Activation of (arachidonyl) phosphatidylinositol turnover in rabbit neutrophils by the calcium ionophore A23187

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The addition of the Ca^{2+} ionophore A23187 to rabbit neutrophils stimulated [^{14}C]arachidonic acid incorporation into phosphatidylinositol and lysosomal enzyme secretion. A significant increase in phosphatidylinositol labelling was observed after a 2 min exposure to $0.1\ \mu\text{M}$ -ionophore A23187. Maximum increases in rate of labelling were obtained with $1\ \mu\text{M}$ -ionophore A23187 within 1 min, declining to basal rates after 15 min. Similarly, maximum rate of enzyme release occurred during the first 2 min of exposure to ionophore and release was essentially complete by 15 min. Threshold and peak ionophore A23187 concentrations for stimulating both processes were identical. In contrast with the specificity of phosphatidylinositol labelling induced by $1\ \mu\text{M}$ -ionophore A23187 in the absence of cytochalasin B, ionophore also significantly stimulated labelling of phosphatidylserine and phosphatidylethanolamine in the presence of cytochalasin B. With a threshold ionophore concentration ($0.1\ \mu\text{M}$), the enhanced incorporation of arachidonate was relatively specific for phosphatidylinositol in cytochalasin-treated cells. Ionophore A23187 did not accelerate labelling of phosphatidylinositol by [^{14}C]acetate or [^{14}C]glycerol, indicating that ionophore A23187 does not stimulate phosphatidylinositol synthesis *de novo*, although it did promote [^{14}C]palmitate and [^{32}P]P_i incorporation into neutrophil phosphatidylinositol. However, the increase in phosphatidylinositol labelling with these latter precursors was generally slower in onset and much more modest in magnitude than that observed with arachidonic acid. These results support the hypothesis that a Ca^{2+} -dependent phospholipase, which acts on the arachidonate moiety of phosphatidylinositol, is responsible for initiating at least certain of the membrane events coupled to the release of secretory product from the neutrophil.

The growing awareness of the potential importance of arachidonic acid and prostaglandins in cellular function provides a new dimension toward elucidating the molecular mechanism by which Ca^{2+} exerts its key regulatory role as an obligatory modulator of the secretory process. Arachidonic acid turnover and prostaglandin synthesis are intimately linked to the actions of Ca^{2+} as revealed by the fact that Ca^{2+} is required for the activation of phospholipase A_2 , which cleaves arachidonic acid from position-2 of phospholipids, thereby providing the substrate for prostaglandin synthesis (Vogt, 1978). This effect of Ca^{2+} would account for the findings that Ca^{2+} -deprivation blocks and the Ca^{2+} ionophore A23187 stimulates prostaglandin synthesis in several secretory tissues, including the

adrenal cortex, blood platelet and neutrophil (Laychock *et al.*, 1977; Knapp *et al.*, 1977; Wentzell & Epand, 1978). The stimulation of phospholipase A_2 not only leads to the liberation of arachidonic acid and the resulting increase in prostaglandin formation, which may participate in the events associated with exocytotic release, but phospholipids serving as the source of arachidonic acid are converted into lysophospholipids, which may have intracellular actions of their own. Moreover, since lysophospholipids are potentially cytotoxic (Bigon *et al.*, 1979; Mookerjee, 1979), cellular mechanisms must exist for preventing these substances from rising to concentrations detrimental to the cell.

Lands and his associates first provided evidence for the existence of an acyl-CoA transferase that catalyses the reacylation of lysophospholipid to its corresponding phospholipid; these investigators proposed that the deacylation–reacylation sequence of

Abbreviations used: fMet, formylmethionyl.

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phospholipid turnover plays a critical role in the biochemical events associated with cell-membrane perturbations during activation (see Lands & Crawford, 1976). Studies from our own laboratory have provided support for this theory in secretory organs by demonstrating the stimulation of (arachidonyl) phosphatidylinositol turnover in isolated cat adrenocortical cells by corticotropin (Schrey & Rubin, 1979) and in rabbit neutrophils by the synthetic chemotactic peptide fMet-Leu-Phe (Rubin *et al.*, 1979, 1980). The turnover of phosphatidylinositol in these two secretory systems was quantitatively and temporally correlated with secretory activity. The key role of Ca^{2+} in this specific sequence is underscored by the Ca^{2+} requirement for corticotropin- and fMet-Leu-Phe-induced stimulation of (arachidonyl) phosphatidylinositol turnover and by the finding that ionophore A23187, which promotes Ca^{2+} transport across cell membranes, elicited an even greater enhancement of arachidonic acid incorporation into phosphatidylinositol in adrenocortical cells than did corticotropin (Schrey & Rubin, 1979). In the present paper, we show that ionophore A23187 is also capable of producing a very rapid and powerful stimulation of arachidonic acid incorporation into phosphatidylinositol in rabbit neutrophils, which is independent of synthesis of phospholipid *de novo*.

