Multiple Effects of Reserpine on Chromaffin-Granule Membranes†

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ABSTRACT: The tranquilizer reserpine has several effects on adrenal medullary chromaffin-granule membrane vesicles (ghosts). At low concentrations (0.20 ± 0.12 nmol/mg of membrane protein), reserpine inhibits proton-linked epinephrine uptake but does not affect transmembrane pH and electrical potential gradients. Reserpine apparently binds to and blocks the catecholamine translocator. At intermediate concentrations (14.3 ± 4.9 nmol/mg of membrane protein), reserpine abolishes the ATP-dependent enhancement of 8-anilinonaphthalene-1-sulfonate fluorescence without affecting the ATP-dependent membrane potential. At high concentrations (550 ± 390 nmol/mg of membrane protein), reserpine stimulates the efflux of epinephrine from preloaded chromaffin-granule ghosts. Because it is highly hydrophobic, reserpine partitions into the membrane and probably exerts a nonspecific detergent-like action. At high concentrations (74 ± 25 nmol/mg of lipid), reserpine also increases the permeability of phospholipid vesicles to epinephrine. The effectiveness of reserpine in inhibiting epinephrine transport correlates with the reserpine/membrane ratio but not with the molar concentration. This may account for the large variation in reports of effective reserpine concentrations.

Reserpine, a tranquilizer widely used in the treatment of hypertension, exerts its primary effect on amine accumulation in secretory vesicles (Stitzel, 1977). Reserpine specifically inhibits ATP-dependent monoamine transport into adrenal medullary chromaffin granules (Kirshner, 1962), adrenergic synaptic vesicles (Toll & Howard, 1978), and the serotonin-containing granules of platelets (Rudnick et al., 1980). In chromaffin granules, amine transport is driven by an inwardly directed proton-translocating ATPase[1] and is mediated by a translocator which catalyzes an amine/$2H^+$ exchange (Njus & Radda, 1978; Njus et al., 1981). Since reserpine does not inhibit the ATPase activity (Ferris et al., 1970; Bashford et al., 1976; Scherman & Henry, 1980), it is thought to block the amine translocator. If it is a specific inhibitor, reserpine will greatly facilitate biochemical studies of the translocator. It has, in fact, been used to demonstrate reconstitution of reserpine-sensitive transport (Maron et al., 1979) and to distinguish between translocator-mediated transport and unmediated amine permeation (Scherman & Henry, 1980).

There are, however, a number of inconsistencies in the action of reserpine. First, concentrations reported to inhibit transport vary from 16 nM (Kanner et al., 1979) to 300 nM (Phillips, 1974). Second, Phillips (1978) has reported that 10 μM reserpine accelerates, rather than blocks, the efflux of serotonin from chromaffin-granule membrane vesicles (ghosts) previously loaded with the amine. Finally, reserpine inhibits some membrane phenomena completely unrelated to amine transport (Balzer et al., 1968).

Because reserpine is a highly hydrophobic molecule, it partitions into membranes and its effective concentration depends on the reserpine/membrane ratio. Unfortunately, reserpine concentrations are usually reported in molarity, so experiments performed at different protein concentrations cannot be compared directly. This may account for the inconsistency in measurements of effective reserpine concentration. For this reason, too, comparisons of reserpine effects on different activities (amine transport, proton pumping, and amine efflux) are open to question. With these problems in mind, we have systematically examined the concentration dependence of several effects of reserpine on chromaffin-granule ghosts. At low concentrations, reserpine blocks epinephrine transport. At much higher concentrations, reserpine increases the permeability of the membrane.

Materials and Methods

Chromaffin-granule ghosts were prepared as described by Njus & Radda (1979). Experiments were performed within 12 h of the cattle being slaughtered. The [^{3}H]epinephrine content of the ghosts was assayed by collecting ghosts on 0.45-μm pore size cellulose acetate filters and washing the filters with ~2 mL of 0.4 M sucrose–40 mM Hepes, pH 7.4. ΔpH, Δψ, and E_{in}/E_{out} were measured by using ^{14}C-labeled methylamine, thiocyanate, and epinephrine, respectively, as described before (Knoth et al., 1980). Internal volumes were calculated from the protein concentration by using a conversion factor of 3 μL/mg of membrane protein (Knoth et al., 1981).

