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MEMBRANES UNDERGOING PHASE TRANSITIONS ARE PREFERENTIALLY HYDROLYZED BY BETA-BUNGAROTOXIN

PETER N. STRONG and REGIS B. KELLY

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, Calif. 94143 (U.S.A.)

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Summary

β-Bungarotoxin preferentially hydrolyzes choline phospholipids (dilauroyl, dimyristoyl, dipalmitoyl) at their respective gel to liquid crystalline phase transition temperatures. Cholesterol markedly reduces the rate of phospholipid hydrolysis; at 0.33 mol percent cholesterol:phospholipid, the toxin's phospholipase activity is completely inhibited.

Certain hydrolytic enzymes (e.g. proteases, nucleases) have long been powerful tools for the structural biochemist. In comparison, the potential of phospholipases as probes of membrane structure is only just being realized. Studies with phospholipase A_2 have provided some of the most convincing evidence for the existence of an asymmetric distribution of phospholipid across the erythrocyte membrane [1—5]. In addition op den Kamp et al. [6, 7] have shown that pancreatic phospholipase A_2 preferentially hydrolyzes phosphatidylcholine liposomes at the transition temperature (T_m) of the phospholipid. They have explained the phenomenon using the concept of lateral compressibility [8] suggesting that the pancreatic enzyme requires widely spaced lipid molecules at the interface, a situation that is maximally realized at the transition temperature.

β-Bungarotoxin, the presynaptic neurotoxin isolated from the venom of the krait *Bungarus multicinctus* [9,10] has recently been shown to possess phospholipase activity [11—13]. Although phospholipases do not normally modify transmitter release [13,14], electrophysiological and anatomical evidence indicates that β-bungarotoxin affects transmitter release by selective hydrolysis of nerve terminal phospholipids [13,14]. We have studied the substrate requirements of β-bungarotoxin to gain some insight into the possible structure of presynaptic plasma membranes. We have found that β-bungarotoxin

hydrolyzes phosphatidylcholine bilayers optimally at their transition temperature between liquid and gel phases. We have also demonstrated that the presence of cholesterol inhibits phospholipid hydrolysis, a finding which can explain some of the selectivity of toxin action.

Table I demonstrates that β-bungarotoxin preferentially hydrolyzed dilauroyl, dimyristoyl and dipalmitoyl phosphatidylcholines at their respective liquid crystalline transition temperatures. The lack of hydrolysis above the transition temperature of dipalmitoyl phosphatidylcholine is not due to thermal inactivation of the enzyme. In the presence of the detergent sodium deoxycholate, (4 mM), which serves to disperse the lipid substrate [11], dipalmitoyl phosphatidylcholine was completely hydrolyzed by β-bungarotoxin in 15 min at 58°C. In the absence of deoxycholate, the specific activity of the phospholipase was very low (1.9 μequiv./min/mg protein), compared with regular assay conditions in the presence of detergent (250 μequiv./min/mg protein) (Strong and Kelly, in preparation), using dimyristoyl phosphatidylcholine as a substrate. For reasons not yet understood, dimyristoyl phosphatidylcholine liposomes were the best substrate for the toxin when absolute rates of phospholipid hydrolysis were compared.

Table I also demonstrates that β-bungarotoxin preferentially hydrolyzed a binary mixture of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, not at their individual transition temperatures but at an

TABLE I

HYDROLYSIS OF PHOSPHATIDYLCHOLINE LIPOSOMES BY β-BUNGAROTOXIN AT DIFFERENT TEMPERATURES

Dilauroyl, dimyristoyl, and dipalmitoyl phosphatidylcholine were obtained from Sigma Co. [1-^{14}C]-Dimyristoyl phosphatidylcholine was obtained from Applied Science. [1-^{14}C]Dipalmitoyl phosphatidylcholine was obtained from New England Nuclear. Hand-shaken phosphatidylcholine liposomes (2 μmol phospholipid/ml) were dispersed in 100 mM NaCl, 5 mM $CaCl_2$, 0.05 mM EDTA and 100 mM Tris·HCl, pH 7.6. A 200 μl aliquot of liposomes (400 nmol) was incubated with β-bungarotoxin (8 nmol) at the temperatures indicated for either 15 min (dimyristoyl phosphatidylcholine), 30 min (dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine (1:1)) or 2 h (dilauroyl phosphatidylcholine). Incubations were terminated with 1 ml 0.5 M EDTA. If EDTA was added to the incubation mixture before toxin, no hydrolysis occurred. Phospholipids were extracted and chromatographed as previously described [13]. Reaction products were scraped off the thin-layer chromatography plate and either assayed for lipid phosphorus or suspended in Aquasol (New England Nuclear) and counted in a liquid scintillation spectrometer (Beckman LS-233) with an efficiency of 86%. Both methods gave essentially the same results.