Materials and methods

Materials

The following radiolabelled materials were purchased from Amersham/Searle Corp., Arlington Heights, IL, U.S.A.: [$1\text{-}^{14}\text{C}$]arachidonic acid (sp. radioactivity 56 Ci/mol) and palmitic acid (sp. radioactivity 59 Ci/mol). [^{32}P]Orthophosphoric acid (sp. radioactivity 250 Ci/mol), sodium [$1,2\text{-}^{14}\text{C}$]acetate (sp. radioactivity 54 Ci/mol) and [$\text{U}\text{-}^{14}\text{C}$]glycerol (sp. radioactivity 43 Ci/mol) were obtained from New England Nuclear, Boston, MA, U.S.A. The following biochemicals were acquired from Sigma Chemical Co., St. Louis, MO, U.S.A.: cytochalasin B, fatty acid-free bovine serum albumin, *p*-nitrophenyl *N*-acetyl- β -glucosaminide and all phospholipid standards other than phosphatidylinositol. Phosphatidylinositol was purchased from Supelco Inc., Bellefonte, PA, U.S.A. Earle's balanced salt solution, without Ca^{2+} and Mg^{2+} , was obtained from Flow Laboratories, McLean, VA, U.S.A., and thin-layer plates of silica gel H for separation of phospholipids were purchased from Analtech Inc., Newark, DL, U.S.A. The ionophore A23187 was obtained from Calbiochem-Behring, LaJolla, CA, U.S.A., and was dissolved in ethanol and diluted with medium to requisite concentrations immediately before use. The amounts of ethanol in the final incubation medium, which never exceeded

0.03%, had no effect on precursor incorporation into phospholipids. All other chemicals were of the highest grade available.

Methods

Albino rabbits were injected intraperitoneally with 400 ml of sterile iso-osmotic saline containing 0.1% glycogen, and the peritoneal exudate was collected in heparinized flasks 12 h later to obtain neutrophils. Cells used for lysosomal enzyme assay were resuspended directly in a modified Earle's balanced salt solution supplemented to contain 0.5 mM- Ca^{2+} , 0.2 mM- Mg^{2+} , 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate], pH 7.4, and 0.025% bovine serum albumin. The cells used for phospholipid analysis were first washed with buffered ammonium chloride to haemolyse erythrocytes and then resuspended in the same medium as above. This medium was used throughout the remainder of the experiments.

All radiolabel-incorporation studies employed 4×10^6 cells in a final volume of 500 μl of medium incubated at 37°C under an atmosphere of O_2/CO_2 (19:1). Cell suspensions were preincubated in the presence or absence of cytochalasin B (5 $\mu\text{g}/\text{ml}$) for 10 min at 37°C and then were added to incubation tubes containing 250 μl of medium comprising 0.2 μCi of [$1\text{-}^{14}\text{C}$]arachidonic acid, 0.8 μCi of [$1\text{-}^{14}\text{C}$]palmitic acid, 0.25 μCi of [$\text{U}\text{-}^{14}\text{C}$]glycerol or 100 μCi of [^{32}P]P_i in the presence or absence of ionophore A23187. [$1\text{-}^{14}\text{C}$]Acetate (2.5 μCi) was added during a 15 min pre-incubation period to allow time for this precursor to equilibrate with endogenous pools. Incubations were carried out for intervals of 1–30 min. The reaction was terminated by adding chloroform/methanol (1:2, v/v), and the phospholipids were extracted and separated by two-dimensional t.l.c. as previously described (Rubin *et al.*, 1980). Location of each phospholipid was determined by using I_2 vapour staining. The areas corresponding to each standard were scraped and transferred to plastic vials containing 10 ml of a solution of Omnifluor (New England Nuclear) plus toluene (4 g/litre). All samples were counted for radioactivity for 10 min in a liquid-scintillation counter (Nuclear-Chicago; Unilux series). The counting efficiency for ^{14}C was 88%, as determined with an unquenched standard, and there was no decrease when ^{14}C from silica-gel scrapings was counted for radioactivity. Values were subtracted from background, which averaged $34(\pm 1)$ c.p.m. for both ^{14}C and [^{32}P]P_i. Each experiment was carried out in duplicate and was generally replicated on at least four different preparations.

