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LOCAL ANESTHETICS NONCOMPETITIVELY INHIBIT TERBIUM BINDING TO THE EXTERIOR SURFACE OF NERVE MEMBRANE VESICLES

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It has previously been shown that terbium binds to membrane vesicles prepared from the walking leg nerve of the lobster (*Homarus americanus*) with a high affinity K_d of 2.2 μM. Fluorescence of bound Tb^{3+} occurs via energy transfer from the aromatic residues of proteins (γ_{ex} = 280 nm; γ_{em} = 546 nm), and calcium inhibits Tb^{3+} binding competitively with a K_i of 1.8 mM. Displacement studies with EDTA demonstrate that more than 95% of the bound Tb^{3+} is at the vesicle exterior and is not being taken up by the vesicles. To investigate the putative role of Ca^{2+} in the interaction of local anesthetics with axonal membranes, lidocaine and the analogs GX-HCl and QX-314 were tested as inhibitors of Tb^{3+} binding. Inhibition by lidocaine is seen only at considerably higher doses (25 mM) than are required for conduction block of intact nerves (5 mM). Inhibition by lidocaine and the primary amine analog GX-HCl is entirely noncompetitive, whereas the quaternary ammonium derivative QX-314 appears to be a mixed competitive-noncompetitive inhibitor of Tb^{3+} binding. These data are not compatible with the hypothesis that there is a functionally essential cation binding site on the axonal membrane surface for which Ca^{2+} and local anesthetics compete, although local anesthetic action may be modified indirectly by altered calcium concentrations. Evidence is presented for a mechanism by which local anesthetics indirectly displace Tb^{3+} by altering the physical state of the axonal membrane.

The effects of Ca^{2+} on the nerve-blocking action of local anesthetics and the general role of Ca^{2+} in membrane function are still unclear.

Electrophysiological evidence has shown that Ca^{2+} inhibits, and in some cases reverses, nerve block caused by tertiary amine local anesthetics [1], β-adrenergic blocking drugs [2,3], alcohols, tranquilizers and barbiturates [4]. It is not known whether cations compete directly for anionic membrane sites or whether they alter the anesthetic-membrane interaction indirectly, resulting in altered rates of drug uptake [5,6]. It has been proposed, for example, that Ca^{2+}-catecholamine complexes are involved in the uptake and storage of catecholamines [3]. Experiments designed to address the possibility of Ca^{2+}-drug competition by measuring total binding of $^{45}Ca^{2+}$ or other radionuclides to membrane fragments or membrane vesicles are complicated by extensive nonspecific binding, radionuclide uptake and the overall low affinity of cation binding to exposed membrane sites. An alternative approach is to substitute terbium (Tb^{3+}) chloride, a fluorescent lanthanide salt with binding properties similar to Ca^{2+} [7], but not capable of transport [8], to measure the interaction of local anesthetics with a class of Ca^{2+}-binding sites in axonal membranes.

Materials and Methods

Axonal membrane vesicles from the walking leg nerve of the lobster (*Homarus americanus*) were

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Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

prepared by the method of Denburg [9]. Briefly, the nerves were homogenized in 0.32 M sucrose/10 mM Tris-HCl, pH 7.8, at 4°C. This was followed by a series of differential centrifugation steps including hypotonic lysis and resealing with $MgSO_4$ to yield a plasma membrane vesicle preparation enriched in axonal membrane [10]. For the present studies, the vesicles were dialyzed against 2 mM Pipes buffer, pH 6.8, or were recentrifuged at 100 000 × g then resuspended in 2 mM Pipes, pH 6.8, in order to change buffers and lower the pH to a range suitable for the solubility of Tb^{3+} salts. Vesicles were quantitated by their protein content as determined by the Bradford method [11].

