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Cation Ionophores A23187 and Valinomycin Enhance Protein-Mediated Transfer of Rat Liver Microsomal Phosphatidylinositol to Liposomes

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Summary. A standard reaction mixture has been established in which partially purified rat liver phosphatidylinositol exchange proteins sustain a maximal rate of phosphatidylinositol transfer from rat liver microsomes to liposomes. Determination of the transfer kinetics confirms the findings of Brophy et al. (*Biochem J.* **174**:413–420,1978) that under such conditions a maximum 70–80% of the homogenously radiolabeled, microsomal phosphatidylinositol is exchanged with biphasic kinetics. The phosphatidylinositol exchange proteins thus indicate the presence of three microsomal phosphatidylinositol pools: One pool is not subject to protein-mediated exchange; the other two pools are both exchangeable but are exchanged with significantly different half-lives. Both the divalent cation ionophore, A23187, and the monovalent cation ionophore, valinomycin, significantly enhance phosphatidylinositol transfer in the standard reaction mixture at concentrations 1 to 2 orders of magnitude greater than those sufficient for the ionophores to facilitate cation transport across membranes. The stimulatory effect of each ionophore, however, is not a function of the ionophore/microsome mass ratio in the reaction mixture. Although both ionophores increase the relative amount of exchangeable phosphatidylinositol, neither ionophore results in all of the exchangeable phosphatidylinositol being transferred with single-state kinetics. The evidence demonstrates that A23187 and valinomycin are the first substances found to markedly enhance the reactivity of a microsomal phospholipid class with phospholipid exchange proteins.

Key Words phosphatidylinositol · phospholipid exchange proteins · A23187 · valinomycin · microsomes · liposomes

Introduction

Phospholipid exchange proteins are cytosolic proteins that catalyze the exchange of phospholipids between membrane systems under *in vitro* conditions (Wirtz & Zilversmit, 1968, 1969). Many of these phospholipid exchange proteins exhibit an absolute or preferential specificity for one of the phospholipid classes, such as phosphatidylcholine or phosphatidylinositol (Wirtz, Kamp & van Deenen, 1972; Kamp, Wirtz & van Deenen, 1973; Helmkamp, Harvey, Wirtz & van Deenen, 1974; Wirtz, 1974; Lumb, Kloosterman, Wirtz & van Deenen, 1976). Studies of phosphatidylcholine exchange proteins have demonstrated the following facts: (*i*) Changes in the polar moiety of phosphatidylcholine affect the interaction of the exchange protein with phosphatidylcholine more markedly than changes in the apolar moiety (Demel et al., 1973; Kamp et al., 1977). (*ii*) Various hydrolytic fragments of phosphatidylcholine (choline chloride, phosphorylcholine, sn-glycerol-3-phosphorylcholine and lysophosphatidylcholine) do not produce a significant reduction of phosphatidylcholine exchange activity (Johnson & Zilversmit, 1975). (*iii*) Phosphatidylcholine exchange activity decreases with increasing ionic strength of the reaction mixture (Johnson & Zilversmit, 1975). (*iv*) The exchange proteins interact only with phosphatidylcholine residing in the outer layer of bilamellar liposomes (Johnson, Hughes & Zilversmit, 1975; Rothman & Dawidowicz, 1975). Finally, a study of phosphatidylinositol exchange proteins has shown that the amounts of phosphatidylinositol exchanged between rat liver microsomes and liposomes vary with the phosphatidylinositol content of the liposomes (Harvey, Helmkamp, Wirtz & van Deenen, 1974). All these finding emphasize the fact that those factors responsible for the organization (such as the transbilayer distribution and rate and extent of transposition) and relative content of phospholipid classes within a donor membrane system are significant determinants of the rate and extent of protein-mediated transfer of a particular phospholipid class to an acceptor membrane system. There is, however, no evidence that the specific activity of the phospholipid exchange proteins themselves can be regulated or that particular substances, through specific and reversible interaction with membranous components, can serve to regulate the protein-mediated exchangeability of various phospholipid classes.

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We report here that two ionophores, A23187 and valinomycin, markedly stimulate protein-mediated transfer of phosphatidylinositol (PI)[1] from rat liver microsomes to liposomes. A23187 is a lipophilic, carboxylic acid antibiotic which can complex calcium and magnesium ions and transport them across membranes (Reed & Lardy, 1972); valinomycin is a neutral ionophorous substance which can form charged complexes with monovalent cations (Pressman, 1973). We have confirmed the findings of Brophy et al. (1978) that in a standard reaction mixture in which partially purified rat liver PI exchange proteins sustain a maximal rate of PI transfer from rat liver microsomes to liposomes (with a phospholipid composition of 98 mole % phosphatidylcholine and 2 mole % PI), a maximal 70–80% of the homogeneously radiolabeled, microsomal PI is exchanged with biphasic kinetics. Although two or more pools of exchangeable microsomal PI are still evident when either ionophore, at concentrations of 100–200 μM, is added to the standard reaction mixture, each ionophore effects a significant increase in the maximal rate of PI transfer and a decrease in the relative amount of nonexchangeable microsomal PI. The evidence that A23187 and valinomycin are the first substances found to markedly enhance the apparent activity of phospholipid exchange proteins raises the possibility that there exist endogenous substances with similar ionophorous properties which associate with either phospholipid exchange proteins or particular components in the membranes of intact cells and thereby regulate the rate and extent to which the phospholipid exchange proteins transfer individual phospholipid classes among various cellular membrane systems.

