Effect of calmodulin antagonists on lysosomal enzyme secretion and phospholipid metabolism in guinea-pig macrophages

Tadaomi TAKENAWA, Yoshimi HOMMA and Yoshitaka NAGAI

Institute of Basic Medical Science, University of Tsukuba, Niihari-gun, Ibaraki-ken and Institute of Medical Sciences, University of Tokyo, Shiroganedai, Minato-ku, Tokyo 108, Japan

(Received 14 April 1982/Accepted 13 September 1982)

The effects of calmodulin antagonists on the secretion of lysosomal enzyme and lipid metabolism in guinea-pig peritoneal macrophages were studied. Calmodulin antagonists, such as trifluoperazine, dibucaine and quinacrine, inhibited the secretion of *N*-acetyl- β -D-glucosaminidase from cytochalasin B-treated macrophages when the macrophages were stimulated by the chemotactic peptide, formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) or the Ca^{2+} ionophore A23187. The effect of calmodulin antagonists on the incorporation of [^{32}P]P_i or [^3H]glycerol into glycerolipids as well as on the redistribution of [^{14}C]glycerol or [^3H]arachidonic acid in [^{14}C]glycerol- or [^3H]arachidonic acid-prelabelled lipids were examined. Trifluoperazine, dibucaine or quinacrine stimulated [^{32}P]P_i incorporation into phosphatidic acid (PtdA) and phosphatidylinositol (PtdIns) without significant effect on the labelling of phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), lysophosphatidylcholine (lyso-PtdCho) and lysophosphatidylethanolamine (lyso-PtdEtn). The incorporation of [^{32}P]P_i into phosphatidylcholine (PtdCho) was, on the contrary, inhibited. When calmodulin antagonists were added to macrophages stimulated by fMet-Leu-Phe, [^{32}P]P_i incorporation into PtdIns and PtdA was synergistically increased compared with that induced only by calmodulin antagonists. Trifluoperazine inhibited the incorporation of [^3H]glycerol into PtdCho, triacylglycerol and PtdEtn. Also in this case, the incorporation of [^3H]glycerol into PtdA and PtdIns was greatly enhanced. But [^3H]glycerol incorporation into PtdSer, lyso-PtdEtn and lyso-PtdCho was not affected by the drug. On the other hand, diacylglycerol labelling with [^3H]glycerol was maximally activated by 10 μM -trifluoperazine and levelled off with the increasing concentration. When the effect of calmodulin antagonists on the redistribution of [^{14}C]glycerol among lipids was examined in pulse-chase experiments, no significant effect on [^{14}C]glycerol redistribution in PtdEtn, PtdCho, PtdIns, PtdSer, PtdA and tri- and di-acylglycerol could be detected. When macrophages prelabelled with [^3H]arachidonic acid were treated with trifluoperazine, dibucaine or quinacrine, the [^3H]arachidonic acid moiety in PtdEtn and PtdCho was decreased and that in PtdA was increased. The formation of [*arachidonate*- ^3H]diacylglycerol and non-esterified [^3H]arachidonic acid was also enhanced, but the increase in [^3H]arachidonic acid was only observed at concentrations between 1 and 50 μM . [*Arachidonate*- ^3H]PtdIns was not significantly affected. The activated formation of [*arachidonate*- ^3H]PtdA, diacylglycerol and non-esterified arachidonic acid by these drugs was synergistically enhanced in the presence of fMet-Leu-Phe.

Abbreviations used: PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdA, phosphatidic acid; lyso-PtdEtn, lysophosphatidylethanolamine; lyso-PtdCho, lysophosphatidylcholine; acyl₃Gro, triacylglycerol; acyl₂Gro, diacylglycerol; acylGro, mono-

acylglycerol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; NEFA, non-esterified fatty acid; MEM, minimal essential medium; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; fMet, *N*-formylmethionyl.

Calmodulin, a ubiquitous Ca^{2+} -binding protein in eukaryotes, is one of the most important modulators, affecting contractile proteins, cyclic nucleotide metabolism, glycogenolysis, secretion, protein phosphorylation, Ca^{2+} transport and lipid metabolism (Cheung, 1980; Stoclet, 1981; Brostrom & Wolff, 1981). But it is still difficult to study the role of calmodulin in the functioning of intact cells directly.

Since Levin & Weiss (1976) first reported that trifluoperazine and related phenothiazine derivatives were potent inhibitors of the Ca^{2+} -sensitive cyclic nucleotide phosphodiesterase, various antipsychotic agents and *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide (W-7) have been shown to have a calmodulin-antagonistic effect (Weiss & Levin, 1978; Hidaka *et al.*, 1979). Thus, certain antipsychotic agents have been used to clarify the role of calmodulin in various cellular processes (Sugden *et al.*, 1979; Nishikawa *et al.*, 1980; Shubart *et al.*, 1980; Naccache *et al.*, 1980; Henquin, 1981). However, the molecules used as calmodulin antagonists are highly hydrophobic and have a membrane-perturbing effect at the same time (Seeman, 1972; Norman *et al.*, 1979; Roufogalis, 1981). It is well known that a large variety of amphiphilic cationic drugs are able to inhibit phosphatidate phosphohydrolase and to stimulate phosphatidate cytidyltransferase (Sturton & Brindley, 1977; Bowley *et al.*, 1977) and that these events lead to the reduction of the synthesis of acyl₃Gro, PtdCho and PtdEtn and production of acidic phospholipids (Allan & Michell, 1975). It is, therefore, important to ascertain whether the effect of calmodulin antagonists are due to their specific action on calmodulin or to their interaction with membrane lipids.

