β-Bungarotoxin-Mediated Liposome Fusion: Spectroscopic Characterization by Fluorescence and ESR†

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ABSTRACT: The capacity of the snake venom neurotoxin β-bungarotoxin to induce fusion of small unilamellar liposomes was demonstrated. The fusion process was studied spectroscopically using three different methods: (i) by resonance energy transfer, using fluorescent lipid analogues; (ii) by the Tb/DPA assay; (iii) by electron spin resonance, using spin-labeled phospholipids. For the latter technique, a new method of analysis based on Fourier-transform component separation was developed. The fusogenic activity was found to be strongly correlated with the known phospholipase A_2 activity of the toxin: both functions were shown to have a specific requirement of Ca^{2+} at almost stoichiometrical concentrations, much below the threshold values found for unspecific divalent cation induced vesicle fusion. Similarly, the presence of phosphatidic acid in the target membrane was essential for both fusogenic and enzymatic activities. The results suggest a molecular mechanism of fusion involving protein binding to negatively charged groups on the membrane surface, followed by local formation of lysophospholipids and as a consequence hereof the creation of point defects in the lipid structure. On the basis of these findings, a model is put forward to explain the specific mode of action of β-bugarotoxin in vivo.

Venoms from certain snakes are known to act by interferring specifically with the presynaptic membrane processes responsible for the storage and release of neurotransmitter from the nerve terminal (Howard & Gundersen, 1980). The mechanism by which presynaptic toxins exert their effects is not known; however, these toxins can be employed as molecular probes in order to gain more information on the mechanism of neurotransmitter release.

β-Bungarotoxin (BUTX),[1] one of the components of the venom of the snake *Bungarus multicinctus*, induces release of neurotransmitter from rat brain synaptosomes together with a decrease of neurotransmitter reuptake, without lysing the synaptosomes (Sen et al., 1976; Spokes & Dolly, 1980; Halliwell et al., 1982). In the neuromuscular junction, BUTX modifies the release of neurotransmitter in two distinct ways: (i) an initial enhancement of spontaneous, evoked, and delayed release is observed, with a maximum within 30 min; (ii) the rate of release declines, and transmission is completely abolished after several hours (Strong et al., 1976).

BUTX is a dimer composed of a 12-kDa A subunit which exhibits Ca^{2+}-dependent phospholipase A_2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) activity and a 7.5-kDa B subunit, which has some sequence homology with protease inhibitors (Abe et al., 1977; Kondo et al., 1981). The reduction of the interchain S–S bonds or the covalent modification of the phospholipase active site leads to a loss of neurotoxicity. Recently, the presence of a toxin high-affinity binding site, with a K_d ranging between 0.4 and 1.9 nM, has been demonstrated both in chick (Rehm & Betz, 1983, 1984) and in guinea pig synaptic membranes (Nicholls et al., 1985). On the basis of these findings, Rhem and Betz (1984) suggested that this protein, possibly together with other similar proteins, plays an important role in transmitter endo- and exocytosis and that the high neurotoxicity of BUTX could be correlated with the presence of these binding sites at presynaptic level. Moreover, Bon's group (Radvanyi et al., 1987) demonstrated that BUTX binds preferentially negatively charged micelles, whereas nonneurotoxic phospholipases A_2 were equally able in binding neutral micelles. These results suggest, in addition to the existence of a possible specific acceptor protein, a major role for the lipid charge in BUTX neurotoxic action. Both enzymatic and neurotoxic activities of the toxin need Ca^{2+}, and Abe and co-workers (Abe et al., 1977) showed that the A subunit possesses one high-affinity Ca^{2+} binding site. Consistently, various other groups have demonstrated a connection between enzymatic and neurotoxic activity of BUTX (Howard & Truog, 1977; Napias & Heilbronn, 1980; Rugolo et al., 1986), and the general opinion is now that the two activities are strongly correlated.

In an early study (Strong et al., 1976) on the mechanism of action of BUTX, a direct involvement of phospholipid hydrolysis (namely, lysophospholipid and free fatty acid production) in the modulation of neurotransmitter release was proposed; this is in good agreement with the hypothesis that lysophospholipids induce membrane fusion (Poole et al., 1970). Following this as a working hypothesis, we have investigated, using both fluorescence and ESR methods, whether BUTX is able to induce fusion in small unilamellar liposomes. On the basis of our results, and taking into account the difficulties of extrapolating results obtained with an artificial system to

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[1] Abbreviations: BUTX, β-bungarotoxin; ESR, electron spin resonance; DMPC, dimyristoylphosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; CA9C, cholesteryl anthracene-9-carboxylate; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; dPC, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; RET, resonance energy transfer; pBPB, *p*-bromophenacyl bromide; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPA, dipicolinic acid; BSA, bovine serum albumin.

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in vivo conditions, we propose that phospholipase activity and fusogenic properties of BUTX are strongly correlated and could modulate the depletion of the neurotransmitter observed in vivo.

Experimental Procedures

Materials

BUTX made up by fraction β-3 of the Abe et al. (1977) preparation was generously provided by Dr. Alemã (Laboratory of Cell Biology, CNR, Rome). The toxin modification with pBPB was carried out according to Abe et al. (1977).

