Tetanus Toxin Is Labeled with Photoactivatable Phospholipids at Low pH†

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Received June 27, 1985

ABSTRACT: The mechanism of cell penetration by tetanus toxin is unknown; it has been suggested that the toxin may penetrate into the lipid bilayer from a low-pH vesicular compartment. In this work, the interaction of tetanus toxin with liposomal model membranes has been studied by following its photoinduced cross-linking with either a nitrene or a carbene photolytically generated from corresponding light-sensitive phosphatidylcholine analogues. The toxin was labeled only at pHs lower than 5.5. The low pH acquired hydrophobicity of tetanus toxin appears to be confined to its light chain and to the 45-kDa NH_2-terminal fragment of the heavy chain. Negatively charged lipids promote the interaction of this toxin with the hydrocarbon chain of phospholipids. The relevance of the present findings to the possible mechanism of nerve cell penetration by tetanus toxin is discussed.

The anaerobic bacterium *Clostridium tetani* produces a powerful neurotoxin, which causes spastic paralysis by blocking neuromediator release at the presynaptic level (Mellanby & Green, 1981; Wellhoner, 1982). Tetanus toxin (TeTx)[1] is composed of two polypeptide chains, L (51.5 kDa) and H (95 kDa), linked by a single disulfide bridge (Craven & Dawson, 1973; Matsuda & Yoneda, 1975). Figure 1 shows a scheme of the structure of TeTx and the pattern of fragments obtained after selective papain cleavage (Helting & Zwisler, 1977).

Very little is known about the mechanism of TeTx action at the cellular level. It has been shown that the 50-kDa COOH-terminal fragment of the H chain, termed C, binds to gangliosides G_{D1b} and G_{T1b} as well as the intact TeTx does but lacks any biological activity (Goldberg et al., 1981; Rogers & Snyder, 1981). On the other hand, the B fragment composed of L chain and of the NH_2-terminal part of the H chain does not bind to gangliosides but is able to induce flaccid paralysis when injected into animals at relatively high concentrations (Helting et al., 1978). Hence, it has been suggested that the active part of TeTx is located within the L chain and/or the H_{45} fragment. However, no enzymatic activity so far has been demonstrated for TeTx or any of its fragments.

We have shown that at low pH TeTx or its B fragment increases the permeability of potassium-loaded asolectin vesicles, and the same activity was also demonstrated for diphtheria toxin (DT)[1] (Boquet & Duflot, 1982), which is known to enter the cell from a low-pH endosomal compartment (Olsnes & Sandvig, 1985). Both toxins form voltage-dependent channels in bilayer lipid membranes at low pH (Hoch et al., 1985).

These data and the inhibition of TeTx biological activity by lysosomotropic agents (Simpson, 1983), which block the acidification of endosomes and lysosomes, suggest that for TeTx, as well as for DT, an obligatory step in the intoxication process is its entry, after cell surface binding, into acidic compartments.

To test the possibility that at low pH TeTx becomes able to interact with the hydrophobic domain of the lipid bilayer, we have used a liposomal model system containing radioactive photoactivatable phospholipids. These lipid analogues are stable in the dark and can be interdispersed in trace amounts among the other components of the membrane. On ultraviolet illumination, the photoactive group is converted into a highly reactive intermediate able to cross-link to neighboring molecules, which then become radioactively labeled (Brunner, 1981; Bayley, 1983; Bisson & Montecucco, 1985a,b). Figure 2 shows the three phosphatidylcholine analogues used in the present study. They bear the photoactivatable group at two different levels of one fatty acid chain in order to probe both superficial and deeper regions of the membrane. These nitrene- and carbene-generating phospholipid probes have been tested in a variety of systems, where they have been shown to label only the lipid-exposed domain of membrane proteins (Bisson et al., 1979; Montecucco et al., 1981, 1983; Hoppe et al., 1983; Brunner et al., 1983).

Materials and Methods

Toxins and Fragments. Tetanus toxin, a kind gift of Dr. Labert (Institut Pasteur Production, Louviers, France), was

† J.B. is supported by a grant from the Swiss National Research Foundation, Bern. C.M. gratefully acknowledges the support of a short-term EMBO fellowship during part of this study.