Recently, it has been proposed that local anaesthetics, such as dibucaine, tetracaine and phenocaine, as well as other drugs with local anaesthetics, such as quinacrine and propranolol, may be included in the category of calmodulin antagonists (Volpi *et al.*, 1981). Among them, quinacrine has been used as an inhibitor for phospholipase A_2 (Blackwell *et al.*, 1977; Hirata *et al.*, 1979; Billah *et al.*, 1981). But its action profile on lipid metabolism in itself is not clear.

It has been thought that calmodulin plays an important role in secretory processes, since calmodulin antagonists, such as trifluoperazine and W-7, inhibited the secretion of insulin (Sugden *et al.*, 1979; Shubart *et al.*, 1980; Henquin, 1981) from pancreatic B-cells and lysosomal enzymes (Naccache *et al.*, 1980) from neutrophils. Increasing evidence has also been provided for a role of phospholipid metabolism in the chemotactic peptide-induced secretory process of neutrophils (Naccache *et al.*, 1979; Bokoch & Reed, 1980, 1981).

In this report, we study the effect of calmodulin

antagonists, such as trifluoperazine, dibucaine and quinacrine, on the lysosomal enzyme secretion and lipid metabolism in macrophages either stimulated or unstimulated by a formylated peptide.

Experimental

Materials

Trifluoperazine was a gift from Yoshitomi Pharmaceutical Co. Dibucaine, chlorpromazine, indomethacin, quinacrine, cytochalasin B, fMet-Leu-Phe and *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide were from Sigma Chemical Co. [^{32}P]P_i (carrier-free), [$2\text{-}^3\text{H}$]glycerol (sp. radioactivity 250 Ci/mol), [$1,3\text{-}^{14}\text{C}$]glycerol (2.5 Ci/mmol) and [$5,6,8,9,11,12,14,15\text{-}^3\text{H}$]arachidonic acid (78.2 Ci/mmol) were obtained from New England Nuclear. PtdA, lyso-PtdEtn, lyso-PtdCho, arachidonic acid, dipalmitoylglycerol and tripalmitoylglycerol were from Sedary Res. Co. PtdIns4P and PtdIns(4,5)P₂ were prepared by the method of Folch (1945).

Macrophages

Female Hartley guinea-pigs (300–400 g) were injected with 20 ml of liquid paraffin. The peritoneal exudate cells were collected 4 days after in Hanks' balanced salt solution. The cells were washed three times with the buffer, suspended in Eagle's MEM containing 15% guinea-pig serum, plated in plastic culture dishes and then incubated for 3 h at 37°C in CO_2/air (1:19). Non-adherent cells were removed by washing with medium and then the remaining macrophages were collected with a rubber policeman. This preparation, containing over 97% macrophages, was suspended in Eagle's MEM and used for the purified macrophages.

Lysosomal enzyme secretion

After macrophages (2×10^6 cells/0.2 ml) in Hanks' balanced salt solution were treated with 5 μg of cytochalasin B/ml for 10 min at 37°C, reaction was started by the addition of stimulants and continued for the indicated time. Cells were quickly chilled to 0°C on ice and centrifuged at 500 *g* for 10 min to stop the reaction. Portions of the supernatant were used to measure secreted *N*-acetyl- β -D-glucosaminidase. The supernatant (50 μl) was incubated with 4 mM-*p*-nitrophenyl *N*-acetyl- β -D-glucosaminide in 0.05 M-sodium citrate buffer (pH 4.5) in a total volume of 500 μl for 30 min at 37°C. Reaction was terminated by the addition of 0.4 M-glycine/NaOH buffer (pH 10.5) and released *p*-nitrophenol absorbance was measured at 410 nm. Total enzyme activities within macrophages were determined by adding 0.1% Triton X-100 to lyse the cells. Results are expressed as the percentage of total activity that was released to the extracellular medium by cells incubated with the stimulus.

Labelling experiments with [^{32}P]P_i, [^3H]glycerol, [^{14}C]glycerol or [^3H]arachidonic acid

Macrophages (10^8 cells/40 ml) were incubated with 1 mCi of [^{32}P]P_i or 100 μCi of [^3H]glycerol in Eagle's MEM containing 5% guinea-pig serum and 20 mM-Hepes/NaOH buffer (pH 7.4) for 45–60 min at 37°C. Then, 0.5 ml of cell suspension was transferred to a siliconized tube. The cells were incubated for various periods with various drugs in the presence or absence of fMet-Leu-Phe and reaction was terminated by adding 3 ml of chloroform/methanol (1:1, v/v).

To examine the effect of various drugs on redistribution of [^{14}C]glycerol in [^{14}C]glycerol-prelabelled macrophages, macrophages were incubated with 25 μCi of [^{14}C]glycerol in Eagle's MEM containing 5% guinea-pig serum and 20 mM-Hepes/NaOH buffer (pH 7.4) for 2 h at 37°C and washed by centrifugation at 500 g for 10 min. The washed cells were resuspended in the fresh medium containing 10 mM-glycerol and incubated for 20 min at 37°C and then reaction was started by the addition of stimulants.