Fluorescence measurements were made on an American Instruments SPF1000 or Perkin-Elmer MPF 44A spectrophotofluorimeter operating in corrected or ratio mode, respectively. In a typical experiment, 100 μg vesicle protein were added to 2 ml buffer (2 mM Pipes, pH 6.8/50 mM NaCl) at 20°C. The excitation wavelength (λ_{ex}) was 280 nm. The emission scan (Fig. 1) was obtained by scanning from 300 to 700 nm and shows a large signal above background, as discussed in detail in an earlier paper [12]. For the inhibition studies with local anesthetics, the vesicles were titrated with $TbCl_3$ while measuring the emission fluorescence (λ_{em}) at 546 nm. In earlier studies, titration of the membrane vesicles with Tb^{3+} in 2 mM Pipes/50 mM NaCl revealed heterogeneous binding in the concentration range 10^{-7}–10^{-4} M. The

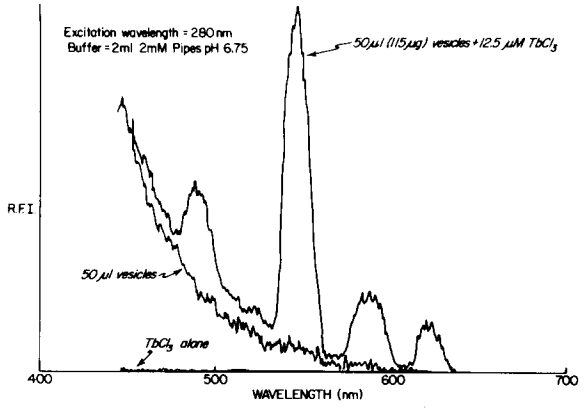


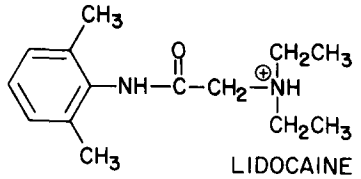
Fig. 1. Fluorescence emission spectra of terbium binding to axonal membrane vesicles. λ_{ex} = 280 nm. Slit widths, excitation = 10 nm; emission = 10 nm. Spectra obtained on an Aminco SPF1000 operating in the corrected mode. T = 20°C. R.F.I., relative fluorescence intensity.

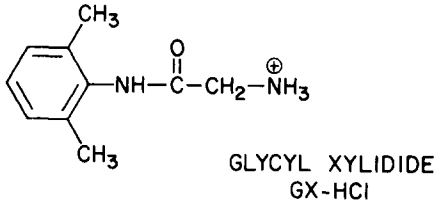
apparent K_d values (2 and 10 μM) were not markedly different, but the biphasic nature of the binding was observed consistently at this ionic strength and measurements by these procedures are reliable to within 5% of the observed values. It was shown that the binding is competitively inhibited by Ca^{2+} (K_I = 1.8 mM) and La^{3+} and noncompetitively inhibited by Mg^{2+} (Deschenes, R.J. and Marquis, J.K., unpublished data). The Tb^{3+} binding assay is based on an energy transfer mechanism from the aromatic regions of proteins and restricts the number of measurable Ca^{2+} sites to those on, or in close proximity to, proteins. This simplifies the analysis by focusing on a subclass of the total membrane Ca^{2+}-binding sites. Displacement studies with EDTA have shown that more than 95% of the bound Tb^{3+} interacts with the vesicle exterior and is not being taken up into the vesicles. This is consistent with uptake studies [8,13] and electron microscopy studies using the lanthanides as electron dense stains [14]. Tb^{3+} binding to the higher affinity sites is not a result of ionic perturbation through a double layer effect [15], because increasing the ionic strength has very little effect on the high affinity binding [12].

The local anesthetics used in these studies were obtained as gifts from Astra Pharmaceuticals. $TbCl_3$ was purchased from Alfa-Ventron and Pipes buffer from Calbiochem.

Results and Discussion

In order to investigate the possible role of Ca^{2+} in the interaction of local anesthetics with the exterior surface of the nerve membrane, lidocaine hydrochloride and two inactive analogs (when applied to the nerve exterior), QX-314 and GX-HCl were tested as inhibitors of Tb^{3+} binding (Fig. 2). None of these xylidide derivatives absorb at 280 nm, the excitation wavelength for these experiments, thus any decrease in fluorescence is not due to quenching by the inhibitor. Lidocaine, QX-314 and GX-HCl all inhibit Tb^{3+} binding to axonal membrane vesicles at concentrations greater than 20 mM (Fig. 3A, B and C). In the Scatchard plots in Fig. 3, the slope = $-1/K_{d(app)}$ and the x-intercept = maximum fluorescence (B_{max}). Based on the values (Table I) calculated by least-squares regression analysis of the plots in Fig. 3A and C, where the $K_{d(app)}$ is unchanged and





