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Fluorometric Analysis of Transferable Membrane Pores†

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ABSTRACT: When pore-forming factors insert into the hyperpolarized membranes of lipid vesicles, ion gradients are rapidly equilibrated, effecting complete depolarization. This process can be conveniently followed with a potentiometric cyanine dye. The generality of the method is demonstrated by applications to three diverse materials. The well-studied gramicidin channel is used to demonstrate that the method is sensitive down to concentrations of 10^{-12} M. An extract from the shark repellent skin secretion of the Red Sea flatfish displays activity in the assay and is used to demonstrate the potential of the method to elucidate some of the characteristics of the pore, including its molecularity. That membrane-active factors can be detected and assayed in crude preparations is demonstrated with an impure extract of "amoebapore" from *Entamoeba histolytica*. In addition, variation of the buffer composition surrounding the vesicles can provide information about the ion selectivity of the pore under investigation.

Recently, we described a simple method for detection of transferable membrane pores (Loew et al., 1983). The principle of the method involved the pore-mediated collapse of a diffusion potential in lipid vesicles that could be conveniently monitored with a potential sensitive dye. In that study multilamellar vesicles (MLV's)[1] were employed and the rate of diffusion potential collapse was limited by the rate of bilayer–bilayer transfer of the pore. We now report that a similar protocol with large unilamellar vesicles (LUV's) can provide sensitivities in the femtomole range and also allows more detailed characterization of the pore properties.

A number of other fluorescence-based methods for studying membrane permeabilizing factors in lipid vesicles have been recently described. Together, they provide a convenient set of additions to electrical measurements on planar bilayers for the study of membrane permeability in reconstituted or model systems. In addition, they make possible the study of the process by which membrane-active agents insert into the lipid bilayer. Clement & Gould (1981a,b) used the fluorescence of vesicle-entrapped pyranine to monitor the influx of H^+, which was limited by the rate of efflux of internal cations. Shanzer et al. (1983), in a study of several synthetic lithium carriers, found that the release of entrapped carboxyfluorescein coincided with the release of Li^+, providing a measure of the relative efficiencies of the carriers. Most recently, Blumenthal et al. (1984) have studied a lytic factor derived from cytotoxic lymphocytes with the carboxyfluorescein method. The method described herein has advantages of high sensitivity and simplicity (e.g., no removal of unentrapped fluorophore is required); it requires only micromolar concentration of the dye and is not sensitive to the pH of the medium.

The scope of the method is demonstrated by application to the analysis of three diverse materials. The sensitivity is assessed with gramicidin, a well-characterized channel (Urry, 1970). Mechanistic details on the action of a newly purified toxin, PX-I, are deduced. A crude pore-forming extract from *Entamoeba histolytica* illustrates the application of the method

