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TERBIUM BINDING TO AXONAL MEMBRANE VESICLES FROM LOBSTER (*HOMARUS AMERICANUS*) PERIPHERAL NERVE

A PROBE OF CALCIUM BINDING SITES *

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Summary

Tb^{3+}, a fluorescent trivalent cation with physicochemical properties similar to Ca^{2+}, binds to peripheral nerve membrane vesicles prepared from the walking leg nerve bundle of the lobster (*Homarus americanus*). Saturable binding is measured for at least two classes of binding site. Bound Tb^{3+} can be displaced by other cations in the order: $Ca^{2+} > Mg^{2+} = Zn^{2+} > NH_4^+$. The binding of Tb^{3+} to the lower affinity site (K_D(app) = 6.0 μM) is inhibitable by Na^+, Mg^{2+} and Ca^{2+}, whereas the higher affinity site (K_D(app) = 2.2 μM) is only sensitive to Ca^{2+}. Using this spectral probe the role of Ca^{2+} in peripheral nerve membrane function can be investigated.

Calcium has been shown to play an essential role in many of the physiological processes of nervous tissue. For example, calcium is important in carrying the message of hormonal interaction with the cell surface [1], excitation-contraction coupling in muscles [2], neurotransmitter release from presynaptic storage vesicles [3], monovalent cation translocation [4], cholinergic receptor function [5,6] and local anesthetic action [7]. This laboratory has

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Abbreviation: Pipes, 1,4-piperazinediethanesulfonic acid.

been interested in the mechanism of calcium and local anesthetic interaction with nerve membranes. Interpretation of $^{45}Ca^{2+}$ binding studies to membranes is hindered by the extensive nonspecific binding to physiologically irrelevant sites, the overall low affinity of extracellular binding sites, and the possibility of uptake into the vesicle interior. An alternative to this approach is the use of the trivalent cation, Tb^{3+}, as a spectroscopic probe of calcium binding sites.

The rare earth lanthanides have been used as Ca^{2+} probes in protein (for a review, see Ref. 8) and membrane binding studies (for a review, see Ref. 9). By virtue of its greater charge density and similar ionic radius, Tb^{3+} binds tightly and competitively to Ca^{2+} binding sites in some purified proteins [10] and, in most cases, is not taken up by Ca^{2+} translocating systems [11]. Electrophysiological studies by Takata et al. [12] have shown that La^{3+}, an ion that has similar properties to Tb^{3+}, affects lobster axons in the same way as Ca^{2+} but at lower concentrations.

The present study was undertaken to investigate the interaction of $TbCl_3$ with membrane vesicles prepared from the walking leg nerves of the lobster. The binding is heterogeneous, inhibited by various cations, and selectively displaced by Ca^{2+} over Mg^{2+}.

Materials and Methods

Axonal membrane vesicles from the walking leg nerve of the lobster (*Homarus americanus*) were prepared by using the method of Denburg [13] as modified by Marquis et al. [14]. Briefly, the nerves were homogenized in 0.32 M sucrose, 10 mM Tris, 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. This membrane fraction was shown to be vesicular by electron microscopy in 10 mM Tris buffer, pH 7.8 [15]. For the present study, 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 terbium salts. Vesicles were quantitated by their protein content as determined by the method of Lowry et al. [16].

Fluorescence measurements were made on either an American Instruments SPF1000 or a Perkin-Elmer 44A spectrophotofluorometer operating in corrected or ratio mode, respectively. In a typical experiment, 100 μg of vesicle protein were added to 2 ml of buffer (2 mM Pipes, pH 6.8, and the specified NaCl concentration) 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. For the kinetic experiments the vesicles were titrated with $TbCl_3$ while measuring the emission fluorescence (λ_{em}) at 546 nm. From these studies a K_D value for Tb^{3+} binding was obtained. The K_I value for calcium inhibition was determined by performing a similar titration in the presence of three Ca^{2+} concentrations: 1, 5 and 10 mM. Cation displacement studies were performed as above, except the $TbCl_3$ concentration remained constant and cations (NH_4^+, Mg^{2+}, Zn^{2+}, Ca^{2+}) were added in the concentration range 0.05—1mM.

$TbCl_3$ was obtained from Alfa-Ventron and Pipes buffer from CalBiochem.

