aliquots (0.2 ml) of each washed prelabeled cell suspension were then transferred to incubation tubes containing the medium with $Ca^{2+}$ or no $Ca^{2+}$ and pre-warmed at 37° for 5 min. Reactions were then initiated by blowing in Fr.D (0.06 $\mu$g in 2 $\mu$l) from a 5-$\mu$l graduated capillary to the mixture and terminated at 2-sec intervals by adding an ice-cold lipid extraction mixture (0.7 ml), consisting of chloroform/methanol/conc. HCl (100/200/2, by vol.) containing 10 $\mu$M 2,6-ditertiary butyl-4-methylphenol to prevent the oxidation of lipids (a team of two trained people can manipulate these rapid procedures easily with more than 90% reproducibility). Then to the extraction mixture were added chloroform (0.2 ml) and 2 M KCl (0.2 ml) [9], and Folch's diphosphoinositide fraction [10], phosphatidylinositol (PI) and phosphatidic acid (PA) as carriers, and the mixture was shaken vigorously for 1 min. The lower organic phase was separated by centrifugation at 1000 *g* for 5 min at 4°, and the aqueous phase was washed once with chloroform (0.8 ml). The combined lower phases were evaporated to dryness at room temperature under a nitrogen stream, and the residue was dissolved in $CHCl_3$/methanol (2/1, v/v) (0.1 ml). Phospholipids were separated by thin-layer chromatography (TLC) on Silica gel G plates impregnated with 1% potassium oxalate containing 2 mM EGTA with $CHCl_3$/methanol/4 N $NH_4OH$ (45/35/10, by vol.) as the developing solvent.

Neutral lipids and fatty acids were separated by TLC on Silica gel G with ligroin/diethyl ether/acetic acid (50/50/1, by vol.) after adding 1,2-diacylglycerol and arachidonic acid as carriers [11]. Lipids were visualized with iodine vapor, scraped from the plates, and counted in 0.5% 2,5-diphenyloxazole (PPO) in toluene (8 ml).

[$^3$H]Inositol phosphates were assayed by the modified method of Downes and Michell [12]. Mast cells ($2 \times 10^7$ cells) prelabeled with [$^3$H]myo-inositol were washed three times with MCM-P (2 ml) with or without $Ca^{2+}$, and then resuspended in the same medium (3 ml). Aliquots (0.2 ml) of the cell suspension were incubated with Fr.D (0.06 $\mu$g in 2 $\mu$l) for several seconds at 37°. The reaction was terminated by adding ice-cold 10% trichloroacetic acid (0.2 ml) and cooling on ice for 10 min. After vigorous shaking for 30 sec, the samples were centrifuged at 1000 *g* at 4° for 5 min. The supernatant fractions were removed, neutralized with NaOH, diluted to 5 ml with water and then loaded on small columns of Dowex $1 \times 8$ (200–400 mesh, formate form, 0.5 ml). Inositol phosphates were eluted successively with water (5 ml), 5 mM disodium tetraborate + 60 mM sodium formate (5 ml), 5 mM disodium tetraborate + 180 mM sodium formate (8 ml), 0.1 M formic acid + 0.4 M ammonium formate (8 ml), and 0.1 M formic acid + 1 M ammonium formate (8 ml) as described by Thomas *et al.* [13]. Aliquots of each fraction were counted in 0.5% PPO dissolved in 33% Triton X-100 in toluene (8 ml). Inositol-1,4-bisphosphate (Ins $P_2$) and inositol-1,4,5-trisphosphates (Ins $P_3$) were identified by co-chromatography of the [$^{32}$P]-labeled Ins $P_2$ and Ins $P_3$, which were prepared from erythrocyte plasma membranes according to the procedure of Downes and Michell [12].