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[1] Abbreviations: MLV, multilamellar vesicle; LUV, large unilamellar vesicle; diS-C_2(5), 3,3′-diethylthiodicarbocyanine iodide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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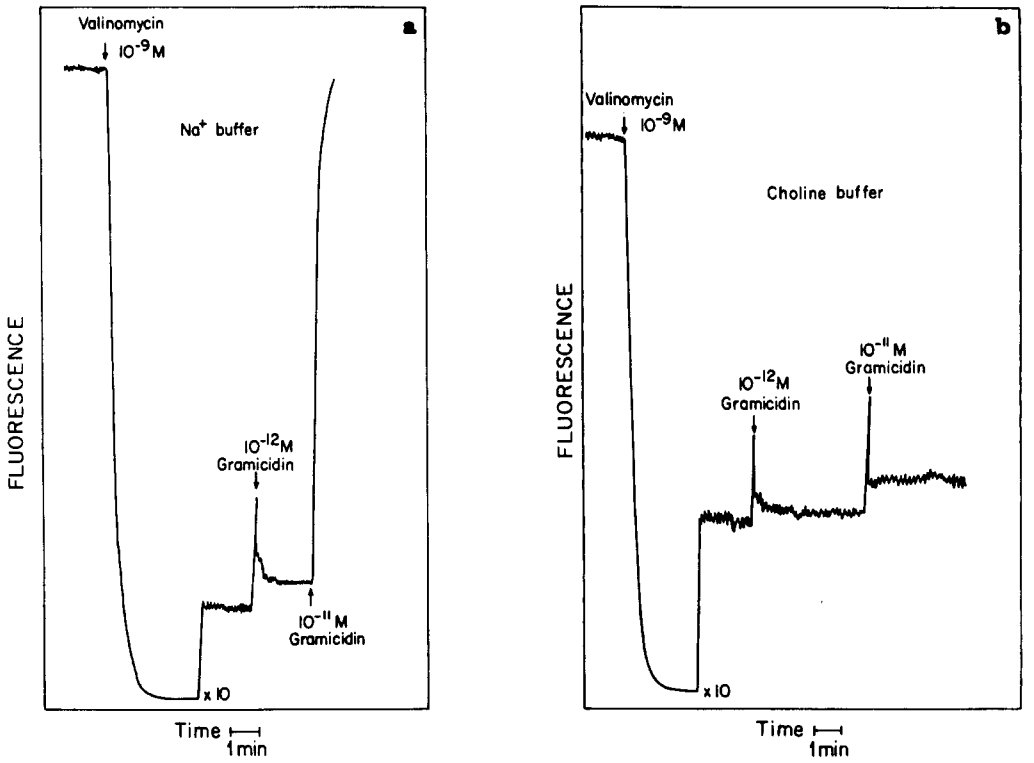

FIGURE 1: Depolarization of LUV's with gramicidin monitored by the fluorescence of diS-$C_2(5)$. A 3-μL aliquot of the LUV preparation is diluted into 3 mL of K^+-free buffer in a fluorescence cuvette that also contains 3 μM diS-$C_2(5)$. In (a) the external buffer contains 50 mM Na_2SO_4 and in (b) it contains 50 mM choline sulfate. Valinomycin (10^{-9} M) and gramicidin are added at the indicated points.

as a simple assay during purifications of membrane-active factors.

Materials and Methods

LUV's are prepared from egg phosphatidylcholine (Sigma Chemical Co., Type XIE) by the method of Mimms et al. (1981) in buffer containing 50 mM K_2SO_4, 10 mM HEPES–SO_4^{2-} (pH 6.8), and 0.1 mM calcein (Hach Chemical Co., Loveland, CO). The final lipid concentration is typically 12 mM. The vesicle concentration can be obtained by combining the results of a total entrapped volume determination (Oku et al., 1982) and the average vesicle volume determined from negatively stained electron micrographs.

In general, the analysis involves monitoring the recovery of fluorescence of a potential sensitive indicator, diS-$C_2(5)$ (Molecular Probes, Junction City, OR), which is quenched inside the hyperpolarized vesicles. A 3-μL aliquot of the vesicles is diluted into 3 mL of isotonic K^+-free buffer in an acrylic fluorescence cuvette (Sarstedt, Inc., Princeton, NJ); the acrylic cuvette was most helpful in reducing drift of the fluorescence resulting from slow binding of the dye to glass or quartz. The optimum sensitivity was obtained with 3 μM dye and 1 nM valinomycin, which were added to the stirred vesicle suspension in this order. Fluorescence was monitored with excitation at 620 nm and emission at 670 nm with either a fluorescence spectrophotometer or a filter fluorometer (Models MPF-44B and 1000 M, respectively, Perkin-Elmer, Wilton, CT) until quenching was complete (1–2 min). At this point the material under investigation could be added to the cuvette.

Gramicidin D was obtained from Sigma; this is a mixture of ~88% gramicidin A, 7% gramicidin B, and 5% gramicidin C. PX-I is a component of pardaxin, the shark repellent secretion of the Red Sea flatfish *Pardachirus marmoratus* (Primor et al., 1983). The details of its purification will be published elsewhere (Lazarovici and Primor, unpublished results). A concentrate of "amoebapore" is obtained from *E. histolytica*, strain HMI:IMSS, as described previously (Lynch et al., 1982); it was further processed to remove ribosomal particles as described below.