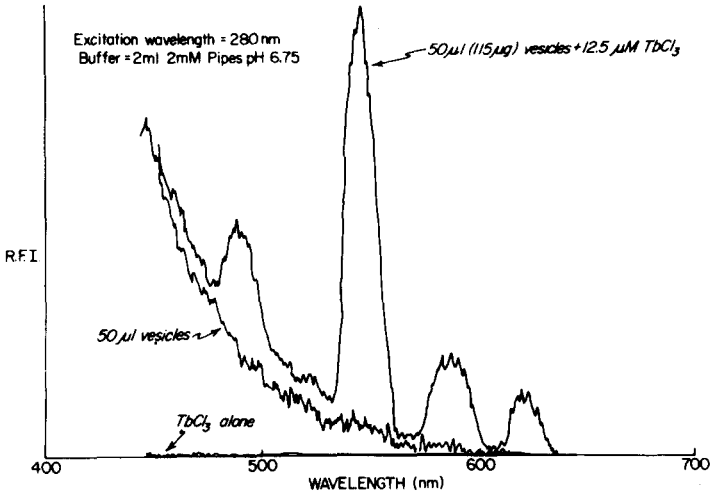

Fig. 1. Fluorescence emission scan of terbium binding to axonal membrane vesicles. λ_{ex} = 280 nm. Slit widths: excitation = 10 nm, emission = 10 nm. These experiments were run on an Aminco SPF 1000 operating on the corrected mode. T = 20° C. R.F.I., relative fluorescence intensity.

All other reagents were purchased from Sigma Chemical Co. Deionized, distilled water was used for all solutions.

Results

The axonal membrane fraction prepared from lobster leg nerves has previously been shown to form sealed vesicles 1000—4000 Å in diameter [15]. Incubation of $TbCl_3$ with the vesicles results in a large increase in fluorescence at the characteristic Tb^{3+} emission maximum (546 nm). The emission spectrum

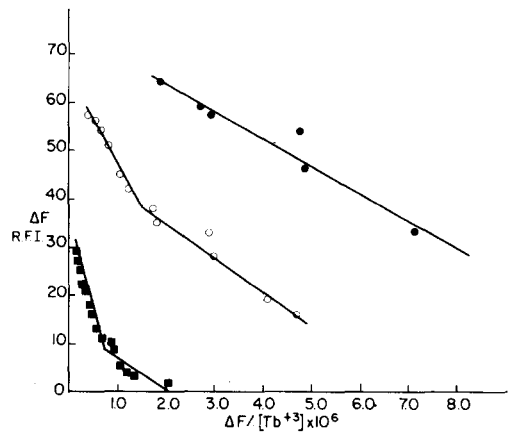

Fig. 2. Eadie-Hofstee plot of terbium binding at different ionic strengths. Terbium is added to 100 µg of vesicle protein in 2 ml of 2 mM Pipes buffer (pH 6.8) containing: 0 (●———●); 50 mM (○———○) or 400 mM (■———■) NaCl. λ_{em} = 546 nm. Experiments were run on a Perkin-Elmer 44A fluorometer operating in the ratio mode. Slit widths: excitation = 4 nm, emission = 2 nm. T = 20° C. R.F.I., relative fluorescence intensity.

(Fig. 1) demonstrates that excitation of 12.5 μM $TbCl_3$ in 2 mM Pipes buffer at 280 nm does not yield a fluorescence signal. Excitation of 115 μg of axonal vesicles at 280 nm results in some scattering but no fluorescence at 546 nm. However, when Tb^{3+} is added to a suspension of vesicles and excited at 280 nm, a peak appears at 546 nm with smaller peaks at 490, 588 and 620 nm. This fluorescence results from non-radiative energy transfer from aromatic amino acids, the primary fluorophores, to bound Tb^{3+}, the acceptor fluorophore. The efficiency of this transfer process is related to the interatomic distance and the degree of spectral overlap of the two fluorophores [17]. Therefore, the relative fluorescence intensity approximates the quantity of bound Tb^{3+}.