*Chemicals*

The chemicals used were obtained from the following sources: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and BSA (fraction V), Sigma Chemical Co. (St. Louis, MO, U.S.A.); Dowex $1 \times 8$, Dow Chemical Co. (Midland, MI, U.S.A.); PPO, Triton X-100 and arachidonic acid, Nakarai Pure Chemicals (Kyoto, Japan); PIPES, EGTA and HEPES, Dojin Laboratories (Kumamoto, Japan); PA, PI and 1,2-diacylglycerol, Serdary Research Laboratories Inc. (London, Ontario, Canada); Siliga gel G plates ($20 \times 20$ cm, 250 $\mu$m layer), Schleicher & Schüll GmbH (Dasel, West Germany); [2(n)-$^3$H]glycerol (500 mCi/mmole) and [5, 6, 8, 9, 11, 12, 14, 15-$^3$H]arachidonic acid (80–120 Ci/mmole), Amersham-Searle (Amersham, England); [$^{32}$P]orthophosphate (carrier free, 24 Ci/ml) in HCl water, Institute for Japan-Atomic Energy (Tokyo, Japan); and myo-[2-$^3$H(N)]inositol (16.5 Ci/mmole) and $^{45}CaCl_2$ (40–50 Ci/g calcium), New England Nuclear (Boston, MA, U.S.A.). Phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4,5-bisphosphate ($PIP_2$) were prepared by the method of Folch [10]. Other chemicals of reagent grade were obtained commercially.

## RESULTS

*Latent period for Fr.D-induced histamine release from mast cells*

As shown in Fig. 1, a latent period of about 10 sec before detectable histamine release from Fr.D-stimulated mast cells was observed in the presence

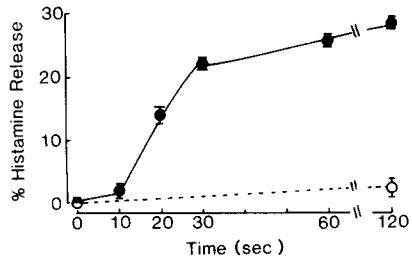


Fig. 1. Time course of Fr.D-induced histamine release from mast cells. Aliquots (1 ml) of a mast cell suspension ($0.5 \times 10^5$ cells), that has been preincubated with (—●—) or without (- -○- -) $Ca^{2+}$ for 5 min at 37°, were incubated with Fr.D (0.3 μg in 10 μl) for the indicated times. Then the histamine released in the supernatant fraction was assayed as described in the text. Each value represents the mean value ± S.E. for three determinations. Similar results were obtained for three more experiments of the same design. The absolute value of 100% is: 0.82 μg.

of $Ca^{2+}$. On the other hand, Fr.D did not induce histamine release in the absence of $Ca^{2+}$.

*Effect of Fr.D on phospholipid metabolism of mast cells*

*Polyphosphoinositides.* During this latent period, within 2–4 sec after the addition of Fr.D in the presence of $Ca^{2+}$, [$^{32}$P]$PIP_2$ in mast cells substantially decreased to the lowest level and then increased to

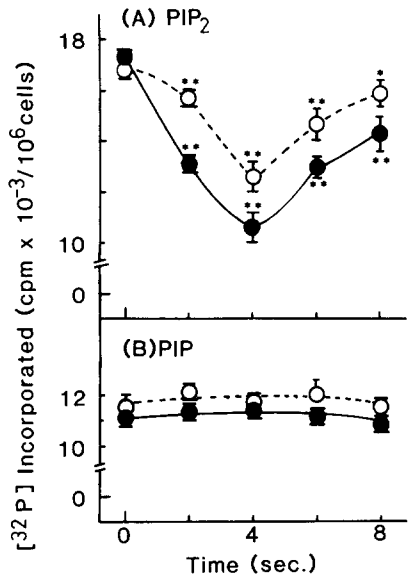


Fig. 2. Effect of Fr.D on polyphosphoinositide metabolism. Aliquots (0.2 ml) of a [$^{32}$P]-labeled mast cell suspension ($6.0 \times 10^5$ cells) were preincubated with (—●—) or without (- -○- -) $Ca^{2+}$ at 37° for 5 min. The reactions were initiated by adding Fr.D (0.06 μg in 2 μl) and terminated at 2-sec intervals by adding 0.7 ml of an ice-cold lipid extraction mixture. The extracted lipids were analyzed as described in the text. Each point represents the mean value ± S.E. for three determinations. Similar results were obtained for three more experiments of the same design. Statistical significance: (*) $P < 0.05$ and (**) $P < 0.01$.