Materials and Methods

Preparation of Rat Liver Microsomes

Rat liver microsomes were prepared according to a modification of the procedure of Wirtz and Zilversmit (1969). Sprague-Dawley rats (weighing 150–250 grams) were fasted overnight and sacrificed by decapitation. The livers were excised, immersed in ice-cold SET (0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4) and minced with scissors. The medium was drained from the tissue mince and the tissue homogenized with a Teflon/glass Potter-Elvehjem homogenizer in fresh ice-cold SET (3 ml SET per gram tissue). The 25% (wt/vol) homogenate was centrifuged at 14,000 rpm ($15{,}300 \times g_{av}$) for 15 min at 2 °C in a Beckman JA-20 rotor to sediment nuclei, mitochondria and heavy microsomes. The supernatant was centrifuged 45,000 rpm ($189{,}000 \times g_{av}$) for 45 min at 2 °C in a Beckman SW50.1 rotor to sediment the microsomes. The microsomal pellets were homogenized in 20 mM Tris-HCl, pH 7.4, and the microsomal PI radiolabeled with [2-^{3}H]myoinositol by isotopic exchange according to the procedure of Helmkamp et al. (1974). Phosphatidyl[2-^{3}H]inositol formed by isotopic exchange is homogeneously distributed throughout the microsomal PI (Brophy et al., 1978).

[1] The abbreviations used are: PI, phosphatidylinositol; DEAE-cellulose, diethyl amino ethyl cellulose; EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate.

Preparation of Partially Purified Rat Liver PI Exchange Proteins

Rat liver PI exchange proteins were partially purified according to a modification of the procedure of Lumb et al. (1976). Liver post-microsomal supernatants were prepared from 25% (wt/vol) homogenates of 2–3 rat livers as described above and dialyzed against 5 mM sodium phosphate, pH 7.4. The dialyzed supernatant was centrifuged at 14,000 rpm for 15 min at 2 °C in a Beckman JA-20 rotor and the supernatant applied to a 1.6×15 cm column of DEAE-cellulose equilibrated with 5 mM sodium phosphate, pH 7.4. The column was then washed with 20 ml 5 mM sodium phosphate, pH 7.4, and 40 ml 25 mM NaCl and 5 mM sodium phosphate, pH 7.4, and the eluants discarded; the first eluant contains phosphatidylcholine exchange protein (Lumb et al., 1976). Thirty ml 50 mM NaCl and 5 mM sodium phosphate, pH 7.4, were applied to the column and the collected eluant saturated to 90% with ammonium sulfate and stirred overnight. The precipitated protein was sedimented by centrifugation at 14,000 rpm for 15 min at 2 °C in a Beckman JA-20 rotor and then dissolved in 1–2 ml 5 mM sodium phosphate, pH 7.4. The resulting protein solution was dialyzed against 50 mM sodium phosphate, pH 7.4, and then assayed for PI exchange activity. Such partially purified PI exchange protein preparations were used in all experiments unless otherwise indicated. The preparations were further purified for some experiments by gel exclusion chromatography in a 2.5×55 cm column of Sephadex G-100 equilibrated in 50 mM sodium phosphate, pH 7.4. Column fractions collected between 1.7 and 1.8 times the void volume of the column contained peak PI exchange activity. The proteins in these fractions were concentrated by ammonium sulfate precipitation as described above, dissolved in and dialyzed against 50 mM sodium phosphate, pH 7.4, and then assayed for PI exchange activity. Both types of PI exchange protein preparations contain two PI exchange proteins (Lumb et al., 1976). Although both proteins can also exchange phosphatidylcholine, they transfer 8–9 times more PI than phosphatidylcholine from rat liver microsomes to liposomes (Lumb et al., 1976). Both types of preparations can be stored at 2 °C for 48 hr without loss of exchange activity.