Redistribution of [^3H]arachidonic acid-labelled lipids in [^3H]arachidonic acid-prelabelled macrophages was detected by the following methods. Macrophages were incubated with 30 μCi of [^3H]arachidonic acid in Eagle's MEM containing 5% guinea-pig serum and 25 mM-Hepes/NaOH buffer (pH 7.4) and the cells were then washed twice to remove non-esterified [^3H]arachidonic acid. Reaction was started by the addition of drugs.

Extraction and separation of lipids

Lipids were extracted by adding 3 ml of chloroform/methanol (1:1, v/v) to the reaction mixture. For the analysis of phospholipids, two-dimensional t.l.c. was carried out. The plates were developed with solvent systems of chloroform/methanol/28% aq. NH₃ (13:7:1, by vol.) for the first dimension and of chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.) for the second dimension. Lipids on the plates were visually detected by exposing the plates to I₂ vapour. Each spot (PtdCho, PtdEtn, PtdIns, PtdSer, PtdA, lyso-PtdEtn and lyso-PtdCho) was scraped off and the radioactivity was measured. For the analysis of neutral lipids and fatty acid, one-dimensional t.l.c. was carried out. The plates were developed with solvent system of light petroleum (b.p. 30–60°C)/diethyl ether/acetic acid (50:50:1, by vol.). Each spot (acyl₃Gro, acyl₂Gro and NEFA) was scraped off, transferred to scintillation vials containing Aquasol (New England Nuclear) and counted for radioactivity in a liquid-scintillation counter. The efficiency of radioactivity counting was 25%. Extraction recovery of lipids was monitored by measuring phosphorus content of each spot scraped from the t.l.c. plate by the method of

Rouser *et al.* (1970) when 25 μg of authentic PtdCho, PtdIns, PtdA and lyso-PtdCho were treated by the same procedures. Extraction recovery of PtdCho and PtdIns was found to be more than 93% and that of PtdA and lyso-PtdCho was 87% and 76% respectively. For the analysis of PtdIns4P and PtdIns(4,5)P₂, the reaction was stopped by adding 1 ml of 10% trichloroacetic acid instead of the organic solvent. Extraction and separation of PtdIns4P and PtdIns(4,5)P₂ was carried out by the modified method of Jolles *et al.* (1981). The plates were developed with chloroform/methanol/4.3 M-NH₃ (18:13:4, by vol.).

Each experiment was repeated at least twice. Amounts of incorporated radioactivity into various glycerolipids in macrophages varied from preparation to preparation, although similar effects of drugs, chemotactic peptide and ionophore A23187 were obtained in each experiment. Therefore, among repeated experiments, a typical result is shown in Figures and Tables.

Results

Effect of calmodulin antagonists on the secretion of N-acetyl- β -D-glucosaminidase induced by fMet-Leu-Phe or Ca²⁺ ionophore A23187

The effect of various concentrations of calmodulin antagonists on 2 μM -fMet-Leu-Phe-induced secretion is shown in Fig. 1(a). Trifluoperazine was a powerful inhibitor of lysosome enzyme release. Dibucaine and quinacrine also inhibited the release moderately. In a similar experiment, the effect of antagonists on 2 μM -ionophore A23187-induced secretion was examined (Fig. 1b). Trifluoperazine also strongly inhibited the secretion, and dibucaine and quinacrine were found to be moderate inhibitors.

Effect of calmodulin antagonists on [^{32}P]P_i incorporation into various phospholipids

The effect of various concentrations of trifluoperazine, dibucaine and quinacrine on [^{32}P]P_i incorporation into various phospholipids are shown in Fig. 2. All these drugs strongly inhibited [^{32}P]P_i incorporation into PtdCho. Addition of 10 μM -trifluoperazine caused a 25% decrease in PtdCho labelling. On the contrary, PtdIns and PtdA labelling was increased by all these drugs at concentrations up to 100 μM . [^{32}P]P_i incorporation into PtdEtn, PtdSer, lyso-PtdEtn and lyso-PtdCho was not affected significantly by these antagonists. When these drugs were added to macrophages stimulated by 2 μM -fMet-Leu-Phe, the combined effect was appreciably larger in PtdCho, PtdIns and PtdA labelling than that of either of its own. Especially, PtdA labelling was enhanced very significantly by a combination of the two. The labelling of PtdEtn,

Fig. 1. Effect of calmodulin antagonists on lysosomal enzyme secretion induced by *f*Met-Leu-Phe (a) or ionophore A23187 (b)

Macrophages were treated with 5 μg of cytochalasin B/ml for 10 min at 37°C and then reaction was started by the addition of stimulants in the presence or absence of various concentrations of trifluoperazine (O), dibucaine (Δ) or quinacrine (●) and continued for 10 min. After cells were quickly chilled to 0°C on ice and centrifuged at 500 g for 10 min, portions of supernatant were used to measure secreted *N*-acetyl-β-D-glucosaminidase. Results are expressed as percentages of total *N*-acetyl-β-D-glucosaminidase. Values are means ± S.E.M. of triplicate determinations.

Fig. 2. Effect of calmodulin antagonists on $[^{32}\text{P}]\text{P}_i$ incorporation into various phospholipids. Macrophages were incubated with $[^{32}\text{P}]\text{P}_i$ for 1 h at 37°C and then reaction was started by the addition of 2 μM-*f*Met-Leu-Phe in the presence or absence of trifluoperazine, dibucaine or quinacrine and continued for 10 min. O and ●, trifluoperazine; Δ and ▲, dibucaine; □ and ■, quinacrine, in the presence (filled symbols) or absence (open symbols) of *f*Met-Leu-Phe. Values represent means of triplicate determinations.