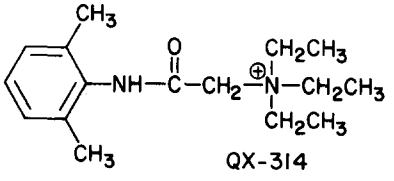


Fig. 2. Chemical structures of the local anesthetics used in these studies.

the B_{max} is decreased, inhibition by lidocaine and GX-HCl is noncompetitive. Inhibition by the quaternary derivative, QX-314, however, appears to be partially competitive (Fig. 3B and Table I). The competitive component of this effect may be attributed to the fixed charge on the quaternary amine. In all cases, the local anesthetics are less potent inhibitors of Tb^{3+} binding than is Ca^{2+} which competitively inhibits with an apparent K_i of 1.8 mM (Fig. 3D).

Several conclusions may be drawn from these data with respect to the role of Ca^{2+} in local anesthetic function. First, the inhibition of Tb^{3+} binding by lidocaine does not correlate with its anesthetic potency. The concentration required to inhibit Tb^{3+} binding to the membrane vesicles is considerably higher than the dose required for lidocaine to block lobster walking leg nerve action potentials. Significant block of the externally recorded action potential occurs with 5 mM lidocaine in 10 min in the whole nerve, where permeability barriers must be overcome (Marquis, J.K. and Deschenes, R.J., unpublished data), while at least 25 mM lidocaine is required to displace Tb^{3+} in lobster axonal membrane vesicles where the permeability barriers have been mostly removed [10]. By comparison, as little as 5 mM Ca^{2+}

TABLE I

EFFECTS OF ANESTHETICS AND CALCIUM ON TERBIUM BINDING

$K_{d(app)}$ and B_{max} values were calculated by least-squares regression analysis of the Scatchard plots in Figs. 3A–D, where the slope = $-1/K_{d(app)}$ and the x-intercept = B_{max}, expressed as relative fluorescence intensity (R.F.I.).

	Higher affinity		Lower affinity	
	K_d (μM)	B_{max} (R.F.I.)	K_d (μM)	B_{max} (R.F.I.)
Control	2.9	49	6.8	64
Lidocaine				
25 mM	3.9	49	8.3	61
50 mM	2.8	32	11	47
100 mM	3.9	32	11	48
GX-HCl				
25 mM	3.2	38	8	50
50 mM	2.9	27	7.4	40
QX-314				
50 mM	5.3	48	9.1	61
100 mM	7.5	42	10.6	48
$CaCl_2$				
0	3.3	35	13.2	54
1 mM	4.5	31	15.6	54
2.5 mM	10.3	35	27.7	54
5 mM	14.7	38	32.3	54

competitively inhibits Tb^{3+} binding to membrane vesicles (Fig. 3D). Secondly, the two physiologically inactive analogs, QX-314 and GX-HCl, are as effective in displacing Tb^{3+} as the potent drug lidocaine. Thirdly, the apparent noncompetitive nature of the inhibition by lidocaine and GX-HCl is not compatible with the theory that there is a functionally essential cation binding site on the outside membrane surface for which Ca^{2+} and local anesthetics compete [1,16]. Based on electrophysiological studies with lobster nerve fibers and externally applied drugs [1], it has been suggested that local anesthetics function by competitive displacement of Ca^{2+} from some membrane sites, possibly phospholipids [16], that control Na^+ permeability. The present studies, carried out on axonal membrane vesicles from the same species, do not confirm this hypothesis for external membrane surface Ca^{2+}-binding sites. If Ca^{2+} and local anesthet-