Preparation of Liposomes

Preparations of egg yolk sn-3-phosphatidylcholine and soybean sn-3-PI dissolved in chloroform were mixed (98 mole % phosphatidylcholine to 2 mole % PI), dried under vacuum at 25 °C, redissolved in diethyl ether, and then dried again under vacuum at 25 °C. The lipids were allowed to swell for 1–2 hr at 2 °C in SET and the suspension then sonified at 2 °C (Branson Sonifier Cell Disruptor 350; 50% duty cycle; output control at 2; four 0.5-min sonifications).

Assay of PI Exchange Activity

PI exchange activity was assayed by determining the *in vitro* transfer of phosphatidyl[2-^{3}H]inositol from rat liver microsomes to liposomes. Aliquots of partially purified PI exchange protein preparations were added to tubes containing (2-^{3}H]myoinositol-labeled microsomes (150 μg microsomal

protein), liposomes (0.5 μmol phospholipid) and SET to a final volume of 0.5 ml. After incubation at 37 °C for 20 min, 2.0 ml ice-cold SET were added to each reaction mixture and the resulting suspension layered atop a step metrizamide gradient in SET consisting of 1.0 ml 10% (wt/vol) metrizamide, 1.0 ml 20% (wt/vol) metrizamide and 0.5 ml 30% (wt/vol) metrizamide. After centrifugation at 20,000 rpm ($37{,}000 \times g_{av}$) for 20 min at 2 °C in a Beckman SW50.1 rotor, the top and bottom 2.5 ml were collected, each fraction mixed with 0.5 ml 5% (wt/vol) SDS and then Aquasol-2 and radioactivity determined with a Beckman liquid scintillation counter.

The metrizamide gradient centrifugation procedure separates liposomes and microsomes by taking advantage of the fact that the two types of membranous vesicles have significantly different average equilibrium densities. Metrizamide, a triiodinated benzamido-derivative of glucose, is a density gradient material which gives dense aqueous solutions of low viscosity and osmolality (Rickwood & Birnie, 1975); membranous organelles and vesicles rapidly attain their equilibrium density in metrizamide gradients centrifuged at relatively low speeds (Morris & Schovanka, 1977; Slaby & Farquhar, 1980). The step metrizamide gradient was designed to permit only the microsomes to equilibrate within the gradient, leaving the liposomes suspended in the layer atop the gradient. The following experiments were conducted to insure that the centrifugation procedure results in a consistently high recovery of liposomes and microsomes in the top and bottom 2.5-ml fractions, respectively, independent of the composition of the reaction mixture and the duration of the incubation period: (1) We found that if a suspension of liposomes labeled with cholesteryl[^{14}C]oleate is layered atop the step metrizamide gradient and centrifuged as described above, 98–99% of the radioactivity is recovered in the top 2.5-ml fraction. This 98–99% liposomal recovery in the top fraction is also obtained after incubation (for periods as long as 3 hr) of the cholesteryl[^{14}C]oleate-labeled liposomes with rat liver microsomes and PI exchange proteins. (2) We found that when suspensions of separate preparations of [2-^{3}H]myoinositol-labeled rat liver microsomes are layered atop the gradient and centrifuged as described above, 75–85% of the radioactivity is recovered in the bottom 2.5-ml fraction. This 75–85% microsomal recovery in the bottom fraction is invariant with respect to the amount of microsomes (6–600 μg microsomal protein) layered atop the gradient. Incubation of [2-^{3}H]myoinositol-labeled microsomes with either liposomes or partially purified rat liver PI exchange proteins results in no more than a 5% shift of the total radioactivity from the bottom to the top fraction. (3) When duplicate aliquots of a [2-^{3}H]myoinositol-labeled microsome suspension are incubated with liposomes for identical periods of time, layered atop gradients, and then centrifuged as described above, the percentages of radioactivity recovered in the bottom 2.5-ml fraction of the two gradients vary by no more than one percentage point. Thus, although the centrifugation procedure is somewhat laborious, it does offer the advantage of providing highly reproducible microsomal recoveries in the bottom gradient fractions from reaction mixtures prepared with the same microsome suspension.

The percentage of microsomal phosphatidyl[2-^{3}H]inositol transferred to liposomes in an experimental reaction mixture was determined in all experiments as follows: Every experimental reaction mixture containing microsomes, liposomes and PI exchange proteins was co-incubated with a control reaction mixture containing only microsomes and liposomes. Following separation of the microsomes and liposomes by the metrizamide gradient centrifugation procedure, the percentage of total [2-^{3}H]myoinositol radioactivity recovered in the top fraction for each reaction mixture was determined. Let A and B be the percentages of the total radioactivity recovered in the top fractions for the control and experimental reaction mixtures, respectively. A represents not only the percentage (15–25%) of the microsomes recovered in the top fraction for the reaction mixture in which there is no protein-mediated PI transfer to liposomes, but also the percentage of microsomes in the experimental reaction mixture for which protein-mediated transfer of radiolabeled PI to liposomes cannot be detected (as this radioactivity is also recovered in the top fraction). The term $(B-A)$ thus represents the percentage of radiolabeled microsomal PI in the experimental reaction mixture which was transferred to liposomes from the percentage $(100-A)$ of microsomes for which transfer of radiolabeled PI to liposomes can be detected. The percentage of microsomal phosphatidyl[2-^{3}H]inositol transferred to the liposomes in the experimental reaction mixture was therefore calculated by the formula

$$\left[\left(\frac{B-A}{100-A}\right)\cdot 100\right].$$

Analytical Procedures

Protein concentration was determined according to the procedure of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard.