PtdSer, lyso-PtdEtn and lyso-PtdCho was not changed by the addition of fMet-Leu-Phe.

Effect of calmodulin antagonists on glycerolipid metabolism

It becomes clear that trifluoperazine is the strongest inhibitor of the secretion and a potent effector of phospholipid metabolism. In an experiment similar to that of Fig. 2, the effect of trifluoperazine on the incorporation of [^3H]glycerol into glycerolipid was measured (Fig. 3). Fig. 3(a) shows the time course of the effect of trifluoperazine. Major glycerolipids, such as PtdCho, acyl₃Gro and PtdEtn, of 50 μM -trifluoperazine-treated macrophages showed a decreased incorporation of [^3H]glycerol. There was also a great increase in the incorporation of [^3H]glycerol into PtdA and PtdIns. In Fig. 3(b), the effect of various concentrations of trifluoperazine on [^3H]glycerol incorporation into glycerolipids is shown. In agreement with [^{32}P]P_i-labelling experiments, trifluoperazine inhibited the incorporation of [^3H]glycerol into major glycerolipids, such as PtdCho, acyl₃Gro and PtdEtn, and stimulated

PtdIns and PtdA labelling in a dose-dependent manner. However, acyl₂Gro labelling reached a maximum at 10 μM and levelled off with increasing concentration. These results suggest that inhibition of PtdCho, acyl₂Gro and PtdEtn synthesis leads to the increased formation of PtdIns and PtdA, which is consistent with previous results in lymphocytes (Allan & Michell, 1975). It has been thought that this re-arrangement of phospholipid synthesis is due to the inhibition of phosphatidate hydrolase. Indeed, there was an inhibition of acyl₂Gro accumulation at high trifluoperazine concentrations. The combination effect of fMet-Leu-Phe and trifluoperazine, which was observed in [^{32}P]P_i-labelling experiments, could not be demonstrated.

When macrophages were labelled with [^{14}C]glycerol, washed and chased with 10 mM-glycerol, effect of trifluoperazine on the redistribution of [^{14}C]glycerol-labelled lipids was studied. Various concentrations of drugs (from 1 to 100 μM) did not affect the redistribution of [^{14}C]glycerol moiety in PtdCho, PtdEtn, PtdSer, PtdIns, PtdA, acyl₂Gro and acyl₃Gro with the incubation up to 10 min. The result suggests that the degradation reaction of glycerolipids is scarcely affected by the drugs.

Fig. 3. Effect of trifluoperazine on glycerolipid metabolism

Macrophages were incubated with [^3H]glycerol for 45 min at 37°C and then reaction was started by the addition of trifluoperazine for the indicated periods. (a) Shows the time course of the effect of 50 μM -trifluoperazine on the incorporation of [^3H]glycerol into glycerolipids. Open symbols represent control cells; filled symbols represent cells treated with 50 μM -trifluoperazine. (b) Shows the dose-response relationship. Incubation was carried out for 10 min. Values represent mean of triplicate determinations.

Fig. 4. Effect of calmodulin antagonists on the redistribution of the $[^3\text{H}]$ arachidonic acid moiety in $[^3\text{H}]$ arachidonic acid-labelled lipids

Macrophages were incubated for 1 h at 37°C and then cells were washed twice by centrifugation. After $[^3\text{H}]$ arachidonic acid-labelled macrophages were resuspended in the medium, reaction was started by the addition of $2 \mu\text{M}$ -fMet-Leu-Phe in the presence or absence of various concentrations of trifluoperazine, dibucaine or quinacrine and continued for 10 min. $\circ$ and $\bullet$, trifluoperazine; $\triangle$ and $\blacktriangle$, dibucaine; $\square$ and $\blacksquare$, quinacrine, with (filled symbols) or without (open symbols) $2 \mu\text{M}$ -fMet-Leu-Phe. Values represent means $\pm$ S.E.M. of triplicate determinations.

Effect of various drugs on the redistribution of [³H]arachidonic acid moiety in [³H]arachidonic acid-labelled lipids

Macrophages were incubated with [³H]arachidonic acid for 1 h at 37°C and then the cells were washed twice to remove non-esterified [³H]arachidonic acid. Under these conditions, more than 99.5% of [³H]arachidonic acid was present in the form of arachidonyl-lipids. By using such [³H]arachidonic acid-labelled macrophages, the effect of various concentrations of trifluoperazine, dibucaine and quinacrine on the redistribution of [³H]arachidonyl-lipids was studied (Fig. 4). [³H]Arachidonic acid in PtdEtn and PtdCho fractions was decreased by the addition of these drugs. On the contrary, these drugs caused a considerable increase in [*arachidonate*-³H]PtdA. The formation of [*arachidonate*-³H]acyl₂Gro and non-esterified [³H]arachidonic acid was also enhanced by the addition of these drugs, but the increase in non-esterified [³H]arachidonic acid was only observed at the concentration of drugs between 1 and 50 μM. Above 50 μM, the accumulation was rather inhibited, whereas the accumulation of [*arachidonate*-³H]acyl₂Gro was increased with drug concentrations up to 100 μM. But [*arachidonate*-³H]PtdIns was not significantly affected by these drugs. In [³H]glycerol-labelling experiments (Fig. 3), we demonstrated that synthesis *de novo* of acyl₂Gro from glycerol 3-phosphate was inhibited by 100 μM-trifluoperazine. But in this experiment (Fig. 4), the accumulation of [*arachidonate*-³H]acyl₂Gro was found to be increased slightly. This discrepancy may reflect the differential features of phosphatidate phospho-