Results and Discussion

The sensitivity of the method is demonstrated in Figure 1 with the channel-forming peptide gramicidin. A valinomycin-mediated diffusion potential is produced across the membrane of K^+-containing LUV's that have been diluted into K^+-free buffer and can be monitored by the fluorescence of diS-$C_2(5)$ (Sims et al., 1974). The dye fluorescence is quenched about 30% upon binding to the vesicles before the addition of valinomycin. Incorporation of gramicidin channels into the vesicle membrane effects immediate collapse of the potential by allowing equilibration of the internal K^+ with external Na^+. Fluorescence recoveries can be detected down to 3×10^{-15} mol (i.e., 1×10^{-12} M in a 3-mL cuvette) of gramicidin (Figure 1a). A control experiment with K^+-containing LUV's diluted into buffer containing K_2SO_4 (i.e., no ion gradients) shows no fluorescence change upon addition of valinomycin or gramicidin. If external Na^+ is replaced by choline, a cation that cannot penetrate the gramicidin channel, even picomolar levels of gramicidin do not lead to any significant fluorescence recovery (Figure 1b). The fluorescence recovery, therefore, is related to the dissipation of existing ionic concentration gradients within the fraction of vesicles that have incorporated channels and not to artifactual interactions between gramicidin and the vesicles or dye.

The high sensitivity can be attributed to the large amplification associated with the release of many dye molecules per channel. As can be seen from Figure 1, the valinomycin-mediated K^+ diffusion potential results in the quenching of more than 98% of the initial fluorescence from the 3 μM solution of the permeant cation diS-$C_2(5)$. Thus, even if one assumes that the residual fluorescence is due to external dye

only, essentially all the dye has become vesicle associated. The vesicle entrapped volume, determined by the method of Oku et al. (1982), is only 5×10^{-5} of the total cuvette volume, meaning that the dye concentration within the vesicles must be approximately 60 mM. This extraordinary concentration of the permeable dye within the vesicles can be understood in terms of the coupled equilibria:

$$\mathrm{Do} \overset{Kv}{\rightleftharpoons} \mathrm{Di} \quad (1)$$

$$n\mathrm{Di} \overset{Ka}{\rightleftharpoons} (\mathrm{Di})_n \quad (2)$$

Equation 1 describes the equilibrium for dye internalization and is governed by an equilibrium constant, *Kv*, which is voltage dependent; in the present case, *Kv* is increased by a factor of 1000 (corresponding to the ratio of internal to external K^+) upon addition of valinomycin. Equation 2 represents the aggregation of the internal dye, a well-known process for cyanine dyes that produces the fluorescence quenching (Sims et al., 1974). The aggregation equilibrium is driven to the right by the voltage-dependent buildup of Di but is not directly voltage dependent (assuming the polycationic aggregates are impermeant). The resulting depletion of monomeric Di, in turn, drives more dye into the vesicles and essentially depletes Do. Therefore, before the addition of gramicidin in Figure 1, approximately 98% of the dye is trapped inside the vesicles in the form of nonfluorescent aggregates. From the total internal concentration of 60 mM, the number of dye molecules in this form inside an average vesicle (having a volume of 4×10^{-18} L from the analysis of electron micrographs) is 1.5×10^5. Thus, when one channel collapses the K^+ gradient across a vesicle membrane, 1.5×10^5 dye molecules are released in the form of a fluorescent monomer.

The optimal concentration of diS-C_2(5), 3 μM, was determined by titrating the hyperpolarized vesicles to the point at which the fluorescence begins to increase. This assures that dye released when a small fraction of the vesicles are depolarized by addition of a permeabilizing agent is not reabsorbed by vesicles that are still hyperpolarized. Under these conditions, the fluorescence recovery is approximately proportional to the fraction of vesicles with channels; in order to fully quantitate the concentration of channel over a wider range, however, a calibration of fluorescence vs. fraction of vesicles depolarized needs to be developed. It should be noted that these partial recoveries cannot be explained in terms of a partial but uniform depolarization of the entire vesicle population; the introduction of enough permeability to allow this would, of necessity, lead to sufficient ion flux to rapidly eliminate all concentration gradients.