Glucose-6-phosphatase activity (as measured by the release of inorganic phosphate from the substrate mannose-6-phosphate) was determined in rat liver microsome preparations before and after addition of 0.4% (wt/vol) sodium taurocholate according to the procedure of Zilversmit and Hughes (1977).

The liposomes and [2-^{3}H]myoinositol-labeled microsomes in control and experimental reaction mixtures were separated by the step metrizamide gradient centrifugation procedure in some experiments and phospholipids extracted from the top and bottom 2.5-ml fractions for thin-layer chromatographic separation of the phospholipids. In these experiments, each 2.5-ml fraction was mixed with 1.25 ml ice-cold 2 mg BSA/ml and then vortexed with 1.25 ml ice-cold 40% (wt/vol) TCA. The precipitable material was pelleted by centrifugation at 14,000 rpm for 10 min in a Beckman JA-20 rotor at 2 °C; for all fractions, the amount of ^{3}H radioactivity recovered in the supernatant represented only 2–3% of the amount of ^{3}H radioactivity in the TCA pellet. The TCA pellet was washed once with 1.0 ml ice-cold 10% (wt/vol) TCA and phospholipids were extracted from the TCA-precipitable material in chloroform/methanol/50 mM KCl (55:28:17, vol/vol/vol) as described by Slaby and Bryan (1976). In all instances, 95–99% of the ^{3}H radioactivity in the TCA-precipitable material was recovered in the phospholipid extract. Phospholipids were separated by two-dimensional, thin-layer chromatography on precoated silica gel G plates according to the procedure of Yavin and Zutra (1977). The PI spot was identified by co-chromatography with commercially available, chromatographically pure PI.

Materials

All reagents used were of the highest purity available. Egg yolk sn-3-phosphatidylcholine, soybean sn-3-PI, valinomycin, bovine serum albumin, DEAE-cellulose and Sephadex G-100 were purchased from Sigma Chemical Co.; metrizamide from Accurate Chemical and Scientific Corp., Hicksville, N.Y.; A23187 from Calbiochem-Behring Corp.; chromatographically pure PI from P-L Biochemicals, Inc.; and [2-^{3}H]myoinositol and cholesteryl[1-^{14}C]oleate from New England Nuclear.