Neutrophils used in the lysosomal enzyme assay were not pretreated with ammonium chloride, since this treatment produced high amounts of basal enzyme secretion; however, the cells were pre-

incubated with cytochalasin B and then added to incubation tubes (4×10^6 cells) in a final incubation volume of $500\mu\text{l}$ in the presence or absence of various concentrations of ionophore A23187. The incubation was carried out for 2–30min and was stopped by immersing the tubes on ice. *N*-Acetylglucosaminidase activity was monitored by a spectrophotometric method previously described (Rubin *et al.*, 1980). Enzyme release is expressed as the percentage of total cellular enzyme activity, or as the rate of enzyme release. When outputs were expressed as rates, spontaneous release was subtracted from the ionophore A23187-stimulated release; this difference was divided by the appropriate time period to obtain the stimulus-induced rate.

The statistical significance of differences between drug-treated and untreated cells was obtained by Student's *t* test for paired or unpaired samples using a Hewlett–Packard programmable calculator (model 10). A value of $P < 0.05$ was taken as the criterion of statistical significance. Values reported are the means $\pm$ S.E.M.

Results

The effect of cytochalasin B on the stimulation of [¹⁴C]arachidonic acid incorporation by ionophore A23187 in neutrophil phospholipids

In a recent study (Rubin *et al.*, 1980) neutrophils preincubated with cytochalasin B, although incorporating less arachidonic acid into phospholipids than untreated cells, exhibited a somewhat similar

pattern of label incorporation, with phosphatidylcholine accounting for the bulk of the label; moreover, cytochalasin B enhanced the stimulant action of fMet-Leu-Phe on arachidonic acid incorporation into phosphatidylinositol. In a similar fashion, cytochalasin B significantly augmented the stimulant effect of $1\mu\text{M}$ -ionophore A23187 on (arachidonyl) phosphatidylinositol turnover (Table 1). Thus, in the absence of cytochalasin B, a 2 min exposure to ionophore A23187 increased arachidonate incorporation from 224 to 327 c.p.m., an average increase of $40(\pm 5)\%$ ($P < 0.05$), whereas in cells pretreated with cytochalasin B, ionophore A23187 stimulated incorporation from 207 to 778 c.p.m. (Table 1), an average increase of 349% (Table 1). In contrast with the specificity of the effect of ionophore A23187 on incorporation of arachidonate into phosphatidylinositol observed in the absence of cytochalasin B, $1\mu\text{M}$ -ionophore A23187 significantly stimulated labelling of phosphatidylserine and phosphatidylethanolamine in the presence of cytochalasin B after 2 min (Table 1); by contrast, label incorporation into phosphatidylcholine was not enhanced (Table 1).

All subsequent experiments were carried out on neutrophils pretreated with cytochalasin B.

Dose-response relationship and time course of ionophore A23187-induced phosphatidylinositol labelling and lysosomal enzyme secretion

A significant increase (79%) in [¹⁴C]arachidonic acid incorporation into phosphatidylinositol was observed after a 2 min exposure of $0.1\mu\text{M}$ -ionophore

Table 1. *Effect of cytochalasin on the ionophore A23187-induced incorporation of arachidonic acid label into neutrophil phospholipids*

Rabbit neutrophils were pre-incubated for 10 min at 37°C in the absence or presence of cytochalasin B ($5\mu\text{g/ml}$). At the end of the first incubation, the cells were transferred to tubes containing $0.2\mu\text{Ci}$ of [¹⁴C]arachidonic acid and incubated for an additional 2 min with or without $1\mu\text{M}$ -ionophore A23187. At the end of this incubation, 3 ml of chloroform/methanol (1:2, v/v) was added to extract the phospholipids and the organic phase was processed for phospholipid analysis as described in the Materials and methods section. Values are means ($\pm$ S.E.M.) for four to seven different determinations, each done in duplicate. The values expressed as a percentage of those in equivalent incubations without ionophore are means ($\pm$ S.E.M.) for six to nine paired experiments, each done in duplicate.