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[1] Abbreviations: TeTx, tetanus toxin; L, light chain of tetanus toxin; H, heavy chain of tetanus toxin; DT, diphtheria toxin; SDS, sodium dodecyl sulfate; G_{D1b}, galactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-*N*-acetylneuraminylgalactosylglucosylceramide; EL, egg lecithin; DML, dimyristoyllecithin; MA, myristic acid; [^{3}H]PC I, 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine; [^{14}C]PC II, 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]phosphocholine; [^{3}H]PC III, 1-palmitoyl-2-[10-[4-[(trifluoromethyl)diazirinyl]phenyl]-8-oxa[9-^{3}H]decanoyl]-*sn*-glycero-3-phosphocholine; Tris, tris(hydroxymethyl)aminomethane.

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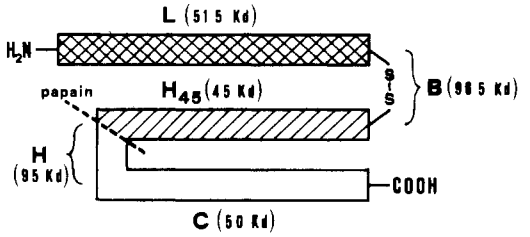

FIGURE 1: Schematic structure of tetanus toxin showing the site of papain cleavage and the nomenclature and molecular mass of the fragments.

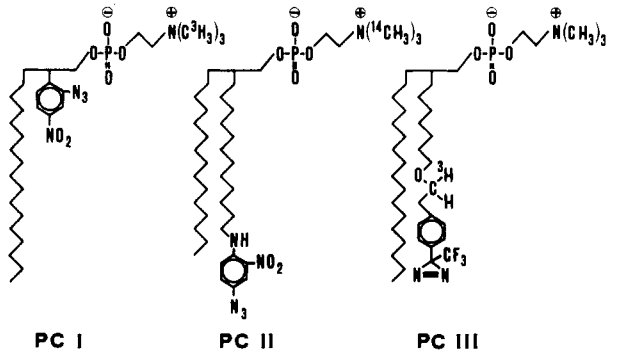

FIGURE 2: Structural formulas of the probes used in the labeling of TeTx.

further purified on DEAE-cellulose DE-52. Fragments B and C were prepared as described by Bizzini et al. (1981). The L subunit of tetanus toxin was purified according to Matsuda and Yoneda (1975) and carboxymethylated according to Chung and Collier (1977). All proteins were dissolved at 0.5–1.0 mg/mL [determined according to Lowry et al. (1951)] in 10 mM sodium phosphate buffer, pH 7.2, containing 100 mM NaCl and stored in aliquots at −80 °C.

Chemicals. Dimyristoyllecithin (DML), egg lecithin (EL), and soy bean asolectin (AL) were from Sigma. After purification according to Kagawa and Racker (1971), asolectin had the following phospholipid composition: phosphatidylcholine, 24%; phosphatidylethanolamine, 39%; phosphatidylserine, 19%; phosphatidic acid, 6%; phosphatidylinositol, 1%; lyso derivatives, 9%; other phospholipids, 2%. Myristic acid was purchased from Koch-Light (U.K.). Highly purified ganglioside G_{D1b} was a kind present of Dr. L. Poletti (Fidia Research Laboratories, Abano Terme, Italy). 1-Palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine ([^{3}H]PC I, sp act. 2.8Ci/mmol), 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]-phosphocholine ([^{24}C]PC II, sp act. 174 Ci/mol), and 1-palmitoyl-2-[10-[4-[(trifluoromethyl)diazirinyl]phenyl]-8-oxa[9-^{3}H]decanoyl-*sn*-glycero-3-phosphocholine ([^{3}H]PC III, sp act. 5 Ci/mmol) were prepared as previously described (Bisson & Montecucco, 1981; Brunner et al., 1983).

Preparation of Liposomes. All subsequent operations were performed under a red safety light. Liposomes were formed by mixing AL, DML, or EL (with MA or ganglioside G_{D1b} when present) with the probes [^{3}H]PC I [^{14}C]PC II or [^{3}H]PC III in chloroform/methanol (2:1), drying under a nitrogen stream, resuspending in ethyl ether, drying, and vacuum pumping for at least 1 h. The molar ratio of probes with respect to the other phospholipids varied in different experiments within the range 1:200 to 1:20 000. The lipids were resuspended at a final concentration of 4 μmol/mL phosphatidylcholines and, when present, 0.1 μmol/mL MA, 0.05 μmol/mL G_{D1b}, in 100 mM Tris–acetate buffer, pH 7.42 (at 20 °C), or in 10 mM citric acid–sodium phosphate buffer, pH 7.50 (at 20 °C), containing 140 mM NaCl, and sonicated in a stoppered vial under nitrogen with a bath-type sonicator (Laboratory Supplies, Hicksville, NJ) until optical clarity.