DMPC, phospholipase A_2 from bee venom, and phospholipase D from cabbage were obtained from Sigma (St. Louis, MO) and used without further purification. Labeled phosphatidylcholine (1-palmitoyl-2-[1-^{14}C]arachidonyl, 58 mCi/mM) was purchased from Amersham Inc. (U.K.). Some experiments were carried out using PC purified from egg yolk according to Bloj and Zilversmit (1976). PA was obtained by the action of phospholipase D on purified PC as described by Yank (1969) and isolated by preparative thin-layer chromatography. CA9C was purchased from Molecular Probes Inc. (Eugene, OR); NBD-PE was prepared as described by Monti et al. (1978). dPC was purchased from Avanti Polar Lipids Inc. (Birmingham, AL) and repurified by thin-layer chromatography. Purification and control of lipids by thin-layer chromatography were carried out according to Spinedi et al. (1987).

Methods

Preparation of Liposomes. Small unilamellar liposomes with an average diameter of 40–50 nm, containing different amounts of PC and PA, were prepared according to Batzri and Korn (1973) taking into account the observation of Kremer et al. (1977) on the variability of the liposome size in relation to the lipid concentration in ethanol. Briefly, a few nanomoles of lipid dissolved in chloroform was dried under a N_2 flow. Ethanol was then added in order to reach a lipid concentration of about 20 μmol/mL. The lipid/ethanol solution was injected with a 20-μL Hamilton syringe into the buffer, giving a final lipid concentration of 300 μM and keeping the ethanol final concentration below 3% (v/v). During injection, the temperature was kept at 50–60 °C, well above the phase transition of the lipid used. Experiments, unless otherwise indicated, were carried out using liposomes diluted in 20 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA to avoid fusion caused by spurious Ca^{2+}. Some experiments were made by using sonicated vesicles prepared according to Huang (1969).

RET Measurements of Fusion. Membrane lipid intermixing was estimated by the method based on RET as described by Struck et al. (1981). CA9C and NBD-PE were used as donor and acceptor fluorescent lipid, respectively, as reported by Vinals et al. (1987), and were incorporated into different vesicle populations. The probes accounted for 1% and 3% of total lipids, respectively. Fusion was induced by adding aliquots of BUTX (5–10 μL) to a phospholipid vesicle suspension (2.5 mL, containing 60 nmol of total phosphorus) directly in the cuvette used for fluorescence determination at 28 °C. Temperature control was achieved by water bath operated circulation around the jacketted cuvette; the temperature of the mixture in the cuvette was routinely checked by a precision thermocouple thermometer.

Fluorescence measurements were done with a Perkin-Elmer LS-5 luminescence spectrometer. Excitation was set at 380 nm, and emission was detected at 460 nm, using a 5-nm band-pass on both light paths. The results are expressed according to Fung and Stryer (1978) as efficiency of energy transfer (E) by the equation:

$$E = 1 - F/F_0$$

where F_0 is the CA9C fluorescence with the acceptor present in a different vesicle population and F is the CA9C fluorescence after fusion.

Tb–DPA Fluorescence Assay. The Tb-DPA method of Wilschut et al. (1980) was followed to estimate mixing of aqueous vesicle contents. Briefly, two liposome populations were prepared by the ethanol injection method in either 10 mM $TbCl_3$ and 100 mM sodium citrate or 100 mM DPA. The medium of both populations contained 10 mM HEPES adjusted to a final pH of 7.4. The liposomes were separated from nonencapsulated material by gel filtration of a Sephadex G-50 column (15 × 1 cm): the elution was obtained with a buffer solution containing 0.2 mM EDTA. A small amount (10 nCi) of [^{14}C]acyl-PC was added to the lipid, in order to follow the elution profile of the liposomes. Aqueous liposome content intermixing experiments were started by adding few microliters of BUTX and/or Ca^{2+} to the reaction mixture (total volume 2.5 mL) containing Tb^{3+} and DPA vesicles at a 1:1 ratio (final lipid concentration 100 μM), directly in the cuvette. Excitation of the Tb/DPA complex was set at 276 nm; fluorescence emission was recorded at 545 nm. The data are expressed as the percentage of maximal fluorescence (100%) with respect to 0.5% (w/v) sodium cholate lysed Tb and DPA–liposomes from which EDTA was removed by gel filtration. Some experiments were made with vesicles containing DPA–Tb (50 and 5 mM) in order to test the ability of BUTX to provoke leakage of liposomes.

ESR Measurments of Fusion. Liposomes for ESR experiments were prepared as described above, but the final concentration of lipid was 500 μM. Two populations were prepared: one unlabeled, containing 80% PC and 20% PA; the other labeled, containing PA and dPC in the same ratio as in the unlabeled liposomes. Samples for ESR experiments were typically prepared by mixing 5 μL of labeled with 45 μL of unlabeled liposomes; after addition of Ca^{2+} and BUTX, the sample was mixed rapidly and drawn into a thin-walled glass capillary, which was then placed in a standard quartz ESR tube. All measurements were done at room temperature with a Bruker ESP 300 spectrometer operating at 9.78 GHz, using a standard TE_{102} cavity. Instrument settings included 2.0-G modulation, 100-mW microwave power, a time constant of 41 ms, and a scan time of 42 s. Normally four single scans of 100-G width were accumulated to improve the signal to noise ratio. The ESR spectrum of the liposomes before fusion consisted of a single broad line, because the high concentration of spin-label led to extensive line broadening. Liposome fusion was determined by the appearance of the unbroadened spectrum of paramagnetically dilute spin-label, superimposed on the broad line. To separate the two spectral components, we developed a new technique, based on the Fourier transformation routines of the Bruker 1600 data system software. The digitized spectra were transformed into the frequency domain, and the undesired line widths wider than 56-MHz parts were deleted. After back-transformation, only the unbroadened spectrum of the diluted spin-labels with narrow (high-frequency) lines was left. The eliminated component represents the remaining population of nonfused liposomes, as well as fusion events among two labeled liposomes. This procedure was possible because the distinct differences in the line widths of the broadened and unbroadened signal (27 and 2 G) made the digital separation very easy. Control measurements showed that the spectral shape and size of the unbroadened component