Phospholipid	¹⁴ C Arachidonic acid incorporation					
	(10 ⁻² × c.p.m./incubation)				(% of control)	
	Without cytochalasin		With cytochalasin		With cytochalasin	
	Control	1 μM-Ionophore	Control	1 μM-Ionophore	0.1 μM-Ionophore	1 μM-Ionophore
Phosphatidylcholine	7.9 ± 1.2	7.4 ± 1.4	6.8 ± 0.8	7.1 ± 1.0	105 ± 4	82 ± 5
Phosphatidylinositol	2.2 ± 0.4	3.3 ± 0.5	2.1 ± 0.3	7.8 ± 0.9*	179 ± 11†	449 ± 74†
Phosphatidylserine	1.7 ± 0.3	1.7 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	123 ± 10†	304 ± 79†
Phosphatidylethanolamine	4.5 ± 0.9	4.7 ± 0.7	1.8 ± 0.3	3.3 ± 0.5	104 ± 6	278 ± 26†

* Significantly different from equivalent incubations without ionophore A23187, calculated by unpaired Student's *t* test ($P < 0.05$).

† Significant stimulation, calculated by paired Student's *t* test ($P < 0.05$).

A23187 (Fig. 1; Table 1), and maximal effects were achieved with $1\text{ }\mu\text{M}$ -ionophore A23187 (Fig. 1). With $0.1\text{ }\mu\text{M}$ -ionophore A23187, a 23% increase in phosphatidylserine was also detected (Table 1), and with $1\text{ }\mu\text{M}$ -ionophore, significant increases in the labelling of all phospholipids except phosphatidylcholine were seen (Table 1).

Analysis of the time course of ionophore A23187-induced arachidonic acid labelling of phospholipids revealed that the greatest increment in phosphatidylinositol labelling (9–10-fold) occurred during the first minute of exposure to $1\text{ }\mu\text{M}$ -ionophore A23187, whether the values were represented in terms of absolute radioactivity counts (Fig. 2) or as a percentage of values derived from control cells (Fig. 3). Utilizing the data depicted in Fig. 2, the rate of label incorporation was determined by dividing the radioactivity (c.p.m.) accumulated during a given time period by the appropriate time interval. Ionophore A23187 increased the rate of labelling from 169 c.p.m./min to 1050 c.p.m./min during the first minute. Over the next 14 min, though basal rate of labelling fell to 76 c.p.m./min, ionophore A23187 still increased it to 133 c.p.m./min. Maximal increments in arachidonic acid incorporation into phosphatidylserine and phosphatidylethanolamine, which were attained within 1 and 2 min respectively after

Fig. 1. Dose-response effects of ionophore A23187 on [^{14}C]arachidonic acid incorporation into neutrophil phosphatidylinositol and lysosomal enzyme secretion.

Neutrophils pretreated with cytochalasin B were incubated for 2 min with $0.2\text{ }\mu\text{Ci}$ of [^{14}C]arachidonic acid with or without various concentrations of ionophore A23187 and analysed for incorporation of arachidonic acid into phosphatidylinositol. Enzyme release was measured on cytochalasin B-treated cells incubated for 15 min. Label incorporation and enzyme release are expressed as a percentage of that in equivalent incubations without the addition of ionophore. Results represent means $\pm$ S.E.M. for four to twelve different experiments. Symbols: ●, phosphatidylinositol labelling; □, enzyme release.

Fig. 2. Effect of ionophore A23187 on the time course of arachidonic acid incorporation into phosphatidylinositol.

Cells pretreated with cytochalasin B were incubated with [^{14}C]arachidonic acid in the presence or absence of ionophore A23187 ($1\text{ }\mu\text{M}$) and label incorporation into phosphatidylinositol was determined at various time periods. The results presented are means $\pm$ S.E.M. for four to eight different experiments. Symbols: ▲, incorporation of label in unstimulated cells; ●, incorporation of label in stimulated cells.

Fig. 3. Relative changes in [^{14}C]arachidonic acid incorporation into neutrophil phospholipids elicited by ionophore A23187.