Liposomes were prepared by the method of Deamer & Bangham (1976). Four milligrams of egg lecithin and 0.4 mg of dicetyl phosphate were dissolved in 2 mL of ether and injected slowly (0.2 mL/min) into 4 mL of 50 mM citrate–50 mM potassium phosphate–1 mM EDTA, pH 5.0, at 60 °C. The liposomes were loaded with [^{3}H]epinephrine by titrating the suspension to pH 8 with 2 N NaOH and incubating for 30 min at room temperature with 1 μCi of [^{3}H]epinephrine (Nichols & Deamer, 1976). The [^{3}H]epinephrine content of the liposomes was assayed by collecting liposomes on cellulose acetate filters (0.2-μm pore size) and washing with ~2 mL of 50 mM citrate–50 mM potassium phosphate–1 mM EDTA, pH 8.0.

Fluorescence of ANS was monitored by using a Perkin-Elmer Model 204S fluorescence spectrophotometer equipped with a water-jacketed sample chamber equilibrated to 37 °C. Excitation and emission wavelengths were 380 and 480 nm, respectively.

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; Hepes, 2-[*N*-(2-hydroxyethyl)piperazin-*N*′-yl]ethanesulfonic acid; ΔpH, transmembrane pH gradient; Δψ, transmembrane electrical potential gradient; E_{in}/E_{out}, transmembrane epinephrine concentration gradient.

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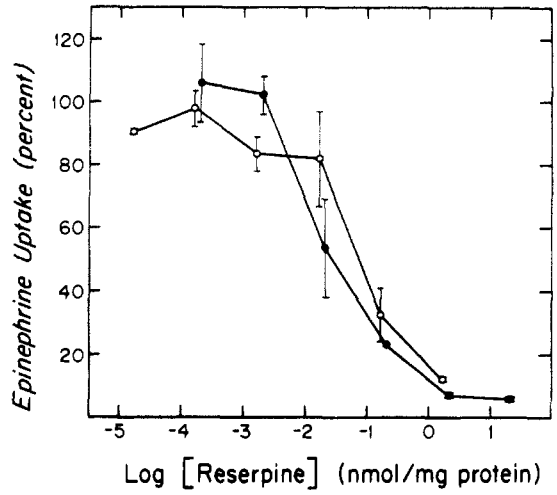

FIGURE 1: Inhibition of epinephrine uptake into chromaffin-granule ghosts. Ghosts [0.27 mg of protein in 50 μL (○) or 2.7 mg in 500 μL (●)] were diluted to 1.0 mL with 0.4 M sucrose–40 mM Hepes, pH 7.4. Reserpine at the concentration indicated was added in 1 μL of solvent. 50 μL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, [^{14}C]epinephrine (0.5 μCi), and ^3H_2O (1 μCi) were added, and the mixture was incubated at 27.5 °C for 20 min. Samples were then centrifuged and processed, and E_{in}/E_{out} values (representing initial rates of uptake) were calculated as described (Knoth et al., 1980). For comparison, values are normalized to E_{in}/E_{out} in the absence of reserpine. These 100% values are 121 ± 21 (○) and 22 ± 1 (●).

DL-[^{3}H]Epinephrine, ^3H_2O, and [^{14}C]methylamine hydrochloride were from New England Nuclear. DL-[^{14}C]Epinephrine bitartrate and potassium thio[^{14}C]cyanate were from Amersham. [^{3}H]Epinephrine was diluted with unlabeled epinephrine to make a 5 mM stock solution (50 μCi/mL) in 25 mM tartaric acid. [^{14}C]Epinephrine (50 mCi/mmol), [^{14}C]methylamine (50 mCi/mmol), and thio[^{14}C]cyanate (59 mCi/mmol) were used at the specific activities delivered. Reserpine was dissolved in acetone–ethanol (60:40). An equal volume of solvent was added to all control samples. Egg lecithin, dicetyl phosphate, and reserpine were purchased from Sigma Chemical Co. ANS (Mg^{2+} salt) was from Eastman Kodak.

Protein was assayed by the biuret method as described (Casey et al., 1976). Error limits are all standard deviations calculated from multiple samples.