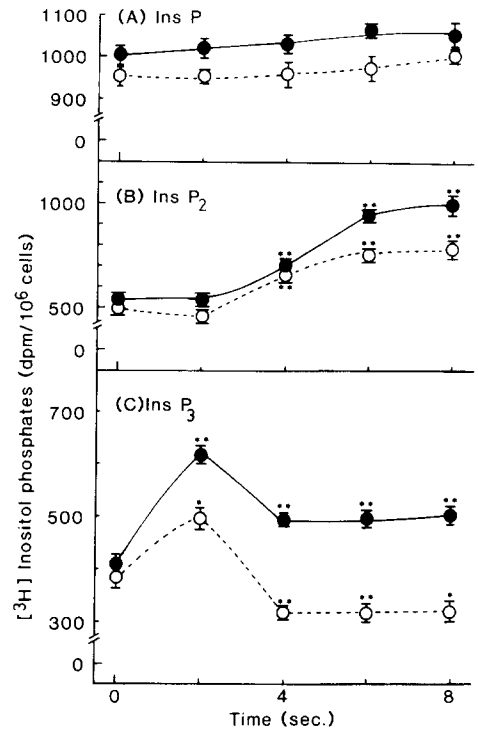


Fig. 3. Effect of Fr.D on the formation of inositol phosphates. Aliquots (0.2 ml) of a [$^3$H]inositol-labeled mast cell suspension ($1.1 \times 10^6$ cells) were preincubated with (—●—) or without (- -○- -) $Ca^{2+}$ at 37° for 5 min. The reactions were initiated by adding Fr.D (0.06 μg in 2 μl) and terminated at 2-sec intervals by adding 0.2 ml of ice-cold 10% trichloroacetic acid. [$^3$H]Inositol phosphates were analyzed as described in the text. Each point represents the mean value ± S.E. for three determinations. Similar results were obtained for three more experiments of the same design. Statistical significance: (*) $P < 0.02$ and (**) $P < 0.01$.

the original, unstimulated level (Fig. 2A), probably due to increased resynthesis of [$^{32}$P]$PIP_2$. The Fr.D-induced reduction of [$^{32}$P]$PIP_2$ in mast cells occurred even in the absence of $Ca^{2+}$ but to a lesser extent than in the presence of $Ca^{2+}$. On the other hand, there was no significant change in the level of [$^{32}$P]PIP during the latent period (Fig. 2B). The time course of [$^{32}$P]$PIP_2$ breakdown coincided with that of the spike increase and subsequent decrease in [$^3$H]Ins $P_3$ (Fig. 3C), but not with the change in the level of [$^3$H]inositol-1-phosphate (Ins P) (Fig. 3A), assayed in the presence of $Li^+$. [$^3$H]Ins $P_3$ rapidly decreased within the next few seconds with a concomitant accumulation of [$^3$H]Ins $P_2$ (Fig. 3B) until the initiation of histamine release.

*Diacylglycerol.* The amounts of [$^3$H]arachidonic acid- or [$^3$H]glycerol-labeled diacylglycerol significantly increased within 4 sec after the addition of Fr.D to the cells in the presence or absence of $Ca^{2+}$, and then rapidly decreased to the original levels during the following 4 sec (Fig. 4).

*Other phospholipids.* In [$^3$H]arachidonic acid-labeled mast cells, marked increases in the radioactivities of the PA, PI and PC fractions, in contrast