Cells pretreated with cytochalasin B were incubated for various time periods with radiolabel in the presence or absence of $1\text{ }\mu\text{M}$ -ionophore A23187. Incorporation of label is expressed as a percentage of that in equivalent incubations without ionophore. The results presented are means for three or four different experiments, each done in duplicate. *Significant stimulation of label incorporation ($P < 0.05$, calculated by paired Student's t test). Symbols: ▽, phosphatidylcholine; ●, phosphatidylinositol; ○, phosphatidylserine; ■, phosphatidylethanolamine.

the addition of $1\mu\text{M}$ -ionophore A23187, did not reach values comparable with those observed with label incorporation into phosphatidylinositol (Fig. 3). By contrast, ionophore A23187 produced a significant decrease in label incorporation into phosphatidylcholine after 15 and 30 min (Fig. 3).

Fig. 4 depicts the temporal profile of ionophore A23187-induced lysosomal enzyme release. The onset of secretion coincided with the onset of arachidonic acid incorporation (cf. Figs. 3 and 4), with the maximal rate of enzyme release ($8.8\%/min$) occurring during the first 2 min. By contrast, ionophore A23187 ($1\mu\text{M}$) failed to promote the release of the cytoplasmic enzyme lactate dehydrogenase (results not shown). The secretory rate of lysosomal enzyme release fell to $1.8\%/min$ between 2 and 15 min, and after 15 min secretion was essentially complete (Fig. 4). Similarly, between 15 and 30 min of incubation, the stimulated rate of phosphatidylinositol labelling (45 c.p.m./min) approached the basal rate (42 c.p.m./min) (Figs. 2 and 3). A comparison of the dose-response relationship for ionophore A23187 stimulation of phosphatidylinositol labelling and enzyme secretion is illustrated in Fig. 1. The correlation between the two responses is striking. Threshold and peak concentrations of ionophore A23187 were identical for both processes such that the dose-response curves were virtually superimposable over a 50-fold range of ionophore concentrations.

Incorporation of other phospholipid precursors

Palmitic acid, the saturated fatty acid that

Fig. 4. Time course of lysosomal enzyme secretion after stimulation with ionophore A23187

Neutrophils pretreated with cytochalasin B were incubated with $1\mu\text{M}$ -ionophore A23187 for indicated time periods. Spontaneous release of enzyme was subtracted from stimulus-induced release at each time point. Results represent means $\pm$ S.E.M. for four to twelve different experiments.

preferentially attaches to C-1 of glycerophospholipids, incorporates into phosphatidylinositol to a lesser extent than does arachidonic acid (Rubin *et al.*, 1980). Ionophore A23187 ($0.1\mu\text{M}$), which exhibited relative selectivity in promoting arachidonic acid incorporation into phosphatidylinositol (Table 1), failed to stimulate palmitate labelling of phosphatidylinositol. Thus palmitate incorporation into phosphatidylinositol was $92(\pm 7)$ and $84(\pm 7)\%$ of control values after 1 and 2 min exposures respectively to $0.1\mu\text{M}$ -ionophore A23187. However, after 15 min the increment in labelling rose to $218(\pm 28)\%$ of control values. None of the other phospholipids exhibited significant alterations in label incorporation elicited by ionophore over a 15 min period. Increasing the ionophore concentration to $1\mu\text{M}$ produced a significant increase (42%) in palmitate incorporation into phosphatidylinositol within 1 min, an increase that continued to develop over the 15 min period of incubation (Fig. 5). Palmitate incorporation into phosphatidylcholine was also enhanced after 1 min (313% of control), thereafter falling to 89% above basal values after 15 min. The incorporation of [^{14}C]palmitate into phosphatidylserine was slightly higher during the early stages of ionophore action, although not statistically significant (Fig. 5). Neutrophils exposed

Fig. 5. Time course of labelling of neutrophil phospholipids with palmitic acid

Cells pretreated with cytochalasin B were incubated with $0.8\mu\text{Ci}$ of [^{14}C]palmitic acid in the presence or absence of $1\mu\text{M}$ -ionophore A23187, and label incorporation was measured at the indicated time periods. Incorporation of label is expressed as a percentage of that in equivalent incubations without ionophore. Results presented are means $\pm$ S.E.M. for four to six different experiments, each done in duplicate. *Significant stimulation ($P < 0.05$, calculated by paired Student's *t* test). Symbols; ∇ , phosphatidylcholine; $\bullet$, phosphatidylinositol; $\circ$, phosphatidylserine; $\blacksquare$, phosphatidylethanolamine.

to ionophore A23187 incorporated less palmitate into phosphatidylethanolamine over the 15 min interval, although only during the 1 min time period was the decrease (29%) statistically significant ($P < 0.05$).