Pardaxin, derived from the toxic skin secretion of the Red Sea flatfish *Pardachirus marmoratus*, has multiple pharmacological effects that were attributed to its ability to interact with membranal phospholipids (Primor et al., 1983). Recently, with anion-exchange and chromatofocusing chromatography, pardaxin was separated into two toxins, PX-I and PX-II, homogeneous by both molecular weight and isofocusing criteria, the former proving to be the primary cytotoxic factor of the crude secretion (P. Lazarovici and N. Primor, unpublished data). PX-I provides a good example of a less well characterized membrane-active polypeptide than gramicidin with which to examine our pore assay.

In Figure 2a, PX-I is shown to induce a fluorescence recovery qualitatively similar to that observed with gramicidin. The fact that small doses lead to abrupt, yet partial, recoveries suggests that the mechanism does not correspond to detergent lysis of the LUV's. Control experiments with external K^+ buffer revealed no significant fluorescence changes upon introduction of PX-I. It is possible that the pore is not a simple membrane channel, however, but acts in a more complex way to disrupt or permeabilize the lipid bilayer. Indeed, these experiments revealed some interesting contrasts between PX-I and gramicidin. PX-I does not discriminate significantly between cations and anions—it is equally effective if sucrose or choline sulfate replaces the Na_2SO_4 in the external buffer. It also requires a much higher dose than gramicidin in order to observe an effect; the 3% fluorescence recovery in Figure 2a is caused by 7×10^{-10} M PX-I added to 1.2×10^{-10} M vesicle. This may be due to either incomplete binding of PX-I to the LUV's or the requirement of many PX-I monomers for the formation of a single pore. To test for binding, PX-I was labeled with ^{125}I (Bolton & Hunter, 1973) and 25 ng was allowed to incubate with 3 mL of 10^{-10} M LUV under the same conditions as in a fluorescence experiment (labeling caused no diminution of pore-forming activity). This solution was passed through a column of Sepharose 4B in order to separate bound and free toxin. It was found that the LUV's obtained in the void volume contained 15% of the original radioactivity. This figure represents the lower limit of the extent of binding since PX-I could be stripped from the LUV's during their passage through the column. The probability of finding *n* monomers in a given vesicle is described by a Poisson distribution:

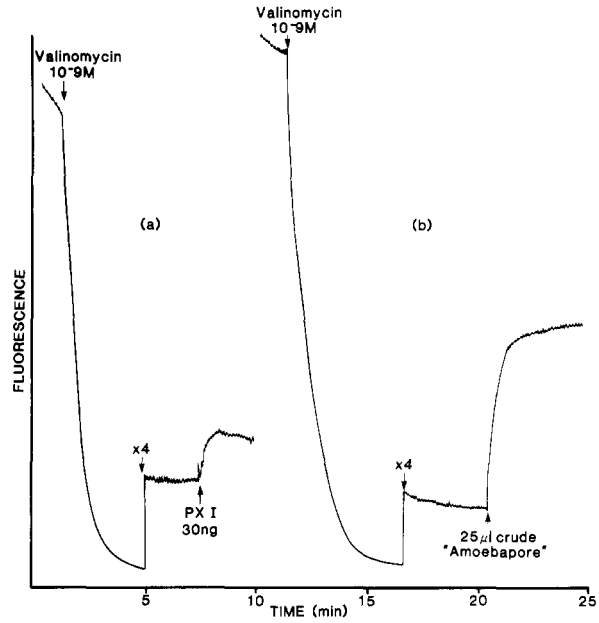

FIGURE 2: Additions of PX-I (a) or amoeba extract (b) effect a partial fluorescence recovery. The conditions are the same as in Figure 1a. The preparation of the amoeba extract is described in the text.