hydrolase and diacylglycerol lipase. Namely, although trifluoperazine could inhibit the activities of both enzymes, diacylglycerol lipase is more sensitive to this drug and slight accumulation of [*arachidonate*-³H]acyl₂Gro may take place. The stimulated accumulation of [*arachidonate*-³H]-PtdA, -acyl₂Gro and non-esterified [³H]arachidonic acid by these drugs was synergistically stimulated in the presence of 2 μM-fMet-Leu-Phe. In particular, the accumulation of [*arachidonate*-³H]PtdA and non-esterified [³H]arachidonic acid was strongly enhanced by the addition of fMet-Leu-Phe. Quinacrine is known to be an inhibitor of phospholipase A₂ (Blackwell *et al.*, 1977; Hirata *et al.*, 1979). However, the drug enhanced the liberation of arachidonic acid when it was added to the macrophages stimulated by fMet-Leu-Phe. Since calmodulin antagonists did not increase the accumulation of [¹⁴C]glycerol-labelled PtdA in macrophages prelabelled with [¹⁴C]glycerol, there may be a system in which the arachidonic acid moiety is transferred effectively from PtdCho or PtdEtn to PtdA and this transesterification may be stimulated by these drugs. The effect of various drugs on the formation of [*arachidonate*-³H]acyl₂Gro from prelabelled macrophages with [³H]arachidonic acid was investigated (Table 1). In the absence of fMet-Leu-Phe, dibucaine, quinacrine, chlorpromazine, trifluoperazine and indomethacin slightly stimulated the accumulation of [*arachidonate*-³H]acyl₂Gro in a dose-dependent manner. On the other hand, in the presence of fMet-Leu-Phe, such activation was synergistically stimulated, although high concentrations of chlorpromazine rather suppressed the combination effect.

Table 1. *Effect of various drugs on the formation of [*arachidonate*-³H]diacylglycerol in [³H]arachidonic acid-labelled macrophages*

Macrophages labelled with [³H]arachidonic acid were incubated with various drugs in the presence or absence of 2 μM-fMet-Leu-Phe for 10 min at 37°C. Acyl₂Gro was separated by t.l.c. as described in the Experimental section. Values are means ± s.e. of triplicate determinations. Values in parentheses indicate the concentration of drug in μM.

		[<i>Arachidonate</i> - ³ H]diacylglycerol radioactivity			
		Without fMet-Leu-Phe		With fMet-Leu-Phe	
Treatment		(d.p.m./10 ⁶ cells)	(%)	(d.p.m./10 ⁶ cells)	(%)
None		629 ± 15	100	750 ± 12	119
Dibucaine	(10)	811 ± 82	129	891 ± 25	142
	(100)	940 ± 21	149	1618 ± 166	257
Quinacrine	(10)	747 ± 17	119	839 ± 36	132
	(100)	959 ± 70	152	1174 ± 53	187
Chlorpromazine	(10)	627 ± 41	100	869 ± 11	138
	(100)	769 ± 50	122	641 ± 9	102
Trifluoperazine	(10)	669 ± 11	106	843 ± 11	134
	(100)	909 ± 55	145	1297 ± 20	206
Indomethacin	(10)	673 ± 30	107	934 ± 34	148
	(100)	864 ± 52	137	1001 ± 46	159
Cytochalasin B	(1)	646 ± 16	103	880 ± 26	140
	(10)	639 ± 85	102	897 ± 22	143

Table 2. Effect of various drugs on the liberation of [^3H]arachidonic acid from [^3H]arachidonic acid-labelled macrophages

Macrophages labelled with [^3H]arachidonic acid were incubated with various drugs in the presence or absence of $1\text{ }\mu\text{M}$ -fMet-Leu-Phe for 10 min at 37°C and then released [^3H]arachidonic acid was measured after separation from other lipids as described in the Experimental section. Values are means $\pm$ S.E. of triplicate determinations. Values in parentheses indicate the concentration of drug in μM .

Treatment		Non-esterified [^3H]arachidonic acid radioactivity			
		Without fMet-Leu-Phe		With fMet-Leu-Phe	
		(d.p.m./ 10^6 cells)	(%)	(d.p.m./ 10^6 cells)	(%)
None		1069 $\pm$ 99	100	5345 $\pm$ 170	500
Dibucaine	(10)	1267 $\pm$ 79	118	9778 $\pm$ 987	914
	(100)	822 $\pm$ 173	77	4843 $\pm$ 601	453
Quinacrine	(10)	1217 $\pm$ 175	114	7148 $\pm$ 148	669
	(100)	813 $\pm$ 40	76	2540 $\pm$ 30	238
Chlorpromazine	(10)	1330 $\pm$ 284	124	7316 $\pm$ 283	684
	(100)	722 $\pm$ 41	68	1083 $\pm$ 159	101
Trifluoperazine	(10)	1537 $\pm$ 99	144	8612 $\pm$ 220	806
	(100)	969 $\pm$ 43	91	2355 $\pm$ 680	220
Indomethacin	(10)	1005 $\pm$ 39	94	5099 $\pm$ 59	477
	(100)	1157 $\pm$ 15	108	5504 $\pm$ 27	515
Cytochalasin B	(1)	1263 $\pm$ 73	118	8323 $\pm$ 347	779
	(10)	1322 $\pm$ 47	124	10517 $\pm$ 590	983