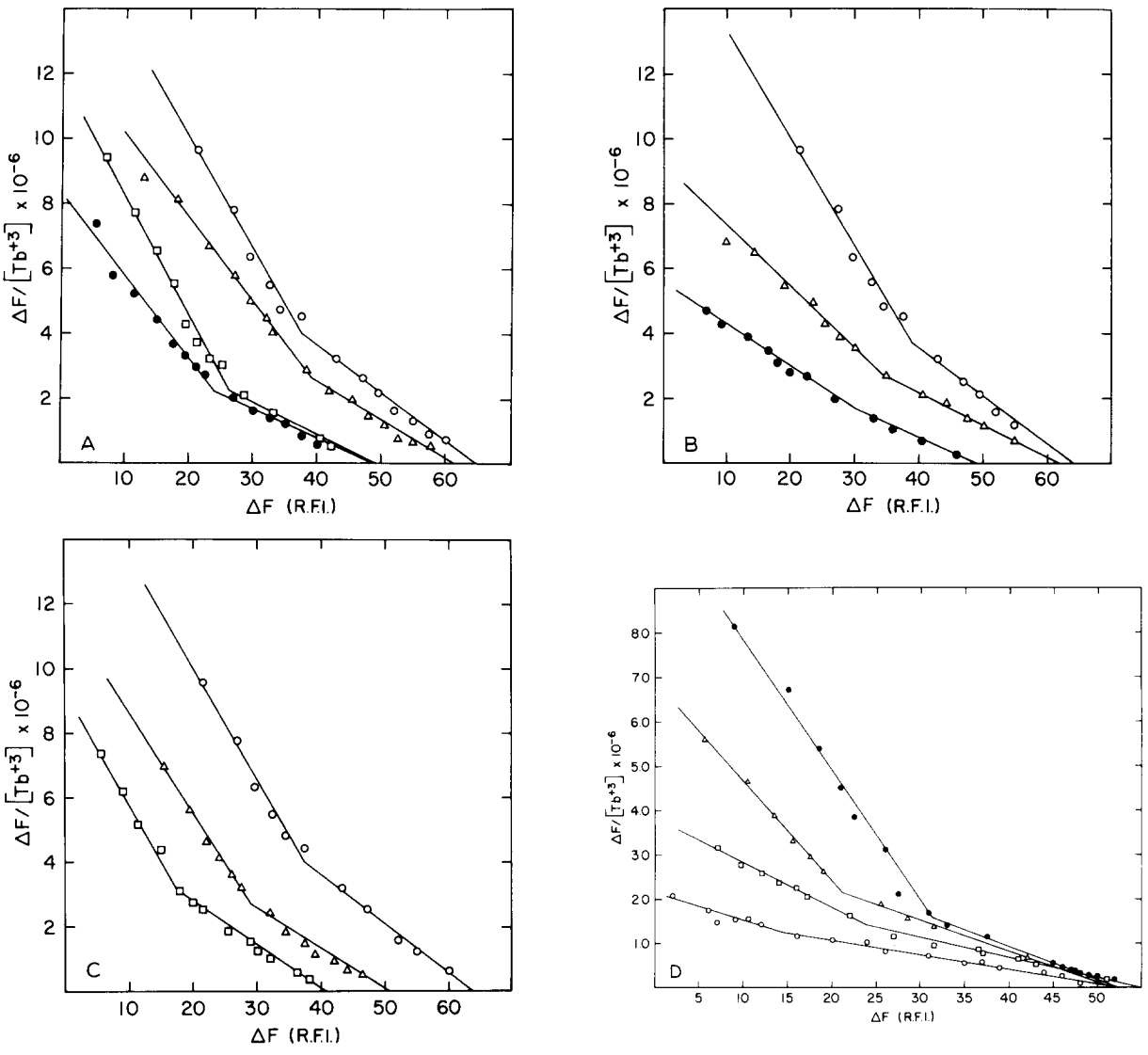


Fig. 3. Scatchard plots showing inhibition of Tb^{3+} binding by lidocaine, the analogs QX-314 and GX-HCl, and Ca^{2+}. (A) 100 μg vesicle protein titrated with $TbCl_3$ in the concentration range 10^{-7}–10^{-4} M. Tb^{3+} binding is measured following 20 min preincubation in 2 mM Pipes/50 mM NaCl, pH 6.8 (○———○); buffer containing 25 mM lidocaine, (△———△); 50 mM lidocaine (□———□); or 100 mM lidocaine (●———●). (B) Same as (A) except preincubation with 50 mM QX-314 (△———△); or 100 mM QX-314 (○———○). (C) Same as (A) except preincubation with 25 mM GX-HCl (△———△); or 50 mM GX-HCl (□———□). (D) Same as (A) except preincubation with buffer alone (●———●); 1 mM $CaCl_2$ (△———△); 2.5 mM $CaCl_2$ (□———□); 5 mM $CaCl_2$ (○———○). Experiments were run on a Perkin-Elmer MPF-44A operating in the ratio mode. λ_{ex} = 280 nm; λ_{em} = 546 nm; slit widths: excitation = 4 nm, emission = 2 nm; T = 20°C. R.F.I., relative fluorescence intensity.