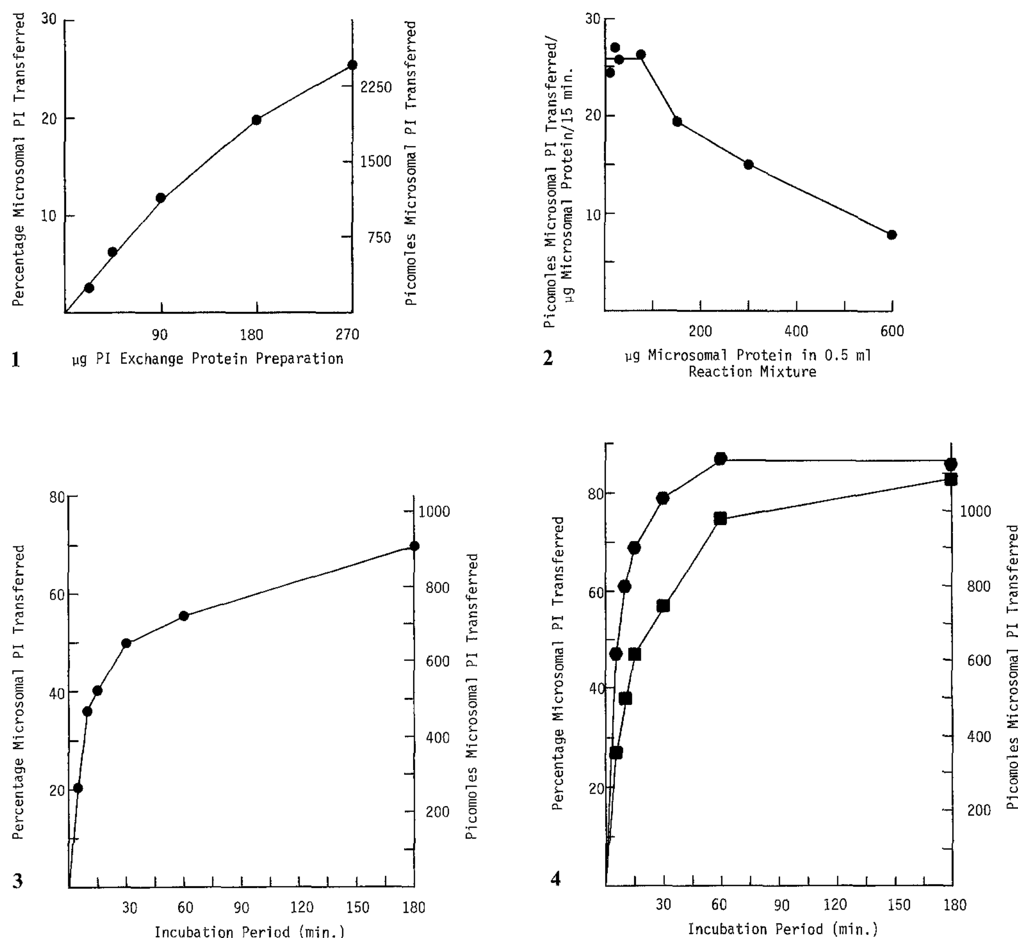

Fig. 1. Percentage and picomoles of microsomal PI transferred to liposomes in the assay reaction mixture as a function of transfer protein concentration. PI exchange activity was assayed as described in Materials and Methods; the picomoles of microsomal PI transferred were calculated on the basis of the presence of 65 picomoles PI/μg microsomal protein (average of three separate determinations)

Fig. 2. Picomoles of microsomal PI per μg microsomal protein transferred to liposomes as a function of microsome concentration. Reaction mixtures containing 0.5 μmol liposomal phospholipid, 100 units of PI exchange activity and varying concentrations of microsomal protein were incubated for 15 min and the amount of microsomal PI transferred determined as described in Materials and Methods

Fig. 3. Kinetics of microsomal PI transfer to liposomes in the standard reaction mixture. The results are the average of three separate experiments

Fig. 4. Kinetics of microsomal PI transfer to liposomes in the standard reaction mixture containing 200 μg/ml A23187 (●——●) or 200 μg/ml valinomycin (■——■). The results are the average of two separate experiments

Results

Determination of Conditions Necessary and Sufficient for Maximum Rate of Protein-Mediated Transfer of Microsomal Phosphatidyl[2-^{3}H]inositol to Liposomes

Previous studies of protein-mediated PI transfer from rat liver microsomes to either mitochondria or liposomes have demonstrated that there is maximal transfer of microsomal PI after 2–3 hr incubation, but not necessarily that there is a maximal rate of PI transfer throughout the incubation period under the *in vitro* conditions employed (Zilversmit & Hughes, 1977; Brophy et al., 1978). Our studies therefore began with an empirical determination of the conditions necessary to sustain a maximal rate of PI transfer. First, we established that PI exchange activity as measured in our assay procedure (*see* Materials and Methods) is directly proportional to protein content (of partially puri-

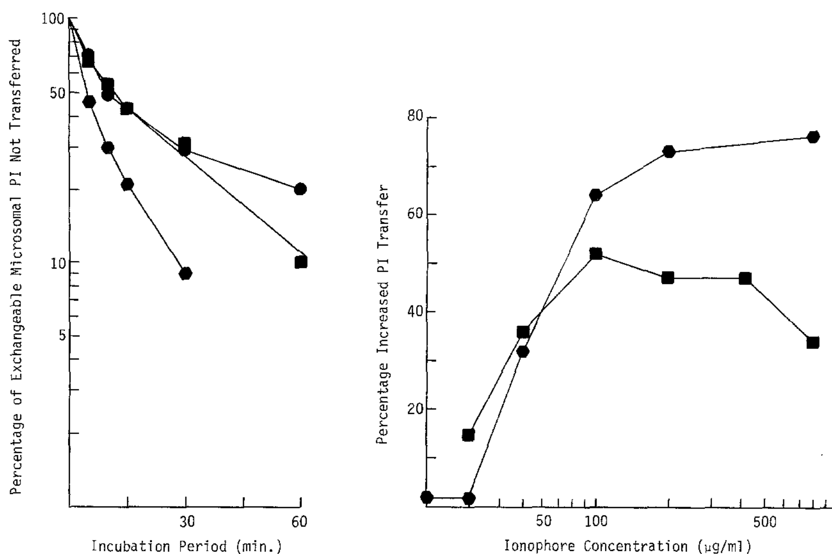

Fig. 5. Semi-logarithmic graphs of exchangeable microsomal PI during the first hour of incubation in a standard reaction mixture (●—●), in a standard reaction mixture containing 200 μg/ml A23187 (●—●), and in a standard reaction mixture containing 200 μg/ml valinomycin (■—■). The kinetic data shown in Figs. 3 and 4 were used in the determination of these graphs. 70% of the microsomal PI is exchangeable in the standard reaction mixture, 87% in the standard reaction mixture containing 200 μg/ml A23187, and 83% in the standard reaction mixture containing 200 μg/ml valinomycin