Results

The reserpine concentration required to inhibit epinephrine transport into chromaffin-granule ghosts depends on the membrane concentration (Figure 1). If the ghost concentration is increased by a factor of 10, 10 times more reserpine is required to achieve a comparable level of inhibition. In three experiments at low membrane concentrations (0.1–0.5 mg of protein/mL), the initial rate of uptake was inhibited 50% by 72 ± 71 nM reserpine. At high membrane concentrations (1–5 mg of protein/mL), 570 ± 370 nM reserpine was required. The inhibitory reserpine concentration is consistent, however, if expressed as the reserpine/membrane ratio. At low membrane concentrations, 50% inhibition required 0.22 ± 0.15 nmol of reserpine/mg of membrane protein. At high protein concentrations, 0.19 ± 0.08 nmol/mg was needed. The average reserpine concentration causing half-maximal inhibition of epinephrine uptake is 0.2 nmol/mg of protein (Table I).

In chromaffin-granule ghosts, an inwardly directed proton-translocating ATPase generates transmembrane gradients in electrical potential and pH. If the ghosts are suspended in a medium free of permeant anions (sucrose), ATP hydrolysis generates a membrane potential (inside positive) but does not change the pH gradient (Knoth et al., 1980). On the other hand, if ghosts are suspended in a medium containing the permeant Cl^- ion (KCl medium), ATP hydrolysis generates a pH gradient (inside acidic) but the membrane potential is much smaller. Epinephrine transport occurs in response to both the membrane potential and the pH gradient. As shown in Tables II and III, reserpine inhibits ATP-dependent epinephrine transport whether it is driven by the pH gradient (KCl medium) or by the membrane potential (sucrose medium). Moreover, the pH gradients and the membrane potentials themselves are not affected when measured at the same reserpine and membrane concentrations.

The ATP-dependent membrane potential causes an ANS fluorescence enhancement, and this is inhibited by reserpine at intermediate concentrations (Figure 2). Reserpine does not quench ANS fluorescence since it does not affect fluorescence in the absence of ATP (Figure 3). However, the drug abolishes the enhancement caused by the ATP-dependent membrane potential. Only about 80% of the fluorescence enhancement produced by Mg^{2+}-ATP is caused by the mem-

Table I: Concentration Dependence of Reserpine Effects[a]

effect	reserpine concn (nmol/mg of protein)	no. of expt
epinephrine uptake (50% inhibn)	0.20 ± 0.12	6
ANS fluorescence enhancement (40% inhibn)	14.3 ± 4.9	3
epinephrine efflux (20%/10 min)	550 ± 390	5

[a] Inhibition of epinephrine uptake, inhibition of ANS fluorescence enhancement, and epinephrine efflux were measured as shown in Figures 1, 3, and 4, respectively. Each experiment was performed the indicated number of times by using different ghost preparations. Inhibitory reserpine concentrations are averages of the values obtained in replicate experiments.

Table II: Effect of Reserpine on ΔpH, Δψ, and Epinephrine Uptake in KCl Medium[a]

addition	ΔpH	Δψ	E_{in}/E_{out}
none	0.48 ± 0.01	0 ± 2	2.8 ± 0.3
ATP	0.68 ± 0.05	17 ± 1	33.5 ± 4.4
reserpine	0.49 ± 0.02	1 ± 5	2.1 ± 0.2
reserpine + ATP	0.74 ± 0.01	13 ± 0	2.5 ± 0.0

[a] Ghosts (3.4 mg of protein in 0.25 mL) were added to 0.25 mL of 250 mM KCl–150 mM sucrose–40 mM Hepes, pH 7.4. 25 μL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, 1 μL of 5 mM reserpine (final concentration = 1.5 nmol/mg of protein), and appropriate radioactive tracers were added as indicated. After incubation at 25 °C for 30 min, samples were centrifuged and processed as described (Knoth et al., 1980). Values are each the average of two replicate samples.