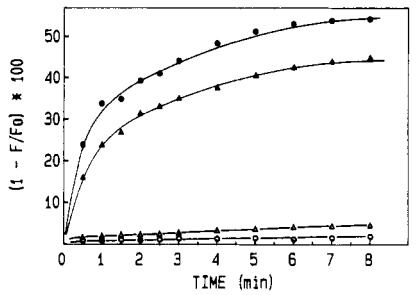

FIGURE 1: Time course of BUTX-induced liposome fusion monitored by RET. CA9C- and NBD-PE-labeled vesicles of DMPC/PA (80:20) were mixed in 20 mM Tris/0.1 mM EGTA, pH 7.4, at a final lipid concentration of 60 nmol in 2.5 mL at 28 °C. The fluorescence of samples containing 62 pmol of BUTX (○), 0.3 mM Ca^{2+} (△), 62 pmol of BUTX + 0.3 mM Ca^{2+} (●), or 5 mM Ca^{2+} (▲) was monitored as described under Experimental Procedures.

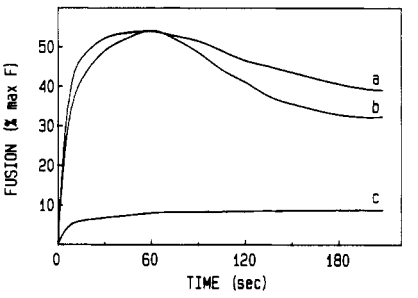

FIGURE 2: Time course of BUTX-induced vesicle aqueous contents intermixing monitored by Tb/DPA assay. Tb^{3+}- and DPA-containing liposomes of DMPC/PA (80:20) were mixed in 20 mM HEPES/0.2 mM EDTA, pH 7.4, at a final lipid concentration of 100 μM. Tb^{3+} fluorescence of samples containing 62 pmol of BUTX + 0.3 mM Ca^{2+} (a), 7.5 mM Ca^{2+} (b), or 0.3 mM Ca^{2+} (c) was followed as described under Experimental Procedures.

were unaffected by these manipulations, apart from a slight distortion of the base line at the extreme ends of the spectrum.

The Fourier transformation method is fast, exact and simple, compared to the conventional methods of spectral separation based on subtraction of differently weighted standards for each spectrum until one of the two components appears to be eliminated [for a discussion of spectral subtraction methods, see Devaux and Seigneuret (1985)]. Furthermore, the conventional methods require better signal to noise ratios than could be obtained in these measurements, where the percentage of the two components changes rapidly. A detailed methodological treatment of the Fourier transformation separation procedure will be presented elsewhere.

Phospholipase Activity Assay. Liposomes for enzyme activity assays were prepared under identical conditions as for the RET studies, except that 5% of [^{14}C]acyl-PC was used to monitor phospholipase activity. The liposomes were incubated with a few microliters of BUTX, and the reaction was stopped by adding methanol/chloroform (2:1) in order to get a final methanol/chloroform/H_2O composition of 2:1:0.8. Lipids were then extracted by partitioning in a two-phase system of one part 0.1 M KCl and one part chloroform. The chloroformic phase was concentrated under N_2 flow and spotted on a high-performance thin-layer chromatography aluminum plate. The plate was resolved in the following system: chloroform/methanol/acetic acid/water (60:27:8:2). The bands corresponding to free fatty acid, PC, and lyso-PC (to monitor possible phospholipase A_1 activity) were scraped, added to 400 μL of ethanol, and transferred to plastic vials containing Opti-fluor (Packard Instrument Co., Downers Grove, IL), and radioactivity (as DPM) was determined by a Packard Tri-Carb 1500 liquid scintillation counter. The enzymatic activity was expressed as the percentage of radioactivity present in the free fatty acids fraction with respect to total radioactivity.

RESULTS

Divalent cations are able to induce fusion in lecithin liposomes containing PA (Papahadjopoulus et al., 1976; Vinals et al., 1987). Figure 1 shows typical time courses of lipid intermixing, demonstrating the Ca^{2+}-induced fusion of DMPC/PA liposomes, monitored by the NBD-PE/CA9C RET assay. The same figure shows the kinetics of liposome fusion induced by BUTX at nonfusogenic levels of Ca^{2+}. Mg^{2+}, which in our system exhibits a fusogenic activity in the same fashion as Ca^{2+} (data not shown), failed to mimic Ca^{2+} in BUTX-induced fusion. Other experiments were carried out using different molecular species of PC and PA, or liposomes obtained by sonication instead of by microinjection. The results obtained with liposomes containing phospholipids with acyl groups others than myristic acid are comparable to but not identical with those reported in Figure 1; a similar finding was made for liposomes prepared by different methods (data not shown). However, toxin-mediated fusion was always observed, irrespective of the acyl group composition and liposome preparation chosen.

The influence of both length and saturation degree of the phospholipid acyl chains on the rate of fusion has been interpreted, according to a "molecular shape" hypothesis, as a different ability to stabilize hexagonal or bilayer phase by different lipid molecular species (Cullis & De Kruijff, 1978; Scheule, 1987). As far as liposome preparation is concerned, we preferred the microinjection method of Batzri and Korn (1973) with respect to the sonication method, because of its higher reproducibility. Several authors stress the possibility that a different length of sonication, using various species of phospholipids, could produce liposomes with "membrane defects" in a different fashion (Lawaczek et al., 1976). This might be the cause of less reproducible results in experiments using Huang liposomes.