Photolabeling. A total of 15–40 μg of tetanus toxin (corresponding to a lipid to protein molar ratio varying from 20000 to 7500) or equivalent amounts of its fragments or subunits were incubated for 10 min with 0.5 mL of liposomal suspension at 20 °C. Since the amount of probe covalently bound to the protein after irradiation is dependent on the lipid to protein ratio and on the percentage of probe, these parameters were kept constant when the labeling with different liposomes was compared. pH was adjusted at the desired value by addition of 25% acetic acid or 7% phosphoric acid and the incubation prolonged for a further 5 min. When required, the pH was returned from 4.00 or 4.33 to 7.40 by addition of 2 M Tris. The samples were illuminated for 10 ([^{3}H]PC I and [^{14}C]PC II) or 20 min ([^{3}H]PC III) with a long-wave ultraviolet lamp (Minera-light, San Gabriel, CA) at room temperature with the protection of a glass–water filter. The protein was recovered by cold acetone precipitation as before (Tomasi & Montecucco, 1981) or by addition of 50% trichloroacetic acid to a final concentration of 5%. The pellets obtained after centrifugation were dissolved in 25–30 μL of 8% SDS, 3% β-mercaptoethanol, and 10% glycerol, and the pH was brought to a basic value with 2 M Tris as estimated from the change of color of bromophenol blue.

Electrophoresis and Radioactivity Determinations. The samples were subjected to SDS gel electrophoresis according to Kadenbach et al. (1983) in 13% polyacrylamide gels. After Coomassie Blue staining, destaining, and densitometric recording, the gels were cut in 1.8 mm thick slices. After overnight incubation with a tissue solubilizer (Packard Soluene 350) and a further 10 h with a scintillation cocktail, the slices were counted in a Packard Tri-Carb 300C scintillation counter. Radioactivity associated with the gel slices was estimated in dpm and corrected for differences in protein content among the different samples as determined by integration of the recorded Coomassie Blue peaks.

RESULTS

Hydrophobic Photolabeling of TeTx. Liposomal model systems have been widely used to study the membrane interaction of several proteic toxins (Duncan, 1984), and in many cases this approach has provided information relevant to their cellular mechanism of action (Olsnes & Sandvig, 1983). Previous results have suggested that TeTx could interact with the hydrophobic phase of the lipid bilayer (Boquet & Duflot, 1982; Boquet et al., 1984; Roa & Boquet, 1985; Hoch et al., 1985).

The membrane penetration of a protein can be most conveniently assayed by the covalent reaction of the protein with a membrane-sequestered radioactive photoactivatable probe (Brunner, 1981; Bayley, 1983; Bisson & Montecucco, 1985a,b).

Figure 3A shows the densitometric trace of a SDS–polyacrylamide gel, under reducing conditions, of TeTx incubated with asolectin vesicles containing [^{3}H]PC I and [^{14}C]PC II and illuminated with long-wave ultraviolet radiations. TeTx appears to be highly pure and well resolved into its two chains; it still contains a small amount of unreduced toxin. Figure 3B,C shows that both subunits become labeled only at low pH. The labeling occurs efficiently both at a superficial and at a deeper level of the lipid bilayer. The lack of labeling at neutral pH provides further and related evidence that these probes are exclusively restricted to the membrane.