Cytochalasin B also enhanced the accumulation only in the presence of fMet-Leu-Phe. Table 2 indicates the effect of various drugs on the liberation of [^3H]arachidonic acid from prelabelled macrophages. The release of [^3H]arachidonic acid was slightly activated by $10\text{ }\mu\text{M}$ -dibucaine, -quinacrine, -chlorpromazine, -trifluoperazine and -cytochalasin B. But at higher concentrations of these drugs ($100\text{ }\mu\text{M}$), the release was, on the contrary, inhibited. The addition of fMet-Leu-Phe stimulated the release of arachidonic acid five times compared with no addition. This increase was synergistically enhanced by the addition of $10\text{ }\mu\text{M}$ -dibucaine, -quinacrine, -chlorpromazine, -trifluoperazine or -cytochalasin B. Thus, not only calmodulin antagonists, but also cytochalasin B, an agent inhibiting the contractile microfilament system, appear to enhance the fMet-Leu-Phe-induced effect. Considering the fact that fMet-Leu-Phe stimulates the release of arachidonic acid through the pathway $\text{PtdIns} \rightarrow \text{acyl}_2\text{Gro} \rightarrow \text{NEFA}$ and the result that the formation of lysophospholipids is not enhanced, it seems that these drugs enhance the liberation of arachidonic acid at the appropriate concentration in macrophages treated with fMet-Leu-Phe through the activation of diacylglycerol lipase.

Effect of calmodulin antagonists on [^{32}P]P_i incorporation into PtdIns4P and PtdIns(4,5)P₂ and the redistribution of [^3H]arachidonic acid in PtdIns4P and PtdIns(4,5)P₂

Calmodulin antagonists, such as trifluoperazine, dibucaine and quinacrine did not show a significant effect on [^{32}P]P_i incorporation into

PtdIns4P, although higher concentrations of trifluoperazine ($100\text{ }\mu\text{M}$) caused an increase in PtdIns4P (Table 3). The presence of fMet-Leu-Phe or ionophore A23187 decreased the labelling of PtdIns4P. This decrease appeared to be suppressed by the addition of these drugs. At the same time, there was a marked increase in the labelling of PtdIns(4,5)P₂ by the treatment of calmodulin antagonists. Although ionophore A23187 also enhanced the labelling of PtdIns(4,5)P₂, the combination with ionophore A23187 and these drugs caused a synergistic activation of [^{32}P]P_i incorporation into PtdIns(4,5)P₂. On the other hand, fMet-Leu-Phe inhibited the formation of PtdIns(4,5)P₂ slightly but this inhibition was suppressed by these drugs.

These drugs also increased the accumulation of [^3H]arachidonate- ^3H]PtdIns(4,5)P₂ to a lesser extent compared with that in [^{32}P]P_i-labelling experiments when macrophages prelabelled with [^3H]arachidonic acid were used (Table 4). The chemotactic peptide fMet-Leu-Phe caused a great decrease in [^3H]arachidonate- ^3H]PtdIns4P and a slight decrease in [^3H]arachidonate- ^3H]PtdIns(4,5)P₂. This decrease was blocked by the addition of these drugs. On the other hand, ionophore A23187 decreased [^3H]arachidonate- ^3H]PtdIns4P very significantly without a marked effect on [^3H]arachidonate- ^3H]PtdIns(4,5)P₂. In combinations with ionophore A23187, these drugs always increased [^3H]arachidonate- ^3H]PtdIns4P and -PtdIns(4,5)P₂. Thus, it appeared that the pathway $\text{PtdIns} \rightarrow \text{PtdIns4P} \rightarrow \text{PtdIns(4,5)P}_2$ was activated and the effect of fMet-Leu-Phe was suppressed by calmodulin antagonists.

Table 3. *Effect of calmodulin antagonists on [32 P] P_i incorporation into PtdIns4P and PtdIns(4,5) P_2 in the presence or absence of fMet-Leu-Phe*

Macrophages were incubated with [32 P] P_i for 1 h at 37°C and then reaction was started by the addition of trifluoperazine, dibucaine or quinacrine in the presence or absence of 2 μ M-fMet-Leu-Phe or 2 μ M-ionophore A23187 and continued for 10 min. The method for extraction and separation of PtdIns4P and PtdIns(4,5) P_2 was as described in the Experimental section. Values are means $\pm$ s.e. of triplicate determinations. Numbers in parentheses indicate the concentration of drug in μ M.