Fig. 6. Stimulatory effects of A23187 and valinomycin on protein-mediated PI transfer as a function of ionophore concentration. Standard reaction mixtures containing A23187 were incubated for 15 min and those containing valinomycin 60 min

fied PI exchange protein preparations) up to a concentration of 90 μg/ml reaction mixture (Fig. 1). This determination permitted us to define one unit of PI exchange activity as the transfer of 1% of the microsomal PI after 20-min incubation in the assay reaction mixture. Secondly, we measured the amount of microsomal PI transferred in reaction mixtures containing 0.5 μmol liposomal phospholipid and 100 units of exchange activity as a function of microsome concentration. Figure 2 shows that under these conditions there is maximal PI transfer per μg microsomal protein after 15-min incubation if 75 μg microsomal protein or less are in the reaction mixture.

We therefore established that incubation of a 0.5-ml reaction mixture containing 100 units of PI exchange activity, 0.5 μmol liposomal phospholipid and 6–75 μg microsomal protein yields maximal kinetics of PI transfer. Figure 3 shows the rate of PI transfer in such a reaction mixture containing 20 μg microsomal protein (hereafter referred to as the standard reaction mixture). Comparison of this Figure with Fig. 2 in the article by Brophy et al. (1978) shows that the kinetics of PI transfer in our standard reaction mixture are similar to those observed by Brophy et al. (1978).

A23187 and Valinomycin Enhance Protein-Mediated Transfer of Microsomal Phosphatidyl[2-^{3}H]inositol to Liposomes

Figure 4 shows the stimulatory effects of A23187 and valinomycin (each at a concentration of 200 μg/ml) on protein-mediated PI transfer in the standard reaction mixture. A23187 is the more effective ionophore; it approximately doubles PI transfer during the first 10-min incubation and results in a maximal transfer (87%) of the PI after 1-hr incubation. Figure 5 illustrates semi-logarithmic plots of the exchange kinetics shown in Figs. 3 and 4; the nonlinearity of these plots demonstrates that in both the absence and presence of the ionophores there occur at least two major exchangeable pools of microsomal PI with significantly different exchange half-lives.

The two ionophores do not exhibit synergistic nor competitive effects. The percentage of microsomal PI transferred after 15-min incubation of a standard reaction mixture containing both A23187 and valinomycin (each at a concentration of 200 μg/ml) is identical to the percentage transferred in the presence of A23187 alone. A maxi-

Table 1. Effects of microsome and liposome concentrations on the stimulatory effects of 200 µg/ml A23187 and valinomycin[a]

Composition of 0.5 ml reaction mixture containing 100 units PI exchange activity			Percentage increased PI transfer relative to PI transfer in standard reaction mixture
Ionophore concentration (µg/ml)	Microsomal protein content (µg)	Liposome content (µmol)	
200 A23187	20	0.5	73
200 A23187	200	0.5	70
200 A23187	200	1.5	65
200 valinomycin	20	0.5	34
200 valinomycin	200	0.5	23

[a] Reaction mixtures containing A23187 were incubated 15 min and those containing valinomycin 60 min

mum 87% of the microsomal PI is transferred after 3-hr incubation in the presence of both ionophores.

Neither ionophore affects the structural integrity of phosphatidyl[2-^{3}H]inositol residing in either the microsomal membranes or the liposomal membranes following protein-mediated transfer. Control standard reaction mixtures and standard reaction mixtures containing 200 µg/ml of either A23187 or valinomycin were incubated for 3 hr, the liposomes and microsomes separated by the step metrizamide gradient centrifugation procedure, and the phospholipids then extracted from the top and bottom 2.5-ml fractions for thin-layer chromatographic separation of the phospholipids. As mentioned in Materials and Methods, 92–97% of all the tritium radioactivity in every fraction is recovered in the phospholipid extract, and 90–95% of this amount of radioactivity co-chromatographs with PI.

A23187 or valinomycin alone do not mediate PI transfer from microsomes to liposomes. Standard reaction mixtures (prepared with cholesteryl-[^{14}C]oleate-labeled liposomes) containing 200 µg/ml of either A23187 or valinomycin but minus PI exchange proteins were incubated for periods as long as 3 hr. At all time points, the percentages of cholesteryl[^{14}C]oleate and phosphatidyl[2-^{3}H]inositol radioactivities recovered in the top and bottom 2.5-ml fractions following the step metrizamide gradient centrifugation are identical to those recovered for a similarly incubated standard reaction mixture containing only microsomes and liposomes. Neither ionophore thus mediates PI transfer nor affects the percentage recovery of liposomes and microsomes in the top and bottom 2.5-ml fractions, respectively.