The radioactivity associated with the L subunit is around

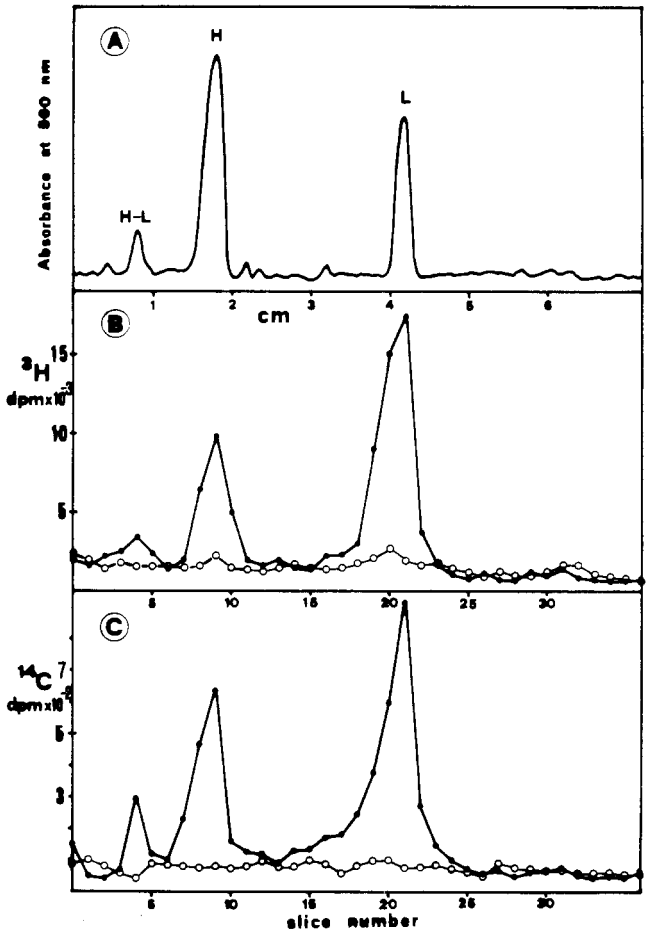

FIGURE 3: Patterns of labeling of TeTx with [^{3}H]PC I and [^{14}C]PC II at pH 7.42 and 4.33. (A) Coomassie Blue staining profile of TeTx (25 μg) run in a SDS–13% polyacrylamide gel after incubation with asolectin vesicles (1.5 mg, containing 15 × 10^6 dopm of [^{3}H]PC I and 0.5 dpm of [^{14}C]PC II, in 0.5 mL of Tris–acetate buffer) and ultraviolet illumination. The gel-associated radioactivity corresponding to samples illuminated at pH 7.42 (○) and pH 4.33 (●) is shown for [^{3}H]PC I in panel B and for [^{14}C]PC II in panel C. The gel was overrun to remove the phospholipids, which migrate just after the tracking dye bromophenol blue, and so lower the radioactivity background.