Ionophore A23187 ($1\mu\text{M}$) increased [^{32}P]P_i incorporation into phosphatidylinositol, as well as phosphatidylcholine and phosphatidylserine, by approx. 50% after 2 min (Fig. 6). Owing to experimental variability, the increase in labelling of the phosphatidylinositol fraction was not statistically significant after 2 min, although the 129 and 176% increases in [^{32}P]P_i incorporation observed after 10 and 30 min respectively were significant. The 33% increase in [^{32}P]P_i incorporation into phosphatidylserine after 10 min was also statistically significant, but no other significant changes in phospholipid labelling were detected (Fig. 6).

To evaluate the effects of ionophore A23187 on phospholipid synthesis *de novo*, [^{14}C]glycerol and [^{14}C]acetate were also employed as precursors. Glycerol rapidly and selectively incorporated into phosphatidylinositol; after 2 min phosphatidylinositol accounted for $87(\pm 3)\%$ of the total label ($n = 5$). However, ionophore A23187 failed to stimulate the

conversion of glycerol into phosphatidylinositol, but in fact appeared to decrease it. Thus the radioactivity in phosphatidylinositol after a 2 min incubation in the absence and presence of $1\mu\text{M}$ -ionophore A23187 was $1151(\pm 212)$ and $826 \pm (215)$ c.p.m. respectively, an average decrease of $34 \pm (11)\%$. Ionophore A23187 appeared to enhance the conversion of glycerol into phosphatidylserine, although owing to the low basal incorporation, it was difficult to make an accurate quantitative assessment of this stimulation. After a 2 min exposure to [^{14}C]glycerol, the phosphatidylserine fraction contained $62(\pm 36)$ c.p.m. ($n = 5$); and ionophore A23187 stimulated the conversion of glycerol into phosphatidylserine by an average of $56(\pm 15)\%$ ($P < 0.05$). The ionophore did not enhance the conversion of glycerol into phospholipid even after 30 min, but inhibited it (results not shown). [^{14}C]Acetate was poorly incorporated into phospholipids, even after a 15 min period of incubation. Phosphatidylethanolamine contained the largest amount of label, averaging $69(\pm 15)$ c.p.m., with the other phospholipids accounting for lesser fractions. Ionophore A23187 ($1\mu\text{M}$) failed to enhance the incorporation of acetate into any of the phospholipids during the 15 min incubation period of two separate experiments, each done in duplicate.

Fig. 6. Time course of labelling of neutrophil phospholipids with [^{32}P]P_i after stimulation with ionophore A23187

Cells pretreated with cytochalasin B were incubated with $100\mu\text{Ci}$ of [^{32}P]P_i in the presence or absence of $1\mu\text{M}$ -ionophore A23187 and label incorporation determined at the indicated time periods. Incorporation of label is expressed as a percentage of that in equivalent incubations without ionophore. Results represent means $\pm$ S.E.M. for four to seven different experiments, each done in duplicate. *Significant stimulation ($P < 0.05$, calculated by paired Student's *t* test). Symbols: ∇ , phosphatidylcholine; $\bullet$, phosphatidylinositol; $\circ$, phosphatidylserine; $\blacksquare$, phosphatidylethanolamine.

Discussion

In a recent study using the formylmethionyl-containing peptide fMet-Leu-Phe to stimulate rabbit neutrophils, we demonstrated that a close correlation exists between the ability of the peptide to enhance arachidonic acid incorporation into phosphatidylinositol and to promote lysosomal enzyme secretion (Rubin *et al.*, 1980). A key role for Ca^{2+} in this sequence was disclosed by the finding that Ca^{2+} deprivation blocked both processes. The present investigation provides additional support for the intimate involvement of Ca^{2+} in these two processes by demonstrating that the Ca^{2+} ionophore A23187, a non-phagocytic secretagogue, also markedly enhanced (arachidonyl)phosphatidylinositol turnover, eliciting almost a 10-fold increase in phosphatidylinositol labelling within 1 min. Moreover, a close quantitative and temporal correlation emerged between ionophore A23187-evoked phosphatidylinositol labelling and enzyme release, implying that the two processes are closely linked.