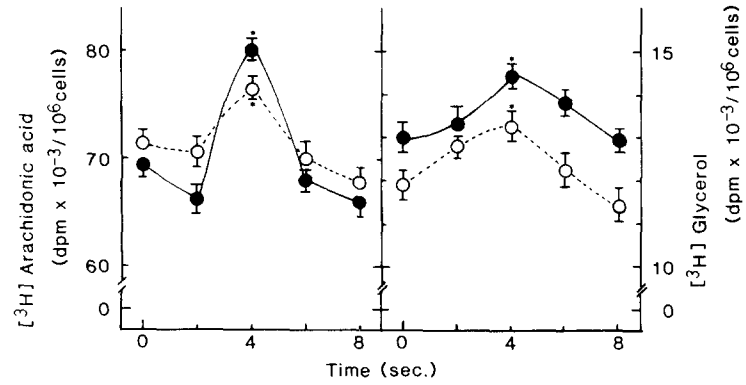


Fig. 4. Effect of Fr.D on diacylglycerol metabolism. Aliquots (0.2 ml) of a [$^3$H]arachidonic acid- or [$^3$H]glycerol-labeled mast cell suspension ($9 \times 10^5$ cells) were preincubated for 5 min with (—●—) or without (– –○– –) $Ca^{2+}$ at 37°. The reactions were initiated by adding Fr.D (0.06 μg in 2 μl) and terminated at 2-sec intervals by adding the lipid extraction mixture. The extracted lipids were analyzed as described in the text. Each point represents the mean value ± S.E. for three determinations. Similar results were obtained for three more experiments of the same design. Statistical significance: (*) $P < 0.05$.

to the decrease in radioactivity in the PE fraction, occurred within 2 sec after Fr.D-induced stimulation of mast cells in the presence of $Ca^{2+}$, but not in the absence of $Ca^{2+}$ (Fig. 5). On the other hand, [$^{32}$P]radioactivity in the PA and PI fractions of [$^{32}$P]-labeled mast cells rapidly increased both in the presence and absence of $Ca^{2+}$, while that in the PC and PE fractions practically did not change (Fig. 6).

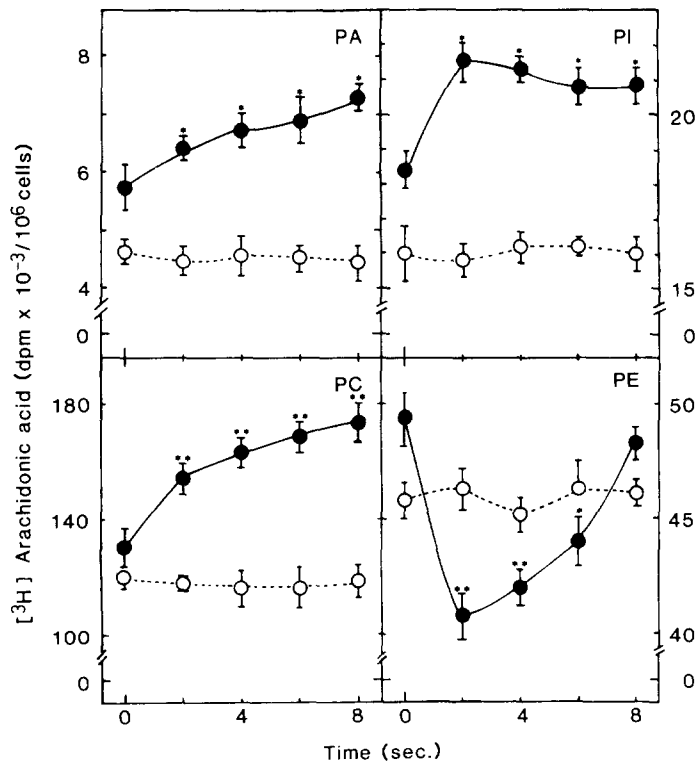


Fig. 5. Effect of Fr.D on [$^3$H]arachidonic acid-labeled PA, PI, PC and PE metabolism. Aliquots (0.2 ml) of a [$^3$H]arachidonic acid-labeled mast cell suspension ($6 \times 10^5$ cells) were preincubated with (—●—) or without (– –○– –) $Ca^{2+}$ at 37° for 5 min. The reactions were initiated by adding Fr.D (0.06 μg in 2 μl) and terminated at 2-sec intervals by adding the lipid extraction mixture. The extracted lipids were analyzed as described in the text. Each point represents the mean value ± S.E. for three determinations. Similar results were obtained for three more experiments of the same design. Statistical significance: (*) $P < 0.05$ and (**) $P < 0.01$.