The qualitative trend of aqueous vesicle content intermixing, evoked by BUTX, is shown in Figure 2. The time course of Tb^{3+} fluorescence emission in the presence of a few micromoles of BUTX and equimolar Ca^{2+} is very similar to that observed in the presence of 7.5 mM Ca^{2+} alone. In both experiments, the fluorescence intensity reaches a maximum within 60 s and then declines slowly. The fast phase corresponds to the formation of DPA/Tb complex after liposome fusion, whereas the slow decrease of fluorescence intensity results from the dissociation of the complex, due to its release into bulk solution which contains EDTA and Ca^{2+}, and the entry of outside solution into fused vesicles (Wilschut et al., 1980). The leakage of the vesicle content was also tested by using liposomes containing DPA/Tb as described by Bentz et al. (1983). A rapid decrease of fluorescence (data not shown) in the presence of both Ca^{2+} and BUTX confirms the result obtained with two distinct populations. Although the Tb fluorescence method is probably the most reliable to study the kinetics of liposome fusion, we preferred to use the RET method in our experiments to avoid the problem of the direct interaction of Tb^{3+} with BUTX (Chu & Chen, 1989). Actually, Tb^{3+} and others lanthanides can mimic Ca^{2+} in the activation of phospholipase activity, and we cannot exclude that the affinity of the Tb^{3+} for the binding site of BUTX may overrule its affinity for EDTA. Because of the leakage of Tb, it will be very difficult to single out the effect of Ca^{2+} in the fusion process, if measured with the Tb/DPA system.

The results obtained with the fluorescence techniques are supported by experiments in which ESR spectroscopy is used

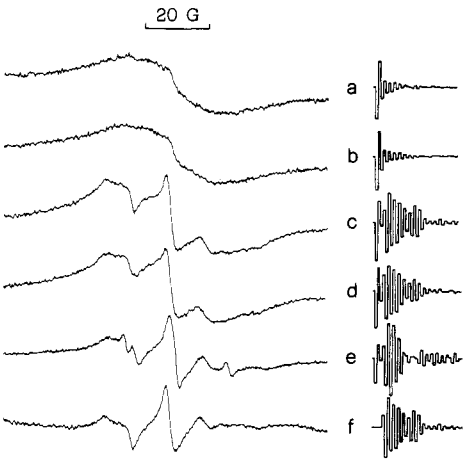

FIGURE 3: ESR spectra of spin-labeled PC liposome (left) and excerpts of their corresponding Fourier transformation (right). The appearance of the unbroadened spin-label signal in the ESR spectrum is indicative of liposome fusion. Samples containing labeled and unlabeled liposomes were prepared and measured as described under Experimental Procedures. (a) 0.3 mM Ca^{2+}; (b) 80 pmol of BUTX; (c) 80 pmol of BUTX + 0.3 mM Ca^{2+}; (d) 5 mM Ca^{2+}; (e) 600 pmol of BUTX + 0.3 mM Ca^{2+}: the sharp lines belong to the fraction of the hydrolysis product 5-doxylstearate present in the aqueous phase; (f) spectrum of unbroadened dPC in fused liposomes, obtained by deleting low-frequency components from spectrum c. The Fourier-transformed spectra are intended only for qualitative comparisons, and only the relevant part of the frequency range is shown.

to follow the fusion process. In analogy with the RET assay, the measurement is based on the spectral changes occurring when probe molecules are diluted as a consequence of vesicle fusion; however, due to the different nature of the two types of spectroscopy, the information obtained from phospholipid spin-labels can be complementary to that obtained from the fluorescent probe.

The ESR spectrum of liposomes containing 80% spin-labeled PC is a single broad line wthout distinct features (Figure 3a), due to the strong magnetic interaction between adjacent nitroxide groups. Upon fusion with normal liposomes, the labels will mix with the unlabeled phospholipids by lateral diffusion in the membrane. As a result, the characteristic anisotropic three-line signal from a magnetically dilute spin-label appears in the ESR spectrum, superimposed on the broad line (Figure 3c). The same changes can occur by direct lipid transfer between the vesicles (Kanda et al., 1982), but under our experimental conditions, no variation in the spectrum is seen in the absence of BUTX. As a control of our experimental system, we can induce the same fusion process by addition of high concentrations of Ca^{2+} (Figure 3d) instead of the toxin. It should be emphasized that the small amount of Ca^{2+} necessary for the fusogenic activity of BUTX does not show any effect by itself and that the toxin does not cause any spectral change in the absence of Ca^{2+} (Figure 3b).

The phospholipase activity of BUTX could be evidenced when the protein was used at high concentrations; under these conditions, three sharp lines appear in the ESR spectrum (Figure 3e). These lines are characteristic of a spin-label moving isotropically in aqueous solution; they are caused by spin-labeled stearic acids released into the medium due to BUTX-mediated hydrolysis of the labeled phospholipids. Although the water/lipid partition coefficient of the stearic acid spin-labels strongly favors the location in the membrane (Gaffney et al., 1983), a significant water signal is seen here because of the low lipid concentration used. In contrast, the membrane part of the stearic acid label signal cannot be distinguished from the phospholipid spin-label spectrum.