Although it is true that ionophore A23187 elicited other alterations in phospholipid turnover, the effect on (arachidonyl) phosphatidylinositol is deemed the most significant for the following reasons. (a) With threshold ionophore concentrations the enhanced incorporation of arachidonate was relatively specific for phosphatidylinositol, although a small increase in label incorporation into phosphatidylserine was also

noted. (b) With a maximal stimulating concentration of ionophore A23187 ($1\mu\text{M}$), a very rapid and powerful enhancement of (arachidonyl) phosphatidylinositol turnover was demonstrable, whereas lesser increases in stimulated arachidonate incorporation into other phospholipids were also observed. Moreover, neutrophils not treated with cytochalasin B manifested a specificity for phosphatidylinositol even when challenged with $1\mu\text{M}$ -ionophore A23187. (c) Finally, and most importantly, the marked correlation between the dose-response curves of phosphatidylinositol labelling and lysosomal enzyme secretion over a broad range of ionophore concentrations also signifies that the turnover of phosphatidylinositol plays a principal role in membrane function associated with secretory activity. It should also be noted that (arachidonyl) phosphatidylinositol turnover and lysosomal enzyme release also increased in parallel when fMet-Leu-Phe was employed as the stimulating agent (Rubin *et al.*, 1980), indicating that receptor-active agents also utilize this same general Ca^{2+} -dependent pathway. However, we cannot as yet determine whether the selective perturbations in phospholipid metabolism described here are a reflection of membrane permeability changes coincident with Ca^{2+} flux or of fusion of granule membranes with plasma membranes (exocytosis).

Arguments have previously been offered for interpreting the enhanced incorporation of arachidonate into phosphatidylinositol on the basis of a deacylation-reacylation sequence triggered by the activation of a Ca^{2+} -dependent phospholipase A_2 (Schrey & Rubin, 1979; Schrey *et al.*, 1980; Rubin *et al.*, 1980). The present investigation has clearly shown that this biochemical sequence of reactions can be activated in the neutrophil, as in the adrenal cortex (Schrey & Rubin, 1979), simply by increasing 'free' or available Ca^{2+} . Although the ability of ionophore A23187 to stimulate neutrophil phospholipase A_2 was not directly assessed here, there is abundant evidence from other test systems that ionophore A23187 is capable of stimulating phospholipase A_2 activity (Pickett *et al.*, 1977; Förstermann & Hertting, 1979; Vanderhoek & Feinstein, 1979; Zenser *et al.*, 1980). In neutrophils the ionophore increases the formation of cyclo-oxygenase and lipoxygenase products (prostaglandins and dihydroxy acids) from arachidonic acid (Wentzell & Epand, 1978; Borgeat & Samuelsson, 1979; Stenson & Parker, 1979), presumably by stimulating the existing membrane-bound phospholipase A_2 (Franson *et al.*, 1974). Moreover, a correlation between Ca^{2+} availability and phospholipid turnover was demonstrated by the findings that cytochalasin B, which markedly potentiated the action of ionophore A23187 on arachidonic acid incorporation into phosphatidylinositol, also augments iono-

phore A23187-induced $^{45}\text{Ca}^{2+}$ uptake into neutrophils (Naccache *et al.*, 1977). Thus these data taken together suggest that ionophore A23187 activates neutrophil phospholipase A_2 by causing the mobilization of Ca^{2+} to the enzyme site on the plasma membrane. The stimulation of phospholipase A_2 leads to the release of arachidonic acid, which provides the substrate precursor for the formation of prostaglandins and/or other metabolites. Lysophospholipids, also formed as a consequence of the deacylation of phospholipids, have putative cellular functions, including the regulation of adenylate and guanylate cyclase (Shier *et al.*, 1976; Aunis *et al.*, 1978) and of membrane-fusion reactions (Poole *et al.*, 1970). But lysophospholipids must be rapidly reacylated because of their cytotoxic potential (Bigon *et al.*, 1979; Mookerjee, 1979). Acyl-CoA-phospholipid acyltransferase, which has been identified in rabbit neutrophils (Elsbach, 1966), possesses a selectivity that promotes a rapid incorporation of unsaturated fatty acids into specific membrane phospholipids (Lands & Crawford, 1976); and membranes enriched in unsaturated phospholipids manifest enhanced fluidity leading to alterations in their surface properties, among which include permeability (Lands, 1980). But whatever the sequelae, it is the activation of acyl chains of membrane phospholipids, triggered by the Ca^{2+} -induced stimulation of phospholipase A_2 , that plays a pivotal role in the proposed events associated with secretion.