ics compete for binding to a membrane site, that site is one which is not being monitored by Tb^{3+}. These data do not exclude the possibility of Ca^{2+}-binding sites on the cytoplasmic membrane surface, a putative site of local anesthetic action, nor do they exclude the possibility of competitive interaction to

explain the inhibition of many Ca^{2+}-dependent cellular processes by local anesthetics, particularly as the more general actions of local anesthetics are not confined to membrane-dependent reactions. Volpi et al. [17] recently presented evidence that local anesthetics and drugs with local anesthetic-like properties are antagonists of calmodulin, suggesting that local anesthetics may antagonize Ca^{2+}-mediated processes indirectly by modifying the Ca^{2+}-binding protein.

A possible explanation of the mechanism by which these drugs displace Tb^{3+} is shown in Fig. 4. It was noted in the course of these experiments that the addition of drug to the membrane vesicles results in an increase in the background scatter signal monitored before addition of Tb^{3+}. This background signal is attributed to light-scattering caused by overlap of the fluorescence emission maximum of Tb^{3+} (546 nm) with the second-order Rayleigh scatter peak (580 nm) for excitation at 290 nm. The scattering is a rough measure of the physical state and physical properties of the vesicles; for example, their relative size or degree of aggregation. When the background scatter is plotted vs. maximum Tb^{3+} fluorescence (Fig. 4) a straight line with a correlation coefficient of 0.97 results. This suggests that the ability of the drug to displace or inhibit Tb^{3+} binding is related to its ability to alter the physical state of the membrane. It is well-documented that local anesthetics increase membrane volume [18]. It is also known that if the distance between the anion groups of a Ca^{2+} chelate is greater than 9.6 Å, then Ca^{2+} will no longer bind. The displacement of Tb^{3+} measured here and the displacement of Ca^{2+} observed by others with local anesthetics may be related to the expansion of the membrane Ca^{2+} chelating groups to a distance greater than 9.6 Å.

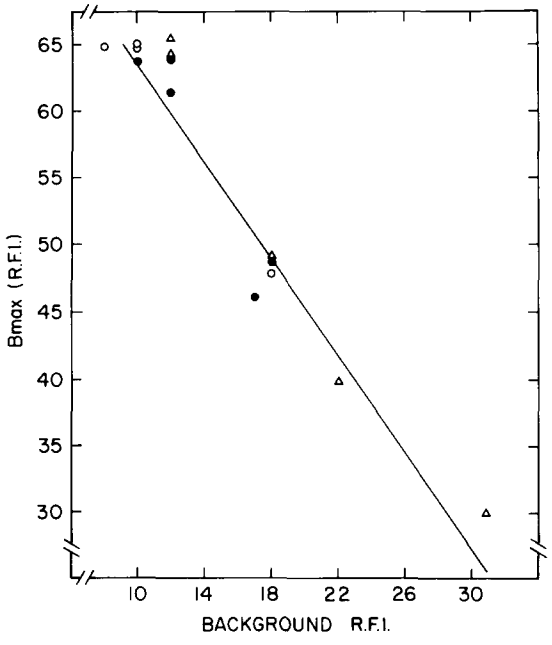


Fig. 4. Maximum Tb^{3+} binding to axonal membrane vesicles plotted as a function of the degree of vesicle light-scattering induced by local anesthetics. B_{max} was determined for Tb^{3+} bound to vesicles incubated for 20 min with lidocaine (●———●); QX-314 (○———○) or GX-HCl (△———△). Background relative fluorescence intensity (R.F.I.) is a measure of vesicle scatter and was determined just prior to incubation with Tb^{3+}. λ_{ex} = 290 nm, λ_{em} = 546 nm; T = 20°C.

In addition to confirming the value of Tb^{3+} as a probe of physiologically relevant Ca^{2+}-binding sites, these studies contribute additional data to support an alternative explanation for the Ca^{2+} effects observed by Blaustein and Goldman [1] and further emphasize the need to reevaluate the molecular mechanism of local anesthesia.

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