A23187 and valinomycin enhancement of PI transfer does not appear to be mediated by cytosolic proteins other than the PI exchange proteins. The stimulatory effects of the two ionophores (each at a concentration of 200 µg/ml) on protein-mediated PI transfer were determined in the standard reaction mixture containing rat liver PI exchange protein preparations purified (as described in Materials and Methods) by both ion exchange and gel exclusion chromatography. Comparison of the exchange kinetics obtained (*results not shown*) with the corresponding curves in Fig. 4 demonstrates that the use of such purified PI exchange protein preparations does not affect the kinetics of ionophore-enhanced PI transfer.

Figure 6 shows the stimulatory effects of A23187 and valinomycin as a function of ionophore concentration. The incubation period selected for A23187 was 15 min and for valinomycin 60 min. Both ionophores exhibit near-maximal stimulatory effects on protein-mediated PI transfer at a concentration of 200 µg/ml. At a concentration of 20 µg/ml, A23187 exhibits no stimulatory effect and that of valinomycin is reduced by about 70%. Expressed in terms of molarity, the results of Fig. 6 show that A23187 and valinomycin do not enhance PI transfer at concentrations equal to or less than 38 and 9 µM, respectively. The lowest concentration at which each ionophore exhibits near-maximal stimulatory effect is approximately 200 µM; such a concentration is about 40 times greater than that commonly used to increase cation transport across cellular membranes by each ionophore (Pressman, 1973; Christophe et al., 1976; Kondo & Schulz, 1976).

The requirement of relatively high ionophore concentrations to stimulate protein-mediated PI transfer could signify that the stimulatory effect of each ionophore is mediated through a mass action effect on the components of the microsomes and/or liposomes. To test this hypothesis, the stimulatory effect of 200 µg A23187 or valinomycin per ml was determined in a standard reaction mixture containing 200 µg microsomal protein. In such a reaction mixture the microsome/ionophore mass ratio is the same as that in a standard reaction mixture containing 20 µg ionophore per ml, and at this ionophore concentration, as shown in Fig. 6, A23187 exerts no stimulatory effect and that of valinomycin is decreased by 70%. However, the results displayed in Table 1 show that a 10-fold increase of the microsome concentration does not measurably diminish the stimulatory effect of 200 µg A23187/ml and decreases that of 200 µg va-

linomycin/ml by only 30%. A 10-fold increase of the microsome concentration plus a threefold increase of the liposome concentration diminishes the stimulatory effect of 200 µg A23187/ml by only about 10%. Neither ionophore therefore appears to enhance PI transfer through a mass action effect on the donor and receptor membranous vesicles.

Finally, we examined whether the stimulatory effect of each ionophore may be mediated through disruption of the structural integrity of the microsomal membranes. Our test for structural integrity was the latency of glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) as measured with the substrate mannose-6-phosphate. Arion, Carlson, Wallin and Lange (1972) have demonstrated that this enzyme is located on the cisternal side of rat liver microsomal membranes, since hydrolysis of the impermeable substrate mannose-6-phosphate is markedly enhanced by solubilization of the membranous components with sodium taurocholate. Table 2 displays the results of a typical experiment in which rat liver microsome suspensions (containing 100 µg microsomal protein per ml) were incubated at 37 °C in SET containing 200 µg A23187 or valinomycin per ml for 15, 30 and 60 min prior to determining mannose-6-phosphatase activity in both the absence and presence of 0.4% (wt/vol) sodium taurocholate. The data shows that incubation of the microsomes in SET containing 200 µg valinomycin per ml does not significantly affect the enzymatic activity in either the absence or presence of the detergent as compared to the control activity measured with microsomes incubated under control conditions in SET. Although incubation of the microsomes in SET containing 200 µg A23187 per ml does not significantly affect mannose-6-phosphatase activity measured in the absence of detergent, it does lead to a progressive loss of activity measured in the presence of the detergent. A progressive loss of activity measured in the presence of the detergent also occurs during incubation of the microsomes in SET containing 40 µg A23187 per ml but not during incubation in SET containing 20 µg A23187 per ml (*results not shown*). The findings thus show that neither ionophore alters the structural integrity of the microsomal membranes sufficiently either to render them permeable to the substrate mannose-6-phosphate or to destroy the activity of mannose-6-phosphatase in intact microsomes. However, incubation of the microsomes in SET containing 200 µg A23187 per ml does progressively decrease the activity of the detergent-solubilized enzyme.