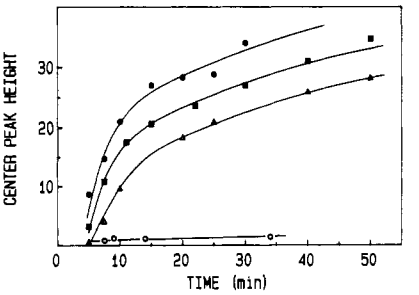

FIGURE 4: Time course of liposome fusion measured by ESR. The increase of the center peak height (arbitrary units) in the spectrum of fused dPC was determined as shown in Figure 3. The reaction was followed in the absence (○) or presence of 20 (△), 80 (■), or 160 pmol (●) of BUTX. All samples contained 0.3 mM Ca^{2+}; other experimental conditions were as described under Experimental Procedures.

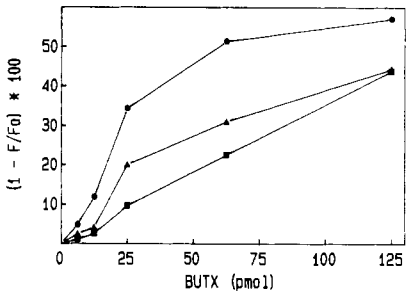

FIGURE 5: Rate of fusion of DMPC/PA (80:20) liposomes as a function of BUTX concentration, assayed by RET. Experiments were made as described in Figure 1. Fusion between vesicles was started by the addition of 0.3 mM $CaCl_2$ and BUTX. The process was stopped by addition of 1 mM EGTA after 30 (■), 60 (▲), or 180 s (●).

The broadened and unbroadened spectral components are separated by Fourier-transform data manipulation, and the height of the center peak of the unbroadened spectral component (Figure 3f) is used as a direct measure of the progress of the fusion process. In Figure 4, the fusion-dependent increase in ESR signal height is shown after addition of BUTX and equimolar Ca^{2+} to a suspension of labeled and unlabeled liposomes. The time course of fusion is biphasic; the fast initial phase is not resolved very well under our experimental conditions. This part corresponds to the actual fusion process and is equivalent to the results obtained with the RET assay. (Figure 1). The slow phase is found to be substantially unaffected by the BUTX concentration, indicating that this phase does not reflect the initial events of fusion. The slow increase must be due to a further rearrangement of the labeled phospholipids in the fused system, and may also reflect the production of membrane-bound labeled fatty acids by the toxin. Similar kinetics have been reported in other fusion studies using spin-labels (Kuroda et al., 1980, 1985). In the absence of Ca^{2+}, no fusion can be detected, in agreement with the result from the RET experiment.

The rate of BUTX-induced fusion in liposomes of DMPC/PA as measured by RET as a function of toxin concentration is reported in Figure 5. In fluorescence experiments, we have taken into account only the BUTX concentrations able to evoke extensive lipid intermixing in relative short times, namely, between 30 s and 3 min: this is to avoid the problem of the occurrence of other events besides fusion, i.e., lipid exchange. The Ca^{2+} requirement for the fusion events in the absence or presence of toxin is described in Figure 6. It is clear that the toxin lowers by 2 orders of magnitude the concentration of Ca^{2+} needed to induce fusion. Moreover, divalent ion chelating agents like EGTA and EDTA inhibit

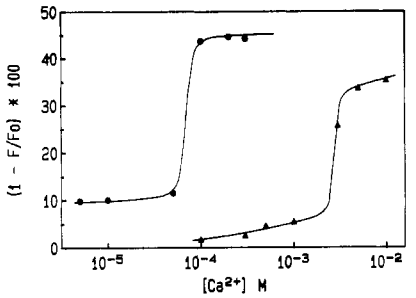

FIGURE 6: BUTX reduces the Ca^{2+} concentration needed to trigger liposome fusion. Experiments were done as described in Figure 1 except for the absence of EGTA. Lipid intermixing was monitored after 3 min by RET as described under Experimental Procedures in the absence (▲) or presence of 62 pmol of BUTX (●).

Table I: Correlation between Fusogenic and Phospholipasic Activities of BUTX[a]

liposome system	% activity: fusogen	% activity: phospholipase
PC/PA liposomes		
no addition	2	3
+BUTX	9	5
+BUTX + 0.1 mM Ca^{2+}	100	100
+BUTX + 0.1 mM Mg^{2+}	2	3
+BUTX + 0.1 mM Gd^{3+}	120	108
+BUTX + 0.1 mM Tb^{3+}	118	110
+BUTX + 0.1 mM Ca^{2+} + 0.2 mM EGTA	0	4
+BUTX + BSA + 0.1 mM Ca^{2+}	97	94
+pBPB-treated BUTX + 0.1 mM Ca^{2+}	8	6
PC liposomes		
+BUTX + 0.1 mM Ca^{2+}	9	5
+BUTX + 5.0 mM Ca^{2+}	ND[b]	6

[a] Fusion was determined with the RET assay using 60 pmol of BUTX and 50 μmol of total lipids in a sample volume of 2.5 mL; phospholipase activity was measured by using 1-[^{14}C]arachidonyl-stearoyl-PC as described under Experimental Procedures. The BSA concentration was 100 μg/mL. All activities were measured after 5 min of incubation at 28 °C. [b] Not determined.

completely the process (Table I). Other divalent ions such as Mg^{2+} are unable to induce BUTX-mediated fusion, but even traces of Ca^{2+}, present as impurities in twice-distilled water or in protein preparations, are sufficient to trigger detectable liposome fusion.