Table III: Effect of Reserpine on ΔpH, Δψ, and Epinephrine Uptake in Sucrose Medium[a]

addition	ΔpH	Δψ	E_{in}/E_{out}
none	0.75 ± 0.12	−6 ± 7	5.8 ± 0.3
ATP	0.75 ± 0.11	+32 ± 3	32.1 ± 0.6
reserpine	0.87 ± 0.04	−7 ± 14	4.8 ± 0.3
reserpine + ATP	0.89 ± 0.02	+37 ± 6	2.6 ± 0.0

[a] Ghosts (1.25 mg of protein in 0.25 mL) were added to 0.25 mL of 400 mM sucrose–40 mM Hepes, pH 7.4. 25 μL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, 1 μL of 5 mM reserpine (final concentration = 4 nmol/mg of protein), and appropriate radioactive tracers were added as indicated. After incubation at 26 °C for 30 min, samples were centrifuged and processed as described (Knoth et al., 1980). Values are each the average of two replicate samples.

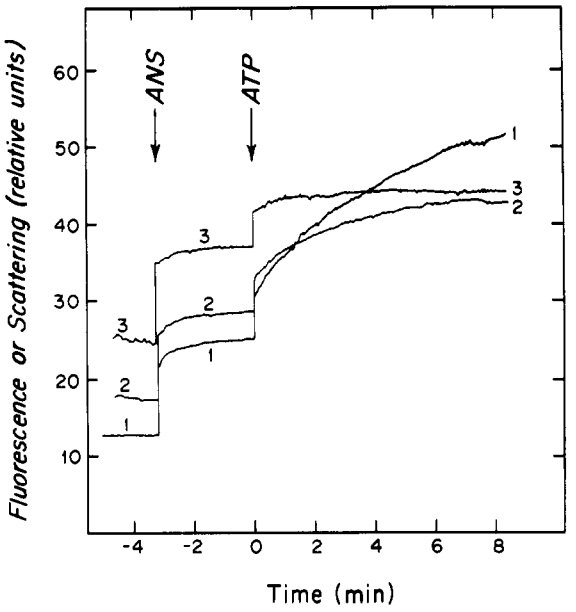

FIGURE 2: ATP-dependent enhancement of ANS fluorescence in the presence of reserpine. Chromaffin-granule ghosts (1.15 mg of protein in 100 μL) were added to 2 mL of 0.4 M sucrose–10 mM Hepes, pH 7.0, and 10 μL of acetone–ethanol (60:40) containing the desired amount of reserpine. The fluorescence was monitored, and 10 μL of 1 mM ANS and 100 μL of 100 mM ATP–100 mM $MgSO_4$, pH 7, were added at the times indicated. Traces represent the following amounts of reserpine (nmol/mg of protein): (1) 0; (2) 13; (3) 43.

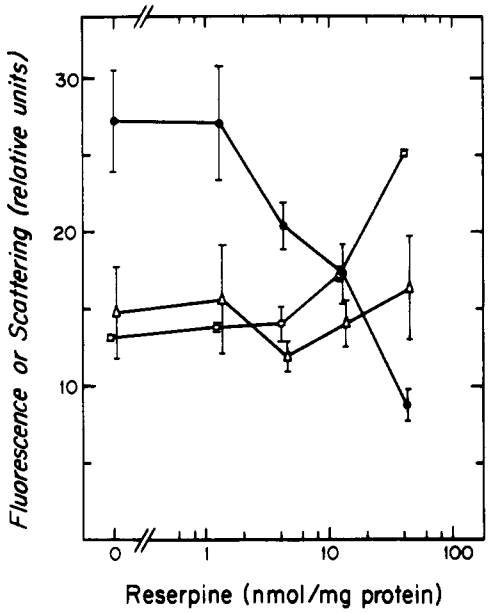

FIGURE 3: Effect of reserpine on ANS fluorescence. Four traces of the kind shown in Figure 2 were obtained at each reserpine concentration. The following parameters were measured, averaged, and plotted: (○) initial signal (scattering), (△) increment caused by ANS addition, and (●) increment caused by ATP measured 8 min after addition.

brane potential (i.e., only 80% is inhibitable by mitochondrial uncouplers). The other 20% is attributable to the change in membrane surface charge caused by Mg^{2+} binding. For that reason, we have taken the reserpine concentration causing 40% inhibition as the index of effectiveness. Inhibiting the ANS fluorescence enhancement by 40% requires 14.3 nmol of reserpine/mg of membrane protein (Table I). This concentration of reserpine also greatly increases the amount of light scattered by the ghosts (Figure 3).

Phillips (1978) reported that reserpine accelerates serotonin efflux from ghosts which have been preloaded with the amine.