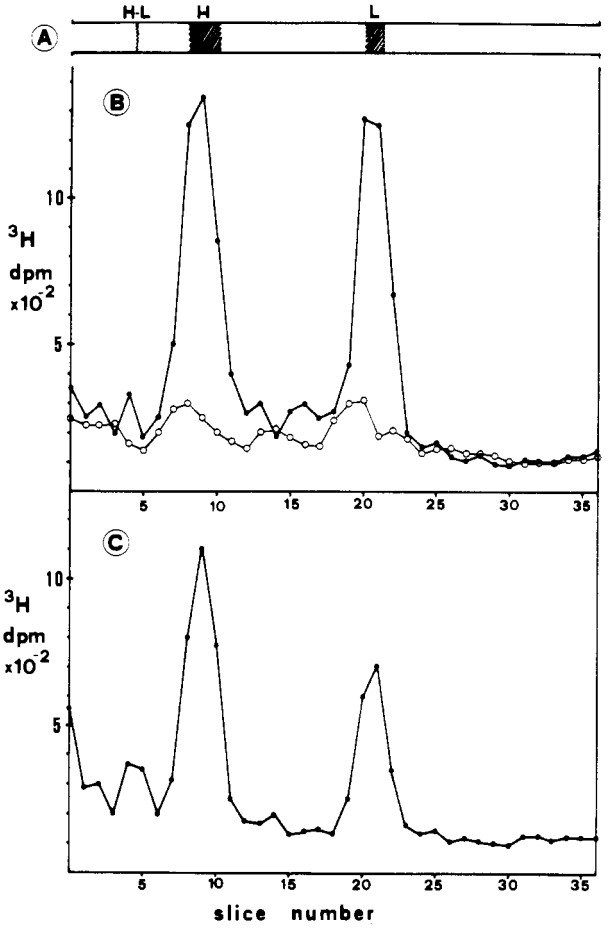

FIGURE 4: TeTx labeling profile with [^{3}H]PC III in asolectin and G_{D1b}–DML liposomes. (A) position of the protein bands (25 μg) on a 13% polyacrylamide gel stained with Coomassie Blue. (B) [^{3}H]PC III radioactivity bound to TeTx chains after incubation with asolectin liposomes (1.5 mg, containing 100 × 10^6 dpm of [^{3}H]PC III, in Trix–acetate buffer); closed circles refer to a sample illuminated at pH 4.33, while open circles show the result of an experiment in which the sample was first incubated for 5 min at pH 4.33 and then brought back at pH 7.40 for a further 5 min before ultraviolet irradiation. (C) [^{3}H]PC III radioactivity bound to TeTx chains after incubation with G_{D1b}–DML liposomes (2 μmol, containing 40 × 10^6 dpm of [^{3}H]PC III, in 0.5 mL of Tris–acetate buffer) and illumination at pH 4.33. G_{D1b}–DML molar ratio as under Materials and Methods.

65% of that linked to the whole TeTx (both with [^{3}H]PC I and [^{14}C]PC II). This differential labeling cannot be directly related to the extent of protein surface exposed to lipids by the two polypeptide chains, because of a possible difference in reactivity vs. the nitrene intermediate between the two chains. In fact, it has been shown that the yield of protein cross-linking to the nitroaryl nitrene is highly dependent on the kind of lateral residues present on the surface of the hydrophobic domain of integral membrane proteins (Bisson & Montecucco, 1985a,b; Hoppe et al., 1983). In agreement with this interpretation is the result shown in Figure 4A obtained with [^{3}H]PC III, which generates the more reactive, yet selective, (trifluoromethyl)aryl carbene (Brunner et al., 1983). With respect to the L chain, the H chain is more labeled with [^{3}H]PC III other than with [^{14}C]PC II (55% of the total radioactivity linked to TeTx compared with 35% in the previous experiment), suggesting that it does expose to lipids a surface at least as large as that of the L chain. Figure 4A also shows that the lipid interaction of the TeTx chains is largely reversible (more than 90%) when the pH is brought back from 4.33 to 7.40 before illumination. This holds true also for both [^{3}H]PC I and [^{14}C]PC II (not shown). The distribution of radioactivity among the toxin subunits is not dependent on the lipid to protein ratio, on the buffer used, or on the molar fraction of phospholipid probes.

Table I: Labeling of TeTx in Liposomes of Different Lipid Compositions Expressed as Percentage of Radioactivity Bound to Toxin When Incubated with Asolectin Vesicles[a]

	H chain	L chain
asolectin	100	100
EL	12–18	7–10
DML	7–10	10–14
DML + MA	35–39	20–26
DML + G_{D1b}	46–48	30–34

[a] A total of 25 μg of TeTx was incubated with 0.5 mL of Tris–acetate buffer, pH 4.33, containing 1.5 mg of purified soy bean asolectin and 0.6 × 10^6 dpm of PC II, at 20 °C.

Effect of the Lipid Composition on the TeTx–Liposome Interaction. The interaction of DT with liposomes is largely affected by their lipid composition; negatively charged lipids increase its insertion into the vesicles (Alving et al., 1980; Donovan et al., 1982; Montecucco et al., 1985).

Table I shows that phosphatidylcholine liposomes, which lack a net negative charge, incorporate much less TeTx than asolectin as seen by the amount of labeling. This can be at least partially attributed to the lack of net charge, since a