Although the experiments employing [^{14}C]acetate and [^{14}C]glycerol as precursors indicate that ionophore A23187 did not stimulate phosphatidylinositol synthesis *do novo* during the course of this study, one cannot discount the findings that ionophore also enhanced the incorporation of other phospholipid precursors into phosphatidylinositol, particularly when a maximal stimulating concentration was used. This indicates that the actions of ionophore A23187 on the enzymes that alter fatty-acid content of membrane phospholipids become less selective at higher ionophore concentrations. Thus, $1\mu\text{M}$ -ionophore A23187 was also capable of promoting the incorporation of palmitic acid into phosphatidylinositol, as well as phosphatidylcholine, although with regard to phosphatidylinositol, palmitate incorporation was generally slower in onset and much more modest in magnitude than arachidonate incorporation. The likelihood that the ionophore A23187-induced palmitate turnover in neutrophils represents the action of phospholipase A_1 rests with the strong preference for palmitate at the 1-position of phospholipids (Hill & Lands, 1979) and from the findings that this enzyme partially purified from brain (Rooke & Webster, 1976) and identified in plasma membrane from liver (Newkirk & Waite, 1971) is Ca^{2+}

sensitive. Moreover, the marked preference of palmitate for neutrophil phosphatidylcholine resembles the substrate specificity exhibited by rat brain phospholipase A₁ (Gatt, 1968). It is noteworthy, however, that there are species of phospholipase A₁ that are either unaffected or inhibited by Ca²⁺ (McMurray & Magee, 1972) and possess selectivity towards phosphatidylethanolamine (Rooke & Webster, 1976). Nevertheless, on the existing evidence, it appears that ionophore A23187 concentrations that are capable of maximally stimulating phospholipase A₂ are also capable of activating phospholipase A₁. On the other hand, it is arguable whether substrate specificity for the reverse reaction involving acyl-CoA-phospholipid acyltransferase could also account for the findings with palmitate (Lands & Crawford, 1976; Irvine & Dawson, 1979).

Stimulation by 1 μM-ionophore A23187 was also accompanied by a relatively modest and slowly developing increase in the rate of [³²P]P_i incorporation into phosphatidylinositol, as well as into phosphatidylcholine and phosphatidylserine. These data confirm previous reports delineating the Ca²⁺-dependence of this reaction in rabbit neutrophils, as assessed by fMet-Leu-Phe-induced turnover of [³²P]P_i in phosphatidylinositol (Cockcroft *et al.*, 1980). The Ca²⁺-dependent [³²P]P_i turnover in rabbit neutrophils, which seems to be an exception rather than the rule in secretory cells (see Cockcroft *et al.*, 1980), appears to cast doubt on any unitary theory formulated to develop a simple causal relationship between stimulated [³²P]phosphatidylinositol turnover, activation of Ca²⁺ channels and secretory activity (Michell, 1975). Finally, it should be noted that, although there was no hard evidence favouring phosphatidylinositol synthesis *de novo* during ionophore A23187 action, the conversion of glycerol into phosphatidylserine was apparently accelerated; this suggests that at least a portion of the enhanced incorporation of precursors, including arachidonic acid in this phospholipid, might be explained on the basis of synthesis *de novo* rather than the turnover of a fatty acid or a polar head group.

In conclusion, our current and previous investigations have provided compelling evidence that a deacylating-reacylating mechanism that acts on a single fatty acid moiety (arachidonate) of phosphatidylinositol appears to be responsible for initiating at least certain of the membrane events coupled to the release of secretory product from the cell. This mechanism has been amply demonstrated not only in the neutrophil but in the steroid-secreting cells of the adrenal cortex (Schrey & Rubin, 1979). It is also most relevant to cite here a recent study that has revealed that arachidonic acid and/or its metabolites may be involved in the mechanisms regulating Ca²⁺ permeability in the neutrophil (Volpi *et al.*,

1980). These findings prompt speculation that Ca²⁺ not only plays a pivotal role in arachidonate metabolism as expressed through its regulation of phospholipase A₂ activity, but that arachidonate or its metabolites, in turn, participates in a feedback mechanism to mediate changes in Ca²⁺ turnover. Such speculation implies the existence of multiple Ca²⁺ pools involved in stimulus-secretion coupling in the neutrophil. Thus it is anticipated that a better understanding of the functional role of arachidonic acid turnover in secretory tissues will provide insight into the mechanism by which Ca²⁺ enters the cell and perhaps even into the mechanism by which this cation elicits secretion.

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