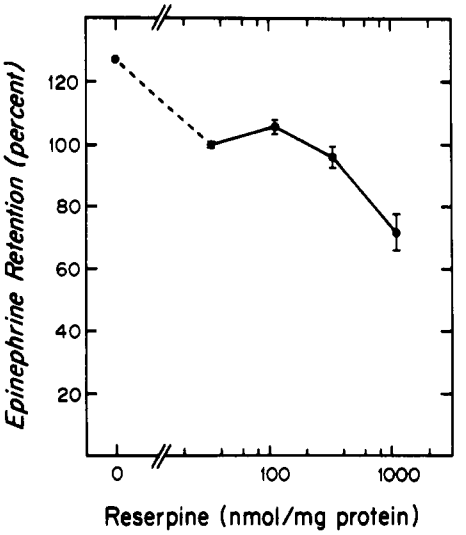

FIGURE 4: Acceleration of epinephrine efflux from chromaffin-granule ghosts. Ghosts (2.4 mg of protein in 150 μL) were added to 1.5 mL of 0.4 M sucrose–40 mM Hepes, pH 7.4. 75 μL of 100 mM ATP, 100 mM $MgSO_4$, pH 7, and 1.5 μCi of [^{3}H]epinephrine were added, and the mixture was incubated at 37 °C for 30 min. Epinephrine uptake was terminated by diluting the ghosts 10-fold with 0.4 M sucrose–40 mM Hepes, pH 7.4. 1-mL aliquots were mixed with 10 μL of reserpine, incubated for 10 min at 37 °C, and collected by filtration. Each point is the average of two samples. 100% = 10.4 nmol/mg of membrane protein.

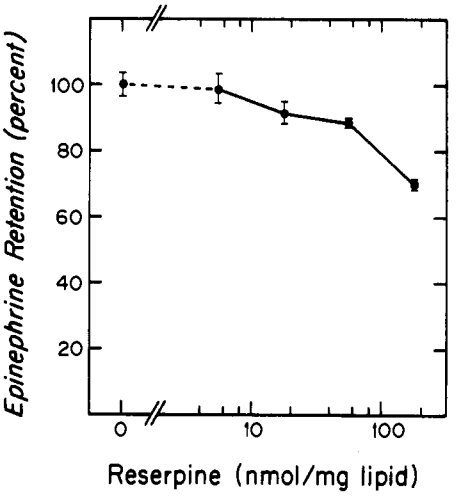

FIGURE 5: Acceleration of epinephrine efflux from liposomes. Phospholipid vesicles were prepared and loaded with [^{3}H]epinephrine as described under Materials and Methods. External [^{3}H]epinephrine was diluted by mixing 0.25 mL of liposomes with 0.75 mL of buffer and 10 μL of acetone–ethanol (60:40) containing a specified amount of reserpine. After 10 min at room temperature, samples were collected by filtration. Each point is the average of two replicate samples. 100% retention = 0.73 nmol/mg of lipid.

A similar effect can be observed by using [^{3}H]epinephrine (Figure 4). In the complete absence of reserpine, some residual active transport occurs, increasing epinephrine retention by about 20%. This uptake is inhibited by the lowest reserpine concentration (~30 nmol/mg of protein). No significant efflux is observed, however, until the reserpine concentration is greater than 100 nmol/mg of protein. Induction of 20% epinephrine efflux in 10 min requires 550 nmol of reserpine/mg of membrane protein (Table I).

A similar reserpine-dependent epinephrine efflux occurs from egg lecithin liposomes in which [^{3}H]epinephrine has been trapped (Figure 5). In Figure 5, a reserpine concentration of 91 nmol/mg of lipid causes 20% efflux in 10 min. The average value obtained in four separate experiments was 74

± 25 nmol of reserpine/mg of lipid.

Discussion

When Kirshner (1962) first discovered that chromaffin granules have an ATP-dependent catecholamine uptake system, he also observed that reserpine was a potent and apparently irreversible inhibitor. Kanner et al. (1979) have recently demonstrated that this apparent irreversibility is a consequence of the hydrophobicity of the reserpine molecule. Reserpine partitions into membranes so strongly that it cannot be removed by dialysis. If, however, phospholipid vesicles are added to the ghost suspension, reserpine partitions into the vesicles and epinephrine transport into the ghosts recovers.