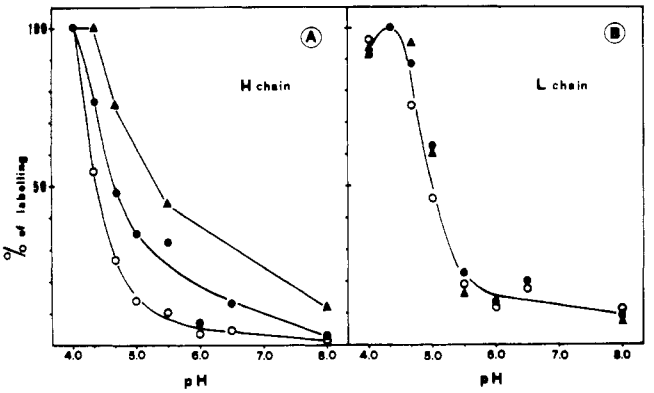

FIGURE 5: pH dependence of the TeTx subunits labeling with [^{3}H]PC I and [^{14}C]PC II. The amount of radioactivity associated with the two TeTx subunits in the whole toxin molecule incubated with asolectin liposomes, as determined by gel slicing and counting, is plotted as a function of pH. In order to compare different experiments in which different amounts of probes and varying lipid to protein ratios were used, the radioactivity is expressed as percentage of the maximal labeling obtained in each experiment. The average of three experiments in Tris–acetate buffer and one in citric acid–sodium phosphate buffer is reported. Subunit H is shown in panel A and subunit L in panel B. (○) [^{3}H]PC I, (●) [^{14}C]PC II, and (▲) [^{14}C]PC II in G_{D1b}–asolectin vesicles (molar ratio as under Materials and Methods).

negatively charged lipid as simple as myristic acid is able to increase the lipid interaction of TeTx. There appears to be a minor contribution of the fluidity of the lipid bilayer since the amount and pattern of labeling is very similar for egg lecithin and dimyristoyllecithin, which are respectively above and below their transition temperatures at 20 °C. This similarity also tends to exclude an influence of the double bonds of the unsaturated fatty acid chains present in egg lecithin and in soy bean asolectin. This latter result is expected on the basis of the low reactivity of aryl nitrenes vs. C–H bonds. G_{D1b}, which has been proposed to act as a receptor for TeTx, promotes its lipid interaction, but still is less effective than the asolectin lipid mixture. This effect cannot be attributed to a differential scavenging effect of the different head groups because any nitrene or carbene reaching the polar surface of the membrane would be effectively quenched by water. It is noteworthy that in the presence of G_{D1b} or MA the low pH induced labeling of TeTx with [^{14}C]PC II is more pronounced for H than for L chain. This is also clearly shown by the pattern of labeling of Figure 4B obtained with [^{3}H]PC III in DML–G_{D1b} liposomes, where 68% of the probe is bound to the H chain.

pH Dependence of the Hydrophobic Photolabeling of TeTx Subunits. Figure 5 shows the pH dependence of the TeTx chains labeling in the whole toxin molecule with [^{3}H]PC I and [^{14}C]PC II probes. The labeling of the H chain is greatly dependent on the pH below 5.5. Protein regions of different reactivity appear to interact with the two probes, since they do not fit the same curve. The L chain is maximally labeled at pH 4.33, and it shows a similar pH dependence for the two probes with a half-maximal labeling around pH 5. A further difference in the behavior of the two subunits with respect to their lipid interaction is shown by the effect of ganglioside G_{D1b}, which shifts to higher pHs the labeling curve of the H chain while it has no effect on the L chain.

Labeling of Isolated TeTx Fragments. As shown in Figure 1, tetanus toxin can be digested and resolved into different fragments; these fragments can be purified to homogeneity and possess some biological activities (Helting & Zwisler, 1977), and hence, their ability to interact with lipids has been tested in asolectin vesicles.

Table II: Labeling of TeTx and Its Subunits and Fragments with [^{14}C]PC II Expressed as dpm/mg of Protein[a]

labeled protein	pH	H	L	H_{45}	C
TeTx	4.33	75 000	277 500		
	4.66	47 370	244 200		
L	4.33		293 100		
	4.66		271 750		
B	4.33		267 450	82 300	
	4.66		239 200	52 800	
C	4.33				3730
	4.66				3450

[a] Conditions as in the legend of Table I.

Table II shows that at low pH the isolated L chain is labeled to the same extent as when it is part of the intact TeTx. However, a large fraction of the L chain (65%) appears to remain associated with lipids after returning the pH from 4.33 to 7.40 before illumination (data not presented). As shown above, this is not the case when L chain is part of the entire TeTx molecule. The amount of radioactivity associated at low pH with the H_{45} fragment is, within experimental error, equal to that of the intact H chain. Together with the absolute lack of labeling of C fragment, this result is in close agreement with previous data indicating that the lipid interaction of the H chain is restricted to its NH_2-terminal fragment H_{45} (Boquet & Duflot, 1982; Boquet et al., 1984; Roa & Boquet, 1985).