Treatment	32 P incorporated (d.p.m./10 ⁶ cells)					
	PtdIns4P fraction			PtdIns(4,5) P_2 fraction		
	Control	+fMet-Leu-Phe	+Ionophore A23187	Control	+fMet-Leu-Phe	+Ionophore A23187
None	290 $\pm$ 10	164 $\pm$ 15	125 $\pm$ 4	180 $\pm$ 8	149 $\pm$ 13	212 $\pm$ 10
Trifluoperazine (10)	306 $\pm$ 7	140 $\pm$ 23	120 $\pm$ 5	182 $\pm$ 13	176 $\pm$ 4	294 $\pm$ 8
(100)	387 $\pm$ 13	227 $\pm$ 11	211 $\pm$ 11	252 $\pm$ 15	224 $\pm$ 13	510 $\pm$ 15
Dibucaine (10)	222 $\pm$ 24	166 $\pm$ 12	135 $\pm$ 6	171 $\pm$ 10	154 $\pm$ 5	278 $\pm$ 12
(100)	214 $\pm$ 38	186 $\pm$ 8	173 $\pm$ 8	292 $\pm$ 13	237 $\pm$ 23	425 $\pm$ 15
Quinacrine (10)	321 $\pm$ 12	178 $\pm$ 13	120 $\pm$ 12	171 $\pm$ 18	164 $\pm$ 7	221 $\pm$ 13
(100)	283 $\pm$ 15	231 $\pm$ 17	143 $\pm$ 15	232 $\pm$ 13	184 $\pm$ 18	290 $\pm$ 7

Table 4. *Effect of calmodulin antagonists on the movement of [3 H]arachidonic acid moiety in PtdIns4P and PtdIns(4,5) P_2*
Experimental procedures were the same as described in the legend to Fig. 4. Values are means $\pm$ s.e. of triplicate determinations. Numbers in parentheses indicate the concentration of drug in μ M.

Treatment	3 H]Arachidonic acid (d.p.m./10 ⁶ cells)					
	PtdIns4P fraction			PtdIns(4,5) P_2 fraction		
	Control	+fMet-Leu-Phe	+Ionophore A23187	Control	+fMet-Leu-Phe	+Ionophore A23187
None	1256 $\pm$ 23	669 $\pm$ 85	596 $\pm$ 67	1245 $\pm$ 65	1087 $\pm$ 57	1473 $\pm$ 53
Trifluoperazine (10)	1257 $\pm$ 31	688 $\pm$ 58	622 $\pm$ 15	1422 $\pm$ 98	1271 $\pm$ 51	1714 $\pm$ 69
(100)	1540 $\pm$ 98	1153 $\pm$ 89	814 $\pm$ 12	1717 $\pm$ 56	1830 $\pm$ 70	2559 $\pm$ 72
Dibucaine (10)	1234 $\pm$ 98	816 $\pm$ 16	657 $\pm$ 65	1210 $\pm$ 23	1107 $\pm$ 57	1761 $\pm$ 38
(100)	1050 $\pm$ 85	936 $\pm$ 28	906 $\pm$ 48	1818 $\pm$ 27	1390 $\pm$ 45	2236 $\pm$ 56
Quinacrine (10)	1322 $\pm$ 25	764 $\pm$ 36	682 $\pm$ 56	1266 $\pm$ 85	1163 $\pm$ 10	1321 $\pm$ 98
(100)	1089 $\pm$ 58	1067 $\pm$ 42	786 $\pm$ 10	1618 $\pm$ 45	1324 $\pm$ 85	1871 $\pm$ 25

Discussion

We demonstrated that macrophages showed enhanced [32 P] P_i -labelling of PtdA and PtdIns when exposed to various calmodulin antagonists. It is thought that the increase in PtdIns- and PtdA-labelling by amphiphilic cationic drugs is caused by the redirection of phospholipid metabolism towards the synthesis of acidic lipids, which is accompanied by a decrease in acyl₂Gro and PtdCho concentrations and it has been suggested that the mechanism by which amphiphilic cations alter the metabolic pathway of phospholipids is due to the inhibition of phosphatidate phosphohydrolase (Allan & Michell, 1975; Sturton & Brindley, 1977; Bowley *et al.*, 1977). The major effect of amphiphilic cations in liver cells was an inhibition of acyl₃Gro and PtdCho synthesis and a marked accumulation of

PtdA (Brindley & Bowley, 1975); in pig lymphocytes, a large increase in the rate of [32 P] P_i incorporation into PtdIns was the most significant event (Allan & Michell, 1975). Thus, responses to amphiphilic cations appear to be different in tissues. In addition, we demonstrated that the accumulation of acyl₂Gro and non-esterified arachidonic acid was also stimulated in macrophages by an appropriate concentration of these drugs (Figs. 3 and 4), although high concentrations of these drugs inhibited the accumulation of non-esterified arachidonic acid. On the other hand, PtdA labelling was increased with concentration up to 100 μ M (Figs. 2, 3 and 4).

The alterations in lipid metabolism induced by calmodulin antagonists appear to be similar to those induced by ionophore A23187 (Y. Homma & T. Takenawa, unpublished work), although ionophore

A23187 stimulated the accumulation of acyl₂Gro more strongly than the drugs did.

It has been reported that fMet-Leu-Phe activates phospholipase C and enhances PtdIns breakdown to acyl₂Gro, which is further degraded to acylGro and NEFA or phosphorylated to PtdA in macrophages (Cockcroft *et al.*, 1981). Therefore, it is clear that the two responses differ fundamentally in their mechanisms. The combination of these different kinds of stimulators increased the accumulation of PtdA, acyl₂Gro and NEFA synergistically as shown in Figs. 1 and 4. In this case, PtdIns breakdown rather than the pathway PtdA → acyl₂Gro → NEFA may be enhanced, since the incorporation of [³H]glycerol into PtdA and acyl₂Gro was not accelerated by the addition of fMet-Leu-Phe.