Because reserpine binds to membranes so completely, its effective concentration is the reserpine/membrane ratio, not the molar concentration. We found that the reserpine concentration causing 50% inhibition of epinephrine uptake is 72 nM at low protein concentrations but 570 nM at higher protein concentrations. Other measurements of effective reserpine concentration also vary widely. For 50% inhibition of l-norepinephrine transport, Phillips (1974) and Pletscher (1977) reported values of about 300 and 40 nM, respectively. Kanner et al. (1979) measured K_i values of 16 nM for inhibition of epinephrine transport and 19 nM for inhibition of serotonin transport. Scherman & Henry (1980), working with intact granules instead of ghosts, found that 100 nM reserpine caused half-maximal norepinephrine uptake. As reported under Results, our concentrations are consistent when expressed as the reserpine to membrane ratio (0.2 nmol/mg of membrane protein).

It has been argued that reserpine is a competitive inhibitor of amine transport because inhibition is diminished at higher substrate concentrations (Jonasson et al., 1964; Phillips, 1974; Kanner et al., 1979). This test, unfortunately, is not definitive because, at higher substrate concentrations, active transport saturates and an unsaturable passive uptake becomes more significant. Therefore, anything inhibiting active transport appears to be more effective at lower and less effective at higher substrate concentrations. For this reason, chlorpromazine, which inhibits transport by acting as an uncoupler, appears to be a competitive inhibitor of epinephrine uptake by the standard Lineweaver–Burk analysis (Drake et al., 1979). The results obtained with reserpine could be similarly misleading.

A better indication that reserpine blocks the catecholamine translocator is that it does not affect the pH gradients or membrane potentials generated by ATP hydrolysis (Tables II and III). Scherman & Henry (1980) made similar observations on intact chromaffin granules. In that study, however, effects of reserpine on ATP-dependent membrane potentials and pH gradients were compared to effects on amine uptake measured in different experiments at different membrane concentrations. By comparing epinephrine uptake, membrane potential, and pH gradient in the same experiment at the same membrane concentration (Tables II and III), we have ruled out the possibility that differences in reserpine binding are causing misleading results. Consequently, reserpine does not block amine transport by affecting the membrane potential and pH gradient. This is consistent with observations that reserpine inhibits amine uptake driven by artificially imposed pH gradients (Phillips, 1978) and membrane diffusion potentials (Njus & Radda, 1979). Therefore, reserpine must specifically inhibit the monoamine translocator. Evidence that the inhibition is competitive, however, is not yet conclusive.

The ATP-dependent membrane potential enhances ANS fluorescence by increasing the amount of ANS bound to the membrane. Reserpine inhibits this potential-dependent binding but does not affect the potential-independent binding of ANS (Figures 2 and 3). These results contrast with an earlier report that reserpine has no effect on ANS fluorescence enhancement (Bashford et al., 1976). Although reserpine abolishes the potential-dependent ANS binding, it has no effect on the membrane potential itself. We have found that reserpine concentrations up to 100 nmol/mg of protein do not diminish the ATP-dependent membrane potential as measured by the thiocyanate distribution method (M. Zallakian and D. Njus, unpublished observations). In this regard, reserpine acts like chlorpromazine (Drake et al., 1979). Both compounds apparently bind to the membrane and block potential-dependent but not potential-independent ANS binding. It is unlikely that the drugs bind directly to ANS binding sites because they do not affect potential-independent ANS binding and they are not anionic like ANS. Rather, reserpine and chlorpromazine may interfere with potential-dependent membrane changes which enhance ANS binding. The increase in light scattering caused by reserpine (Figure 3) also suggests that reserpine is binding to and altering the structure of the chromaffin-granule membrane.