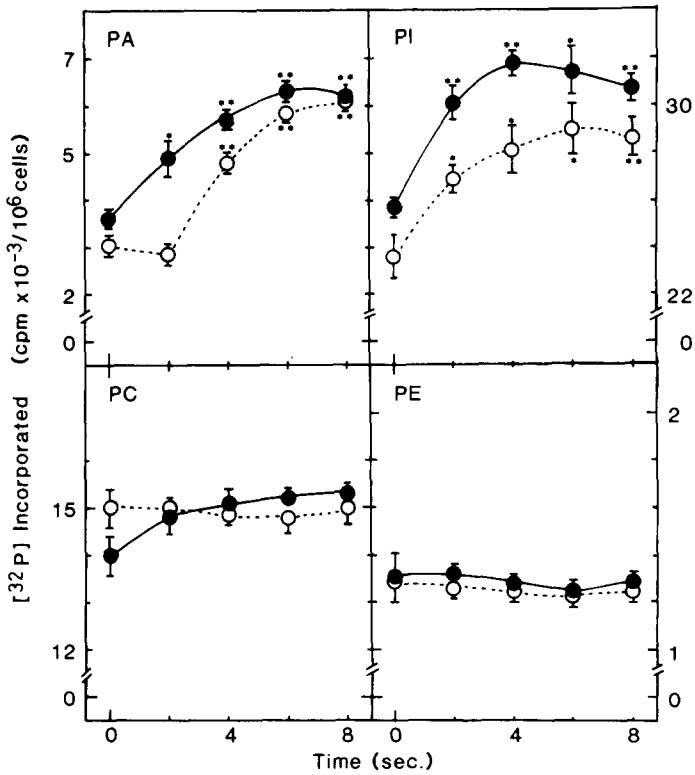


Fig. 6. Effect of Fr.D on [$^{32}$P]-labeled PA, PI, PC and PE metabolism. All the experimental conditions and symbols used are the same as in the legend to Fig. 5, except for the use of [$^{32}$P]-labeled mast cells in place of [$^{3}$H]arachidonic acid-labeled cells. Each point represents the mean value ± S.E. for three determinations. Similar results were obtained for three more experiments of the same design. Statistical significance: (*) $P < 0.05$ and (**) $P < 0.01$.

*Effect of Fr.D on $^{45}Ca^{2+}$ influx*

Fr.D dose-dependently stimulated the incorporation of $^{45}Ca^{2+}$ into mast cells during a 5-sec incubation at 37° but not at 0° (Table 1). These results suggest that the entry of $^{45}Ca^{2+}$ into the intracellular compartment is related to the temperature-dependent histamine releasing response induced by Fr.D but not to the temperature-independent binding of Fr.D to the cells [2].

Table 1. Effect of Fr.D on $^{45}Ca^{2+}$ uptake of mast cells

| Fr.D (μg) | Temperature (°C) | Calcium (nmoles/$10^6$ cells) |
|---|---|---|
| 0 | 37 | 3.08 ± 0.29 |
| | 0 | 3.14 ± 0.33 |
| 0.05 | 37 | 3.35 ± 0.19 |
| | 0 | ND* |
| 0.1 | 37 | 4.14 ± 0.27 |
| | 0 | ND |
| 0.3 | 37 | 6.22 ± 0.30† |
| | 0 | 3.43 ± 0.22 |
| 0.5 | 37 | 7.12 ± 0.12† |
| | 0 | 3.52 ± 0.20 |

Mast cells ($2.4 \times 10^6$ cells/0.5 ml) that had been preloaded with $^{45}CaCl_2$ were incubated with each of the indicated amounts of Fr.D in HEPES medium at 37° or 0° for 1 min. After incubation, aliquots (100 μl) of the cell suspensions were filtered and counted as described in the text. Each value represents the mean ± S.E. for three samples.

* Not determined.

† Statistical significance, $P < 0.05$.