Phosphatidylcholine	T_m (°C)	Incubation temperature (°C)	Percent phospholipid hydrolysis*
Dilauroyl phosphatidylcholine	0	0	95
		15	<5
Dimyristoyl phosphatidylcholine	23	15	<5
		23	60
		31	8
Dipalmitoyl phosphatidylcholine	41	31	9
		41	52
		58	7
Dimyristoyl/dipalmitoyl phosphatidylcholine (1:1)		23	<5
		31	45
		41	10

*The percent phospholipid hydrolysis is the average of three determinations. Triplicate assays were reproducible within 5%.

intermediate temperature (31°C) which is predicted to be the approximate mid-point of the phase transition for an equimolar mixture [15,16]. There was correspondingly little hydrolysis of the mixture at either 23°C (T_m (dimyristoyl phosphatidylcholine)) or 41°C (T_m (dipalmitoyl phosphatidylcholine)). We conclude that the unusual temperature requirements are a property of the substrate, and not the enzyme.

The presence of cholesterol in phosphatidylcholine liposomes markedly reduced the rate of hydrolysis (Table II). At a cholesterol:phospholipid mol ratio of 0.33, hydrolysis of dipalmitoyl phosphatidylcholine was negligible at all temperatures measured. Lower concentrations of cholesterol reduced the rates of hydrolysis, although maximal hydrolysis still occurred at the transition temperature.

The observation that cholesterol containing membranes are poor substrates for the toxin's phospholipase activity may allow us to reconcile earlier findings with the toxin. Mitochondria with a cholesterol:polar lipid mol ratio of 0.03 [17], are inactivated by a 5 min exposure at 25°C to 2 nM β-bungarotoxin [18], sarcoplasmic reticulum membranes (mol ratio = 0.05) show reduced calcium accumulation after exposure to as little as 1 nM β-bungarotoxin for 20 min [19]; bacterial membranes which presumably lack cholesterol are good enzyme substrates [12]; red blood cells with a high cholesterol content (mol ratio = 1.0) are not lysed by 4 h exposure to 5000 nM toxin at 37°C [14]. For comparison, neuromuscular transmission is blocked when the frog neuromuscular junction is exposed to 10 nM β-bungarotoxin, for 2 h at 25°C (Strong and Kelly, unpublished observations). Schwann cell membranes (cholesterol:polar lipid mol ratio = 0.95) have an unaltered morphology even in the presence of 1000—5000 nM β-bungarotoxin for two hours; the nerve terminal membranes disintegrate under these conditions [14,20].

Pancreatic phospholipase A_2 and β-bungarotoxin are similar in that both prefer deoxycholate-solubilized substrates, but will nevertheless hydrolyze phospholipids in liposomes provided that the phospholipids are near their transition temperatures, and that cholesterol is absent [6,7]. Since the natural substrates of pancreatic phospholipase A_2 are probably bile salt-solubilized phospholipids, the physiological significance of this anomalous temperature dependence in the case of the pancreatic enzyme is unclear. The natural substrate of β-bungarotoxin is however almost certainly an intact membrane [13,

TABLE II

EFFECT OF CHOLESTEROL ON β-BUNGAROTOXIN HYDROLYSIS OF DIPALMITOYLPHOSPHATIDYLCHOLINE LIPOSOMES

The experimental protocol was identical to that for Table I except that cholesterol and dipalmitoyl phosphatidylcholine were dissolved in $CHCl_3$:CH_3OH (2:1), evaporated to dryness and the process repeated twice before hand shaken liposomes were prepared by the usual procedure.

Mole ratio cholesterol:dipalmitoyl phosphatidylcholine	Percent dipalmitoyl phosphatidylcholine hydrolysis		
	31°C	41°C (T_m)	58°C
(No cholesterol)	9	52	7
0.18	5	10	<5
0.33	<5	<5	<5

14]; it is reasonable to enquire therefore what might be the significance of the toxin's enzymatic preference for membranes undergoing a phase transition. Hydrolysis of the 2-fatty acyl side chain of the phospholipid requires that the enzyme penetrate at least partially into the hydrophobic region of the membrane. Phillips et al. have shown preferential insertion of the hydrophobic protein, casein, into monolayers at their transition temperature [21]. In addition, van der Bosch and McConnell [22] have suggested that the hydrophobic region of the membrane may be partially exposed at boundaries between solid and fluid phases. Exposure of hydrophobic regions at phase boundaries might facilitate phospholipase action by allowing access to fatty acid chains. While few of the phospholipids in natural membranes may be at their transition temperatures at physiological temperatures, integral membrane proteins can induce phase boundaries in fluid membranes by having an annulus of tightly bound phospholipid, the hydrocarbon chains of which are not free to move. In natural membranes such phase boundaries might be the site of fusion [22] or of phospholipase A_2 action.

We have not yet succeeded in discovering why the plasma membranes of motor nerve terminals, in contrast to the surrounding Schwann cell membrane and postsynaptic muscle membranes, are highly sensitive to β-bungarotoxin. Possibilities include an unusually low level of cholesterol in nerve terminals, an unusually high concentration of phase boundaries or, as suggested previously [23], a protein recognition site to which the toxin binds. After binding, the toxin could hydrolyze phospholipids in the vicinity of this recognition site, perhaps at the phase boundary between the annular lipids of the recognition site and the surrounding lipid bilayer. The irreversibility of the toxin's action, saturation effects at high toxin concentrations [10,20] and the saturable binding of iodinated β-bungarotoxin to synaptosomes [23] encourage us to favor the latter possibility.

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