$$P = 1 - e^{-\lambda} \sum_{x=0}^{n-1} \frac{\lambda^x}{x!}$$

where λ is the ratio of bound monomers to vesicles and *P* is the fraction of vesicles containing *n* or greater monomers. In the experiment of Figure 2a, the fraction of vesicles that contain a sufficient number of PX-I monomers to form a pore is approximately 0.03, assuming the fluorescence recovery is reasonably proportional to the fraction of depolarized vesicles. If all of the PX-I is bound, $\lambda = 6$, and the minimum number of monomers necessary to discharge the ion gradients across a vesicle membrane is 11. If only 15% of the PX-I monomers are bound, this reduces to 4. These figures nicely bracket the value of 6 estimated by Primor et al. (1983) from the dependence of planar bilayer conductance on crude secretion concentration. This analysis illustrates the potential of the

method to probe the molecularity of membrane pores. With the development of a good calibration of fluorescence vs. fraction of depolarized vesicles, we hope to refine the analysis so as to obtain both binding parameters and molecularity from theoretical fits of titrations of the hyperpolarized LUV's with pore-forming materials.

It has been shown that strains of *E. histolytica*, the causative agent of amoebiasis in man, produce a potent pore-forming polypeptide, amoebapore (Lynch et al., 1980, 1982). Since the original finding, much effort has gone into the purification, characterization, and role of amoebapore in the etiology of amoebiasis. Studies using planar lipid bilayers have shown that amoebapore spontaneously incorporates into the bilayer, causing an increase in conductance (Lynch et al., 1982; Young et al., 1982). In past attempts to purify amoebapore activity, the MLV assay system has been employed to monitor relative activity in extracts and column fractions (Loew et al., 1982, 1983). These preparations offered, therefore, a good opportunity to test the applicability of the LUV assay to the detection of small pore concentrations in crude cell extracts.

Amoebapore can be conveniently concentrated from total amoeba homogenate in a 150000*g* pellet of a low-speed centrifugation supernate (Lynch et al., 1982). This fraction contains significant amounts of RNA, presumably in ribosomal particles, that interfere with the assay [diS-C_2(5) fluorescence is quenched by this material in the control experiment where LUV's are diluted into K_2SO_4 buffer]. The amoebapore could be solubilized from the pellet in 1 M ammonium acetate–30 mM $MgCl_2$, while the intact ribosomes could be sedimented by recentrifugation at 150000*g* for 3 h. The buffer is then replaced by passage of the supernate through a short column of Sephadex G-25 preequilibrated with the standard 50 mM Na_2SO_4 buffer. Aliquots of this material showed significant activity in the LUV assay (Figure 2b) and did not affect the fluorescence in control experiments. The results indicate that a 25-μL dose contains 3×10^{-14} mol of pore; on the basis of a count of the number of cells harvested for the preparation, this corresponds to about 10000 monomers per amoeba.

As with pardaxin, choline sulfate or sucrose replacing the external Na_2SO_4 did not significantly alter the magnitude of the fluorescence recovery, indicating that amoebapore allows SO_4^{2-} to escape the LUV's. The data obtained thus far are insufficient to reliably determine the numbers of monomers per pore from the statistical approach used with pardaxin, especially since there is considerable uncertainty with respect to the state of aggregation of amoebapore in solution and the fact that amoebapore has not as yet been purified to homogeneity.

We have demonstrated that this method provides a general, convenient, and sensitive means for the detection of transferable membrane pores. The assay of pore-forming factors in crude cell extracts can be accomplished on a routine basis with a simple fluorometer. Information about the gross selectivity of the pores can be deduced. In addition, the method can provide information about the state of aggregation of individual peptides composing the pore, either in an aqueous environment or in their functional form within the membrane. In principle, kinetic studies of the fluorescence recovery can be used to investigate the rates of channel insertion into lipid vesicle membranes or the rates of carrier-mediated ion translocation. Such kinetic extensions of the method are in progress.

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