## DISCUSSION

The present results show that Fr.D, the most potent $Ca^{2+}$-dependent histamine releasing component of compd 48/80 [1], induced rapid $PIP_2$-breakdown as one of the early biochemical events shortly after binding to mast cells but prior to the initiation of histamine release (Fig. 2). The rapid breakdown and recovery of $PIP_2$ were followed successively by the rapid accumulation and breakdown of [$^{3}$H]Ins $P_3$ (Fig. 3) and [$^{3}$H]glycerol-labeled diacylglycerol (Fig. 4), and also by a steady increase of [$^{32}$P]PA and [$^{32}$P]PI (Fig. 6).

This $PIP_2$ breakdown pathway is generally assumed to be an important trigger for the induction of a hormone- or neuropeptide-stimulated cellular response in various target tissues [14–16]. However, in the absence of extracellular $Ca^{2+}$, Fr.D also induced a similar decrease in $PIP_2$ and similar increases in Ins $P_3$ and diacylglycerol, and [$^{32}$P]-labeled PA and PI fractions in mast cells as observed in the presence of $Ca^{2+}$, although the relative magnitudes of the changes were slightly lower in the

$Ca^{2+}$-free mast cells. The increase of $PIP_2$ breakdown in the presence of $Ca^{2+}$ indicates the involvement of $PIP_2$ phosphodiesterase, since for this enzyme in the rat brain $Ca^{2+}$ was reported not to be essential for the activity but still activating the enzyme [17]. These results indicate that all of these changes related to PI cycle result mainly from modulation of the phospholipid metabolism induced by the interaction of Fr.D with its binding sites which is not $Ca^{2+}$-dependent [2]. These data are consistent with the previous finding by Cockcroft and Gomperts [18] that PI cycle was $Ca^{2+}$-independently induced in Con A-stimulated mast cells. It should be emphasized that [$^3$H]arachidonic acid-labeled PA, PI and PC were $Ca^{2+}$-dependently increased in Fr.D-stimulated mast cells in association with the apparent but transient reduction of [$^3$H]arachidonic acid-labeled PE (Fig. 5). The mechanism of $Ca^{2+}$-dependent changes in the levels of [$^3$H]arachidonic acid-labeled PC and PE fractions but not of [$^{32}$P]-labeled PC and PE fractions is at present unknown. However, it is possible that these changes might be mediated via the deacylation–reacylation pathway, "Lands pathway" [19], or via the deacylation–transacylation pathway, which has been reported in platelets and other mammalian tissues [20–22]. In this respect, we previously demonstrated the rapid $^{45}Ca^{2+}$ accumulation within 2.5 sec after the stimulation by compd 48/80, followed by the subsequent secretion of [$^3$H]arachidonic acid within 10 sec from mast cells preloaded with this labeled fatty acid [7]. This suggests that arachidonic acid might be liberated from phospholipid by $Ca^{2+}$-dependent phospholipase $A_2$ in Fr.D-stimulated mast cells as observed in several tissues [11, 23–25]. However, unexplained is the inconsistent stoichiometry of the levels of [$^3$H]arachidonic acid radioactivity between the phospholipids examined (Fig. 5), suggesting that PE was not the only donor of arachidonic acid in these transfer reactions. It is possible that the accumulation of [$^3$H]arachidonic acid-labeled PA and PI in the presence of $Ca^{2+}$ results from the activation of the PI cycle. However, at present, it is not clear why the increased levels of [$^{32}$P]-radioactivities in PA and PI fractions were not coincident with their changes in the level of [$^3$H]arachidonic acid in $Ca^{2+}$-free mast cells (Fig. 5 vs Fig. 6).

Modulation of PI cycle-related metabolism may not be sufficient for the $Ca^{2+}$-dependent histamine release from Fr.D-stimulated mast cells, and the rapid $Ca^{2+}$-dependent accumulation of arachidonic acid into PC, PI and PA, via a reaction(s) unknown at present, may be important for the subsequent histamine-releasing reactions. Fr.D seems to be a useful reagent to distinguish the extracellular $Ca^{2+}$-dependent reaction(s) from various cellular responses in phospholipid metabolism when mast cells were stimulated by compd 48/80.

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