DISCUSSION

Several lines of evidence suggest that the two most powerful neurotoxins, secreted by bacteria of the *Clostridium* genus, namely, botulinum and tetanus toxins, are very similar in their mechanism of action at the presynaptic level (Simpson, 1981). Recently, it has been shown that botulinum toxin enters into the nerve terminal (Dolly et al., 1984), and this step is required for the toxic activity to be exerted. It is very likely that botulinum toxin is taken up by endocytotic vesicles. The internal pH of endosomal compartments has been estimated to be as low as 5 or less (Steinman et al., 1983). TeTx at low pH becomes able to interact with lipids as assessed by its effect on the permeability of potassium-loaded asolectin vesicles (Boquet & Duflot, 1982; Boquet et al., 1984). Moreover both botulinum and tetanus toxins increase the conductance of bilayer lipid membranes at low pH (Hoch et al., 1985).

The present results show that TeTx at low pH becomes able to interact with the fatty acid portion of the lipid bilayer. Both the H and L subunits are labeled with two reagents ([^{14}C]PC II and [^{3}H]PC III) localized in the hydrophobic core of the lipid bilayer and by a phospholipid analogue ([^{3}H]PC I) probing the superficial region of the membrane. This result does not appear to be related to a liposomal structural alteration induced by low pH, since the C fragment is not labeled at the low pHs tested and asolectin vesicles are impermeable to potassium ions at low pH (Boquet & Duflot, 1982). Moreover, other proteic toxins such as cholera toxin, abrin, and ricin do not show a pH-dependent lipid interaction (unpublished results). Rather, it appears that TeTx, with the lowering of the pH, undergoes a conformational change characterized by the generation of hydrophobic surfaces that render the protein able to penetrate into the membrane and to interact with the hydrocarbon chains of phospholipids. This view is supported by the appearance on TeTx at low pH of many binding sites (about 100 at pH 3.0) for Triton X-100 (Boquet et al., 1984). This low pH acquired hydrophobicity of TeTx appears to be confined to the L chain and the H_{45} portion of the H subunit since only these toxin fragments are labeled. The present results are in good agreement with our

recent observations that a subfragment of H_{45} is protected against proteolysis by asolectin vesicles at low pH (Roa & Boquet, 1985). On the other hand, the lack of photolabeling and Triton X-100 binding of the COOH-terminal part of the H chain suggests that it is located externally to the lipid bilayer.

The membrane insertion of TeTx is favored by the presence of negatively charged lipids, and the highest incorporation is obtained with the asolectin lipid mixture, similarly to that found with DT (Montecucco et al., 1985).

Ganglioside G_{D1b} has been suggested to act as a receptor for TeTx and actively promote the insertion of TeTx into the membrane (Yavin et al., 1981; Borochov-Neori et al., 1984). When included within asolectin liposomes, G_{D1b} does not increase TeTx photolabeling; rather, it shifts the pH dependence of the phenomenon toward slightly higher pHs, in agreement with previous findings on TeTx-induced potassium release from G_{D1b}–asolectin liposomes (Boquet et al., 1984). The G_{D1b} effect appears to be related to the H chain, because the pH dependence of the labeling curve is shifted to higher pHs only for this polypeptide chain and not for the L one. This result also suggests that the two TeTx subunits interact with lipids in a different way.

It was previously found that the isolated H chain, but not the L chain, is able to promote the release of potassium ions from liposomes (Boquet & Duflot, 1982). Here we show that at low pH the isolated L subunit can be labeled. Hence, it must insert into the membrane in such a way as not to change the permeability properties of the lipid bilayer. A similar situation has been found for diphtheria toxin, where the isolated hydrophilic subunit A has been shown to interact with lipids (Montecucco et al., 1985) and yet not to alter the conductance of bilayer lipid membranes (Miesler, 1983).

The labeling of both TeTx chains shows a large transition in the pH range 4–5. Carboxyl residues in proteins have pK_a values in this pH region (Tanford, 1962), and their protonation could be involved in the pH-driven conformational change of TeTx. Since the isoelectric point of TeTx is around 5.9 (L. P. Rosenthal, personal communication), the membrane-interacting form of TeTx has a net positive charge. This could be related to the higher efficiency of labeling with negatively charged liposomes at low pH.

It should be emphasized that the present results were obtained in a range of pHs that have been experimentally observed in endosomal compartments (Steinman et al., 1983) and, hence, are in agreement with the possibility that tetanus toxin, like botulinum and diphtheria toxins, is taken up by the cell within endocytotic vesicles.