Calmodulin antagonists stimulated phospholipid metabolism and arachidonic acid liberation in macrophages. However, they inhibited lysosomal enzyme secretion, although arachidonic acid by itself can induce degranulation of rabbit neutrophils without these drugs (Naccache *et al.*, 1979; Bokoch & Reed, 1980, 1981). These results suggest that there are many steps in the mechanism of release of lysosomal enzymes and a relatively late step, such as protein phosphorylation, may be blocked by calmodulin antagonists, whereas liberation of arachidonic acid, as a relatively early step, may be stimulated. However, it is not certain whether secretion can be blocked when the initial reaction, such as activation of phospholipid metabolism, is inhibited.

Quinacrine is thought to be a potent inhibitor of phospholipase A₂ (Blackwell *et al.*, 1977; Hirata *et al.*, 1979), but in the present paper the drug produced similar effects on phospholipid metabolism to those detected with other calmodulin antagonists. Interestingly, the liberation of arachidonic acid induced by fMet-Leu-Phe was synergistically enhanced by the drug at concentrations between 1 and 50 μM. This result may indicate that fMet-Leu-Phe does not stimulate the liberation of arachidonic acid by phospholipase A₂ activation.

As shown in Fig. 4, the formation of [*arachidonate*-³H]PtdA was increased when macrophages labelled with [³H]arachidonic acid were stimulated by calmodulin antagonists, whereas when [¹⁴C]-glycerol-labelled macrophages were stimulated by these drugs a significant increase in [¹⁴C]glycerol-labelled PtdA was not detected. Furthermore, these drugs did not cause a decrease in [¹⁴C]glycerol-labelled PtdCho and PtdEtn or an increase in lyso-PtdEtn or lyso-PtdCho. These results may support the idea (Billah *et al.*, 1981) that there is a transesterification system between PtdA and other phospholipids, because [*arachidonate*-³H]PtdA is increased neither through the activation of phospholipase A₂ nor through that of phospholipase C.

Thus, transesterification between PtdA and PtdCho or PtdEtn may be enhanced also by these drugs.

References

- Allan, D. & Michell, R. H. (1975) *Biochem. J.* **148**, 471–478
- Billah, M. M., Lapetina, E. G. & Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5399–5403
- Blackwell, G. J., Duncombe, W. G., Flower, R. J., Parsons, M. F. & Vane, J. R. (1977) *Br. J. Pharmacol.* **59**, 353–366
- Bokoch, G. M. & Reed, P. W. (1980) *J. Biol. Chem.* **255**, 10223–10226
- Bokoch, G. M. & Reed, P. W. (1981) *J. Biol. Chem.* **256**, 5317–5320
- Bowley, M., Cooling, J., Burditt, S. L. & Brindley, D. N. (1977) *Biochem. J.* **165**, 447–454
- Brindley, D. N. & Bowley, M. (1975) *Biochem. J.* **148**, 461–469
- Brostrom, C. O. & Wolff, D. J. (1981) *Biochem. Pharmacol.* **30**, 1395–1405
- Cheung, W. Y. (1980) *Science* **207**, 19–27
- Cockcroft, S., Bennett, J. P. & Gomperts, B. D. (1981) *Biochem. J.* **200**, 501–508
- Folch, J. (1945) *J. Biol. Chem.* **177**, 505–519
- Henquin, J.-C. (1981) *Biochem. J.* **196**, 771–780
- Hidaka, H., Yamaki, T., Totsuka, T. & Asano, M. (1979) *Mol. Pharmacol.* **15**, 49–59
- Hirata, F., Corcoran, B. A., Venkatasubramanian, K., Schiffmann, E. & Axelrod, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2640–2643
- Jolles, J., Zwiers, H., Dekker, A., Wirtz, K. W. A. & Gispén, W. H. (1981) *Biochem. J.* **194**, 283–291
- Levin, R. M. & Weiss, B. (1976) *Mol. Pharmacol.* **12**, 581–589
- Naccache, P. H., Showell, H. J., Becker, E. L. & Sha'afi, R. I. (1979) *Biochem. Biophys. Res. Commun.* **87**, 292–299
- Naccache, P. H., Molski, T. F. P., Alobaidi, T., Becka, E. L., Showell, H. J. & Sha'afi, R. I. (1980) *Biochem. Biophys. Res. Commun.* **97**, 62–68
- Nishikawa, M., Tanaka, T. & Hidaka, H. (1980) *Nature (London)* **287**, 863–865
- Norman, J. A., Drummond, A. H. & Moser, P. (1979) *Mol. Pharmacol.* **16**, 1089–1094
- Roufogalis, B. D. (1981) *Biochem. Biophys. Res. Commun.* **98**, 607–613
- Rouser, G., Fleisher, S. & Yamamoto, A. (1970) *Lipids* **5**, 494–496
- Seeman, P. (1972) *Pharmacol. Rev.* **24**, 583–655
- Shubart, U. K., Erlichman, J. & Fleisher, N. (1980) *J. Biol. Chem.* **255**, 4120–4124
- Stoclet, J. C. (1981) *Biochem. Pharmacol.* **30**, 1723–1729
- Sturton, R. G. & Brindley, D. N. (1977) *Biochem. J.* **162**, 25–32
- Sugden, M. C., Christie, M. R. & Ashcroft, S. J. H. (1979) *FEBS Lett.* **105**, 95–100
- Volpi, M., Sha'afi, R. I., Epstein, P. M., Andrenyak, D. M. & Feinstein, M. B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 795–799
- Weiss, B. & Levin, R. M. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 285–303