In Figure 7, we report the correlation between increasing PA content in the liposomes and the ability of Ca^{2+} or BUTX to provoke fusion. In our liposome system, increasing PA levels up to 20% molar produces a proportional rise in Ca^{2+}-induced vesicle fusion. Even at a very low PA:PC ratio (0.01) both Ca^{2+} and BUTX exhibit a residual fusogenic activity, most likely due to the perturbing presence of NBD-PE in the liposomes used for RET experiments.

The enzyme activity of BUTX can be quantified by the production of free fatty acids during fusion process. A time course of the phospholipase activity (Figure 8) closely parallels the kinetics of the fusion process, suggesting that fatty acids or lysophosphatides play an important role in the mechanism of fusion.

The fusogenic and enzymatic activities of BUTX are compared in Table I. Under our conditions, both activities of the toxin seem to be regulated in the same way. It is remarkable that lanthanides such as Tb^{3+} and Gd^{3+} can mimic the Ca^{2+} effects, both in modulating the enzymatic activity and in triggering liposome fusion (Table I). The treatment of BUTX with pBPB, a reagent able to inhibit the enzymatic activity of pancreatic PLA_2 by modifying a histidine residue in the active site (Volwerk et al., 1974), abolishes both the fusogenic

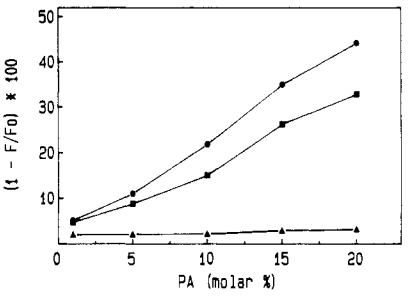

FIGURE 7: Effect of PA on DMPC–liposome fusion induced by BUTX. Experiments were performed as described in Figure 1. Fusion was started by the addition of 124 pmol of BUTX + 0.3 mM Ca^{2+} (●) or 0.3 mM Ca^{2+} (▲) or 5 mM Ca^{2+} (■), and lipid intermixing was monitored after 60 s by RET as described under Experimental Procedures.

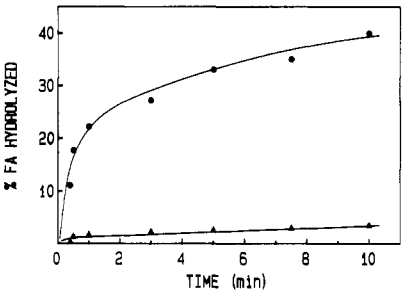

FIGURE 8: Time course of BUTX-induced PC hydrolysis. Liposomes containing PC/PA (80:20), at a final lipid concentration of 60 nmol in 2.5 mL, were incubated with 62 pmol of BUTX in 20 mM Tris buffer (pH 7.4) containing 0.1 mM EGTA, in the presence (●) or absence (▲) of 0.3 mM Ca^{2+}. Reaction was stopped by the addition of a mixture of chloroform/methanol (1:2), and products of hydrolysis were determined as described under Experimental Procedures.

and enzymatic activity of BUTX. In Table I, the relation between the presence of PA in liposomes and enzymatic activity of BUTX is also shown: no detectable phospholipid hydrolysis and only insignificant fusion between liposomes are observed in PA-free vesicles.

Discussion

Fluorescent probes have been often used for studies on membrane fusion (Pagano et al., 1981). Basically, two different approaches exist, based either on the release of intravesicular water-soluble probes or on the intermixing of membrane components. The experiments carried out with the DPA/Tb method clearly show that addition of nanomolar BUTX concentrations leads to fusion of liposomes with a concomitant intermixing of their aqueous contents (Figure 2). However, this method is not very suitable for studies on the mechanistic aspects of the fusion process, because the active site of the toxin has a high affinity for Tb^{3+} (Chu & Chen, 1989) and the Tb–BUTX complex formed is highly active (Table I); leakage of Tb^{3+} from the liposomes will thus give rise to Ca-independent fusion. To avoid the problems of an assay that interferes with the system under study, we have chosen the RET type of assay; however, this assay can be misleading due to fusion-independent effects (Wharton et al., 1986; Mac Donald, 1987) and does not exclude the possibility of vesicle aggregation. Furthermore, there might be some interference of the polar head groups of the probes, even if present at low concentrations. We have therefore confirmed the fluorescence results on intermixing of liposome lipids by an independent spin-label ESR method. This method has been used for fusion studies by the group of Ohnishi (Kuroda et al., 1980, 1985; Maeda et al., 1981), but problems occur due to the superposition of two changing spectral components.

Fourier-transform manipulation in ESR has previously been applied to separate high-frequency components (Pedersen et al., 1988); here we find that the same procedure is excellent to resolve the two phases seen during fusion measurements. The spin-label method in itself has several shortcomings: the amount of label in the membranes is extremely high, up to 80% on a molar basis, and the time resolution is low, due to the low overall lipid concentrations used which makes long measurement times necessary. ESR is therefore not suited for quantitative studies on the initial fusion events or the kinetics of the process, while in contrast the RET approach permits a continuous monitorage. However, the ESR experiments complement the fluorescence results, since the ambiguities of the RET measurements, such as vesicle aggregation, do not occur with the spin-labels. In particular, possible artifactual effects of the probe molecules are less likely when two different spectroscopic techniques give the same qualitative answer (Ford et al., 1982).