ACKNOWLEDGMENTS

We thank Dr. R. Bisson for critical reading of the manuscript and Prof. G. F. Azzone for support and encouragement.

Registry No. PC I, 99657-09-7; PC II, 99657-10-0; PC III, 86013-84-5; dimyristoylphosphatidylcholine, 13699-48-4; myristic acid, 544-63-8; ganglioside G_{D1b}, 19553-76-5.

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Metal Anthracycline Complexes as a New Class of Anthracycline Derivatives. Pd(II)-Adriamycin and Pd(II)-Daunorubicin Complexes: Physicochemical Characteristics and Antitumor Activity†

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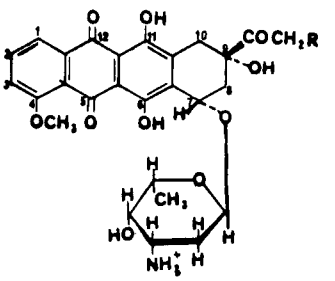
Received July 8, 1985

ABSTRACT: Pd(II) complexes of two anthracyclines, adriamycin and daunorubicin, have been studied. Using potentiometric absorption, fluorescence, and circular dichroism measurements, we have shown that adriamycin can form two complexes with Pd(II). The first complex (I) involves two molecules of drug per Pd(II) ion; one of the molecules is chelated to Pd(II) through the carbonyl oxygen on C_{12} and the phenolate oxygen on C_{11}, and the other one is bound to Pd(II) through the nitrogen of the amino sugar. This complexation induces a stacking of the two molecules of drug. In the second complex (II), two Pd(II) ions are bound to two molecules of drug (A_1 and A_2). One Pd(II) is bound to the oxygen on the carbons C_{11} and C_{12} of molecule A_1 and to the amino sugar of molecule A_2 whereas the second Pd(II) ion is bound to the oxygen on C_{11} and C_{12} of molecule A_2 and to the amino sugar of molecule A_1. The same complexes are formed between Pd(II) and daunorubicin. The stability constant for complex II is $\beta = (1.3 \pm 0.5) \times 10^{22}$. Interaction with DNA has been studied, showing that almost no modification of the complex occurred. This complex displays antitumor activity against P-388 leukemia that compares with that of the free drug. Complex II, unlike adriamycin, does not catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase.

Adriamycin (Adr) and daunorubicin (Dr) (Chart I) are anthracycline antibiotics widely used in the treatment of various human cancers. Clearly, adriamycin is the antitumor agent with the broadest range of activity clinically. However, a major limitation to its use includes its acute and chronic toxicities. The chronic total dose limiting toxicity is cardiotoxicity, which limits the duration of therapy and consequently the duration of remissions in some situations. Impairment of mitochondrial function in myocardiac cells has been implicated in attempts to explain this cardiotoxicity (Bachmann et al., 1975). Several mechanisms of action have been proposed, including inhibition of electron transfer through the respiratory chain and oxidative phosphorylation (Muhammed et al., 1982), complexation of phospholipid cardiolipin (Goormaghtigh & Ruysschaert, 1983), and initiation of lipid peroxidation (Demant, 1983; Demant & Jensen, 1983). Thus the hope of finding a noncardiotoxic yet active anthracycline antibiotic has prompted the search for new naturally occurring anthracyclines and the development of a large number of semisynthetic analogues. The following structural changes are particularly taken into consideration: (i) derivatization at C_{14} in the side chain, (ii) modification and/or substitution of the amino sugar, and (iii) modification of the substituents in the aglycon moiety.

Chart I



R=H Daunorubicin Dr
R=OH Adriamycin Adr

Thus, varying the number and the position of the hydroxyl groups on the aglycon moiety appears to greatly modify the redox chemistry of these compounds (Ashnagar et al., 1984). This is also true when the anthracycline is modified at the quinone, 5-iminodaunorubicin being thus far the only known one. This modification of the redox properties is an important point since recent investigations of Adr-induced cardiotoxicity have focused on the ability of the drug to be reduced by components of the NADH dehydrogenase system (Doroshow, 1981; Thayer, 1977).

The complexation of anthracycline by metal ions appears to be a route to get new compounds modified simultaneously

† This work was supported in part by a PIRMED (CNRS) grant.
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0006-2960/86/0425-0924$01.50/0