Phillips (1978) reported that 10 μM reserpine accelerates serotonin efflux from ghosts that have been preloaded with the amine. Because he was using a very low protein concentration in that experiment, the reserpine/membrane ratio was quite high: 1.4 μmol/mg of membrane protein. We find that 550 nmol of reserpine/mg of protein induces 20% epinephrine leakage in 10 min. Chromaffin-granule ghosts have 2 times as much lipid as protein (Winkler, 1976), so this reserpine concentration is equivalent to about 275 nmol/mg of lipid. At this concentration, reserpine in the membrane is probably exerting a detergent-like action. An indication that this is indeed a nonspecific effect is the fact that a similar phenomenon is observed in phospholipid vesicles (Figure 5) at a concentration of 74 nmol of reserpine/mg of lipid. Liposomes differ from chromaffin-granule membranes in that they contain traces of organic solvent, are composed of different lipids, and do not contain protein. These factors may account for the difference in sensitivity to the permeabilizing effect of reserpine. Since reserpine at high concentrations has nonspecific effects on chromaffin-granule membranes, it may be expected to affect all membranes. An example may be the inhibition by reserpine of Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activity in sarcoplasmic reticulum (Balzer et al., 1968).

Because reserpine has nonspecific membrane effects, some care must be exercised in using it to specifically inhibit the amine translocator. It is particularly important to relate the reserpine concentration to the amount of membrane protein. Given these precautions, however, reserpine can be used to distinguish between translocator-mediated transport and unmediated permeation (Scherman & Henry, 1980) and to demonstrate translocator-mediated amine transport in reconstituted membrane systems (Maron et al., 1979).

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Fluorescence Method for Measuring the Kinetics of Ca^{2+}-Induced Phase Separations in Phosphatidylserine-Containing Lipid Vesicles†

Dick Hoekstra

ABSTRACT: The effects of Ca^{2+} and Mg^{2+} on the fluorescence behavior of the phospholipid analogues 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine in small unilamellar vesicles consisting of phosphatidylserine, mixtures of phosphatidylserine/phosphatidylcholine, and mixtures of phosphatidylserine/cholesterol were studied. Fluorescence quenching was observed when Ca^{2+}, but not Mg^{2+}, was added to phosphatidylserine vesicles containing 5 mol % fluorescent lipid. The quenching process, which could be monitored continuously, was virtually complete within 5–6 min at Ca^{2+} concentrations $\geq$ 1.5 mM and resulted in a decrease of fluorescence intensity of approximately 60%. Fluorescence quenching did not occur in the presence of 0.5 mM Ca^{2+}; however, simultaneous addition of 6 mM Mg^{2+} initiated a quenching process similar in rate and extent to that observed at higher concentrations of Ca^{2+} alone. This quenching of 4-nitro-2,1,3-benzoxadiazole (NBD) fluorescence is best explained in terms of Ca^{2+}-induced separation of lipid phases that leads to an increase in local concentration of NBD–lipid in the bilayer and hence to self-quenching of NBD fluorescence. The kinetics of Ca^{2+}-induced phase separation were also studied in several mixed lipid systems containing phosphatidylserine. In the case of mixtures of phosphatidylserine/cholesterol, the results indicate the presence of phase-separated regions as an intrinsic property of the vesicles in the absence of Ca^{2+}. Finally, results are presented indicating that the kinetics of phase separation is slow compared to vesicle–vesicle fusion.

The importance of divalent cations in promoting the fusion of artificial lipid vesicles composed of acidic phospholipids is well established (Papahadjopoulos et al., 1979). In particular, the involvement of Ca^{2+} in this process has attracted considerable attention because of its presumed role in potentiating various biological fusion events [see Poste & Nicolson (1978)]. Although the molecular basis for Ca^{2+} induction of membrane fusion is not known, several mechanisms have been proposed in which phase transitions (Papahadjopoulos et al., 1973, 1979) and/or formation of nonlamellar lipid particles (Cullis & De Kruijff, 1979) in the fusing membranes play a role in the fusion process. In addition, it has also been suggested that the ability of Ca^{2+} to promote membrane fusion may be related to its potential to induce phase separations of membrane lipids (Papahadjopoulos et al., 1974, 1977). However, because of limitations in current techniques for examining phase separations, such structural changes have only been studied under equilibrium conditions (Ito et al., 1975; Papahadjopoulos et al., 1977; Van Dijck et al., 1978), and potentially important membrane changes during the initial stages of the fusion process may not have been detected.

In this paper a method is presented for continuous monitoring of lipid phase separations during the time course of vesicle–vesicle fusion. The method is based on the use of small amounts of fluorescent phospholipid analogues incorporated into the vesicle bilayer, which, during phase separation, are

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