Having established the fusogenic capacity of BUTX, the obvious question is through which mechanism this activity proceeds. Two aspects of the toxin properties are relevant; the first is the specific requirement for Ca^{2+}, and the second is the phospholipase activity that leads to the formation of lysophospholipids. From our data, it is clear that there is a correlation between enzymatic activity and fusogenic properties of BUTX: the phospholipid hydrolysis proceeds with kinetics very similar to the ones of the fusion reaction (Figures 1 and 8), and every event that reduces or inhibits the phospholipase activity also reduces or inhibits the fusogenic activity of the toxin in a comparable fashion (Table I). In agreement with this observation is the finding that bee venom phospholipase A_2 can mimic both phospholipasic and fusogenic behavior of BUTX in the same liposome system (results not shown). This suggests that lysophosphatides or fatty acids could play a crucial role in the fusion process. Lysophosphatides might induce a nonbilayer structure in specific membrane domains and thereby lower the energy barriers for intermixing of the constituents of closely adjacent membranes. The ability of lysophosphatidylcholine micelles to induce fusion of cell membranes is in fact well established (Howell & Lucy, 1969). On the other hand, it has been reported that even after extensive hydrolysis of membrane phospholipids, the bilayer conformation is maintained (Jain & De Haas, 1981). We failed to demonstrate in the present study that a specific removal of fatty acids and lysophosphatides by fatty acid free bovine serum albumin alters the fusogenic properties of BUTX (Table I). Furthermore, the addition of EGTA or EDTA at any moment during the toxin action immediately blocks the fusion process, thus indicating that the lyso derivatives formed are not by themselves capable of continuing the process. It can be concluded that the presence of phospholipid hydrolysis products, and the formation of lysophospholipid membrane domains with consequent defects in the bilayer structure, is not sufficient to explain the action of the toxin.

Several experiments were made to understand the role of Ca^{2+} in the fusogenic events caused by BUTX. The fusion of liposomes induced by high Ca^{2+} concentrations was spectroscopically indistinguishable from the toxin-mediated fusion; in fact, the action of BUTX might be described as a lowering of the Ca^{2+} concentration threshold in order to trigger liposome fusion. The Ca^{2+} requirement of the toxin is one of the major unresolved problems. Ca^{2+} bound to the high-affinity binding site on the A subunit is essential for the activity (Abe et al., 1977), and cation chelators block both fusogenic and enzymatic activities of BUTX. Moreover, Ca^{2+} concentrations giving a Ca^{2+}:toxin ratio near to 1 are still able to trigger fusion. Finally, the Ca^{2+} requirement is very specific; other divalent cations, like Mg^{2+}, fail to stimulate phospholipase activity or toxin-mediated fusion. It is interesting that Gd^{3+} and Tb^{3+} can activate both toxin activities in our system; in fact, lanthanides are known to be able to mimic Ca^{2+} effects in several biological events (Martin & Richardson, 1979; Chu & Chen, 1989; Bentz et al., 1988). However, Ca^{2+} not only is important for catalytic activity but also seems to be necessary for the interaction of the toxin with its biological target. Abe et al. (1977) demonstrated that toxin binding to the nerve terminal is regulated by Ca^{2+}, and both binding and enzymatic activity were inhibited by treatment with pBPB. There are several ways to envisage this aspect of BUTX function: (i) the toxin could bind to different vesicles and act as a bridge to bring in contact membrane patches rich in acidic phospholipids; the particular charge distribution of the protein could be in favor of such a mechanism; (ii) Ca^{2+} could interact with phospholipids and allow BUTX binding; (iii) Ca^{2+} could stabilize particular membrane conformations needed for liposome fusion and induced by the toxin–phospholipase activity. In this connection, it could be relevant that for a large number of virus types, Ca^{2+} or other divalent cations are required for virus–cell fusion; otherwise, cell lysis rather than fusion occurs (Hart et al., 1976).

Our results show a correlation between the phospholipase activity of BUTX and the presence of acidic phospholipids in PC liposomes. In mixed phospholipid vesicles, PA seems to be at least partially segregated from PC (Galla & Sackman, 1975), and addition of divalent cations increases the extent of phase separation drastically (Massari & Pascolini, 1977). Such phosphatidic acid rich membrane patches could represent the toxin binding site in our liposomes. These results fit with those obtained by Bon's group, which report maximal BUTX binding in micelles of anionic detergent, while little or no binding is observed in nonionic detergent micelles (Radvanyi et al., 1987).

Unfortunately, the dependence of both PC hydrolysis and fusion rate on the PA concentration in the liposomes does not answer the question whether acidic phospholipids are really needed for the fusion event; in other words, it is unclear whether the presence of acidic phospholipids is needed only for toxin binding or its important also for successive steps of the fusion. It is well-known that PA can form a H_{II} nonbilayer structure in the presence of "high concentrations" of divalent cations (Verkleij et al., 1982); this was proposed as an early event in the fusion process. In liposomes composed of PC only, the toxin did not show any effect; however, in these liposomes, also Ca^{2+} and Mg^{2+} failed to trigger fusion, even at high concentrations (>10 mM). It has been reported that lysophospholipids do not act as fusogens in a system of pure dipalmitoyl-PC liposomes (Howell & Lucy, 1969), a further indication that negative surface charges are involved in the fusion events. Alternatively, the only PA involvement in the BUTX-mediated fusion event could be its receptorial function: the phospholipase ability of lipid–surface dehydration (Jain & Vaz, 1987) and lipid perturbation might be sufficient to trigger fusion.

The main conclusive finding of the present study lies in the qualitative agreement between the RET and ESR techniques, which demonstrates unequivocally that BUTX does induce fusion of liposomes at physiological pH. Although the results do not resolve the details of the fusion mechanism, we can conclude that the fusogenic effect of the BUTX is mediated by a complex interplay between BUTX–Ca^{2+}–membrane in-

teractions and, probably, the local formation of lysophospholipid.