Table 2. Effects of A23187 and valinomycin on rat liver microsomal D-glucose-6-phosphate phosphohydrolase activity[a]

Incubation conditions	Period of incubation (min)	µmol inorganic phosphate released/50 µg microsomal protein/30 min	
		Without detergent	With detergent
SET	0	0.015	0.126
	15	0.014	0.127
	30	0.017	0.122
	60	0.021	0.109
SET containing 200 µg/ml A23187	0	0.016	0.114
	15	0.020	0.081
	30	0.027	0.025
	60	0.034	0.006
SET containing 200 µg/ml valinomycin	0	0.017	0.123
	15	0.025	0.121
	30	0.024	0.122
	60	0.029	0.109

[a] Microsome suspensions (containing 100 µg microsomal protein per ml) were incubated at 37 °C in either SET or SET containing 200 µg ionophore/ml for 0, 15, 30 and 60 min prior to determining the phosphohydrolase activity (with the substrate mannose-6-phosphate) in both the absence and presence of 0.4% (wt/vol) sodium taurocholate according to the procedure of Zilversmit and Hughes (1977)

Discussion

The principal finding of this study is that the divalent cation ionophore, A23187, and the monovalent cation ionophore, valinomycin, are the first substances found to significantly enhance the reactivity of a microsomal phospholipid class with phospholipid exchange proteins. Each ionophore's stimulatory effect is a function of the ionophore's absolute concentration in the standard reaction mixture but not of the ionophore/microsome mass ratio. Evaluation of all the findings does not indicate whether the stimulatory effects occur as a result of each ionophore binding to the PI exchange proteins or, alternatively, altering the structural organization of the PI molecules within the microsomal membranes.

Although the mechanism of action by which each ionophore exerts its stimulatory effect is not evident, neither ionophore results in all the exchangeable microsomal PI behaving as a single pool. Neither ionophore, therefore, disrupts those structural factors within the microsomal membranes responsible for the appearance of two or more exchangeable pools under control conditions. In the study by Brophy et al. (1978), where their kinetic

curve of PI transfer is very similar to our curve shown in Fig. 3, they noted that there appear to be two phases of protein-mediated PI transfer, namely a rapid phase and a slow phase. Hence, they extrapolated the slope of their curve between 30 and 120 min to zero time to show that the kinetics can be accounted for by the presence of two major exchangeable pools, one representing 42% of the microsomal PI with an exchange half-life of 5 min and the other representing 38% of the microsomal PI with an exchange half-life of about 1 hr. We can analyze the semi-logarithmic plot punctuated by closed circles in Fig. 5 in an analogous manner to estimate the sizes and exchange half-lives of these two pools in our standard reaction mixture. In other words, we can assume that the slope of the plot between 30 and 60 min represents the exchange half-life of the slowly exchangeable pool and that extrapolation of this part of the plot to zero time indicates the percentage of exchangeable microsomal PI present in this pool. Such analysis indicates that in our standard reaction mixture 40% of the microsomal PI is transferred with an exchange half-life of 5–6 min and 30% with an exchange half-life of 50–60 min. Our estimates of the sizes and exchange half-lives of these two pools thus closely match those determined by Brophy et al. (1978). Now, in order to have some quantitative measure of each ionophore's stimulatory effect on protein-mediated exchangeability of microsomal PI, let us assume that there are still only two major exchangeable pools in the presence of each ionophore at a concentration of 200 μg/ml. In other words, let us analyze the other two semi-logarithmic plots of Fig. 5 in the manner just described above for the plot representative of the standard reaction mixture. Such analysis indicates that in the presence of A23187 44% of the microsomal PI is transferred with an exchange half-life of about 2 min and 44% with an exchange half-life of 10–15 min, and that in the presence of valinomycin 27% of the microsomal PI is transferred with an exchange half-life of about 2 min and 56% with an exchange half-life of 20–25 min. It is important to note that although these estimates of the sizes and exchange half-lives of two major PI pools in the presence of each ionophore closely account for the kinetic curves displayed in Fig. 4, the curves could also be adequately accounted for by an assumption of three or even more exchangeable PI pools. The above estimates therefore indicate only that if there are just two major exchangeable pools in both the absence and presence of the ionophores, the ionophores effect a significant decrease in the exchange half-life of each pool and alter the relative sizes of the pools.

Finally, the *in vitro* stimulatory effects of A23187 and valinomycin suggest that there may be endogenous ionophorous substances which, via interaction with either phospholipid exchange proteins or particular components of cellular membranes, manifest similar stimulatory effects *in vivo*. Possible candidates are phosphatidic acid and di- and trienoic fatty acids, which Serhan et al. (1981) have recently demonstrated act as calcium ion ionophores in multilamellar vesicles. Experimental verification of endogenous stimulants of protein-mediated phospholipid exchangeability would provide one mechanism by which the different phospholipid compositions of various cellular membrane systems can be regulated and maintained in the presence of continuous phospholipid exchange.

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