The above-mentioned considerations of BUTX action are valid for liposomes, and of course cannot be extrapolated directly to in vivo conditions without experimental evidence. However, it has been proposed (Akabas et al., 1984) that the fusion reaction of phospholipid vesicles will be topographically identical with exocytosis, and thus in spite of the difficulty of transferring results obtained with artificial membranes to in vivo conditions, we suggest that the enzymatic activity, and the accompanying fusogenic effect, plays a pivotal role in the initial phase of the neurotoxic function of BUTX. The binding sites in the presynaptic membrane (Rehm & Betz, 1983, 1984) could place the toxin in the condition to exert the enzymatic activity in a crucial domain of the synapse. An ESR study of the interaction of a BUTX-like toxin (*Crotalus scutulatus* toxin) with synaptosomal membranes demonstrated a partial penetration of the phospholipase subunit into the bilayer only when both subunits were present; otherwise, the B subunit alone was unable to perturb the membrane lipids (Harris et al., 1983). The BUTX mechanism of neurotoxicity could be hypothesized as follows: (i) the A subunit acts to direct the toxin to a proteic binding site near to a "critical" point of the nerve terminal; (ii) the B subunit hydrolyzes phospholipids in this "critical" point near to or at the active zone; (iii) products of hydrolysis (i.e., lysoderivatives and/or fatty acids) trigger the fusion of neurotransmitter vesicles with the nerve terminal membrane. In our experimental system, the PA could mimic the proteic binding site present in the nerve terminal. Rugolo et al. (1986) suggested that the toxicity of BUTX could be accounted for by site-specific phospholipase-induced permeabilization of the membrane, in analogy with the effects caused by the diphtheria toxin (Lai et al., 1984). We do not exclude this possibility, but our results suggest that membrane fusion is involved in the initial enhancement of neurotransmitter release. It is likely that increased membrane permeabilization could be responsible for the slow phase of BUTX action, where transmitter release is completely blocked.

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Registry No. BUTX, 12778-32-4; DMPC, 18194-24-6; Ca, 7440-70-2; phospholipase A_2, 9001-84-7.

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Evidence for an *S*-Farnesylcysteine Methyl Ester at the Carboxyl Terminus of the *Saccharomyces cerevisiae RAS2* Protein†

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ABSTRACT: The protein products of yeast and mammalian *ras* genes are posttranslationally modified to give mature forms that are localized to the inner surface of the plasma membrane. We have previously demonstrated that the mature form of the *Saccharomyces cerevisiae RAS2* gene product is methyl esterified at a modified C-terminal cysteine residue. Here we provide evidence that this residue is an *S*-farnesylcysteine α-carboxyl methyl ester. This result establishes common posttranslational modifications for *RAS* proteins and fungal sex factors. These polypeptides exhibit sequence similarities at their C-termini that appear to be the critical recognition elements for a common set of modification enzymes. In mammalian cells, proteins with analogous C-terminal sequences appear to be prenylated and carboxyl methylated by a similar mechanism.

Peptides and proteins translated with C-terminal sequences including a cysteine residue in the fourth position from the terminus are candidates for a series of modification reactions including lipidation, proteolysis, and methyl esterification (Clarke et al., 1988). For example, the genes for the **a** mating factors from *Saccharomyces cerevisiae* encode C-terminal sequences of Cys-Val-Ile-Ala (Brake et al., 1985). The mature form of this factor, however, is lacking the terminal three amino acids, and the newly exposed cysteine residue is farnesylated at the sulfhydryl group and methylated at the carboxyl group (Betz et al., 1987; Anderegg et al., 1988). A similar sequence is present in the gene for the mating pheromone of the basidiomycetous yeast *Rhodosporidium toruloides* (Akada et al., 1989), and a similar C-terminal structure is present, although no evidence has been presented for its methyl esterification (Kamiya et al., 1978). In other proteins where terminal Cys-Xaa-Xaa-Xaa sequences are encoded by the gene, evidence has been obtained for at least some of these modification reactions. In the retinal cGMP phosphodiesterase, a C-terminal cysteine methyl ester has been demonstrated in a membrane binding domain (Ong et al., 1989). Members of the nuclear lamin family have been reported to be methyl esterified at unidentified sites (Chelsky et al., 1987), and evidence has been presented consistent with the farnesylation of the terminal cysteine residues (Beck et al., 1988; Wolda & Glomset, 1988; Farnsworth et al., 1989; Vorburger et al., 1989a). Additionally, several other peptides and proteins have been shown to be either isoprenylated at cysteine residues and/or methyl esterified at C-terminal cysteine residues where no information is available on the original coding region. These include the peptidyl sex factors from the jelly fungi *Tremella mesenterica* (Sakagami et al., 1981) and *Tremella brasiliensis* (Ishibashi et al., 1984), as well as mammalian small molecular weight G-proteins (Yamane & Fung, 1989) and potentially similar species (Backlund & Aksamit, 1988; Ota & Clarke, 1989).

We have been interested in this type of posttranslational processing in the mammalian and yeast ras protooncogene products, all of which contain Cys-Xaa-Xaa-Xaa C-terminal sequences (Barbacid, 1987; Santos & Nebreda, 1989). Evidence has been presented for the methyl esterification of the mammalian H-ras (Clarke et al., 1988) and N-ras (Gutierrez et al., 1989), as well as yeast *RAS2* protein (Deschenes et al., 1989), and for the isoprenylation of mammalian H-ras, K-ras,

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