The ionic strength dependence of binding was examined by changing the buffer NaCl concentration. Terbium was added in increments to obtain a titration curve. Fig. 2 is an Eadie-Hofstee plot of the data where the ordinate is total bound Tb^{3+} and the slope yields the K_D(app) value. Binding of Tb^{3+} at the higher ionic strength appears to involve at least two components. While the higher affinity component is not dependent on ionic strength or sodium, the lower affinity binding is sensitive to ionic strength or sodium. A control experiment demonstrated that Tb^{3+} fluorescence follows Beers law in this range. With $CaCl_2$ as the inhibitor, a different pattern results. Fig. 3a shows that Tb^{3+} binding in 2 mM Pipes, 50 mM NaCl, is biphasic with apparent affinity constants of 2.2 and 6.9 μM. With 5 mM $CaCl_2$ in the medium, the data yield a single apparent K_D value of 8.7 μM. At 10 mM $CaCl_2$, the binding is again biphasic with apparent dissociation constants of 12 and 19 μM. Calcium inhibition of high-affinity Tb^{3+} binding exhibits competitive kinetics (linear K_D(app) vs. $[Ca^{2+}]$ plot) with a K_I value of 1.8 mM (see Fig. 3b). The non-linearity that is observed when the K_D(app) values for the low-affinity component are plotted against Ca^{2+} concentration indicates that this inhibition is not purely competitive.

The data are summarized in Table I. NaCl affects the lower affinity Tb^{3+}

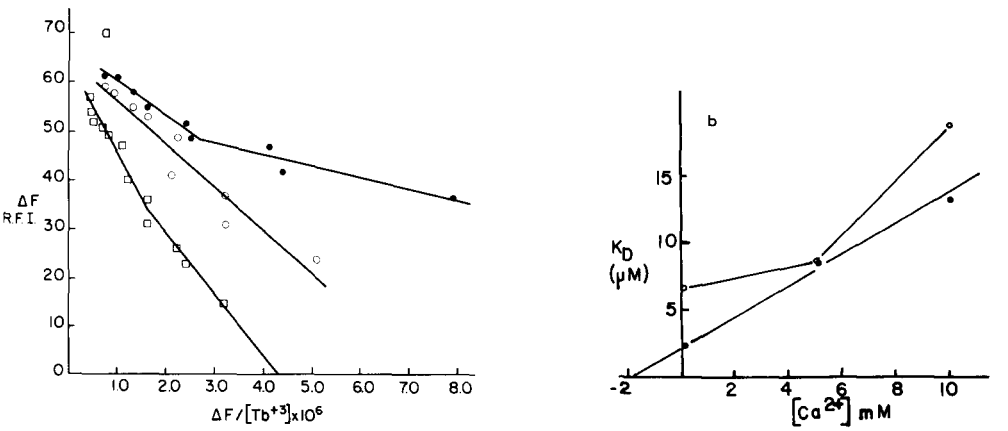

Fig. 3. (a) Calcium inhibition of terbium binding. Terbium is added to 100 μg vesicle protein in 2 ml of 2 mM Pipes, 50 mM NaCl (pH 6.8) containing: 0 (●———●); 5 mM (○———○) or 10 mM (□———□) $CaCl_2$. Experiments were run as in Fig. 2. (b) K_D for Tb^{3+} binding vs. Ca^{2+} concentration. Low-affinity Tb^{3+} binding (○———○); high-affinity binding (●———●). R.F.I., relative fluorescence intensity.

TABLE I

EFFECT OF CALCIUM AND SODIUM ON THE APPARENT AFFINITIES OF TERBIUM BINDING TO AXONAL MEMBRANE VESICLES

	K_D(app) (M) ($\times 10^{-6}$)	
	High affinity	Low affinity
Calcium inhibition		
2 mM Pipes/50 mM NaCl		
+0 $CaCl_2$	2.2	6.9
+5 mM $CaCl_2$	8.7	8.7
+10 mM $CaCl_2$	12.4	19.0
Sodium inhibition		
2 mM Pipes		
+0 NaCl	5.6	5.6
+50 mM NaCl	7.3	18.0
+400 mM NaCl	6.2	40.3

binding without altering the higher affinity sites. Preliminary results suggest that Mg^{2+} also inhibits lower affinity sites without significantly affecting the higher affinity sites. In contrast, Ca^{2+} alters the apparent affinity of both groups of binding sites. It should be noted that Ca^{2+} and Na^{+} inhibition studies were carried out on two separate membrane preparations. Absolute K_D(app) values were found to vary somewhat between membrane preparations, but measurements within one preparation were reproducible.

The sensitivity of bound Tb^{3+} to displacement by various cations was examined in 12 μM $TbCl_3$. The competing cation was added, equilibrated (4—5 min) and the fluorescence emission recorded. Cations displaced Tb^{3+} in the order $Ca^{2+} > Mg^{2+} = Zn^{2+} > NH_4^+$ (Fig. 4) with Ca^{2+} displacing about 26% of the bound Tb^{3+} while Mg^{2+} displaced 10%. This selectivity is consistent with that of physiologically relevant Ca^{2+} binding sites [18].

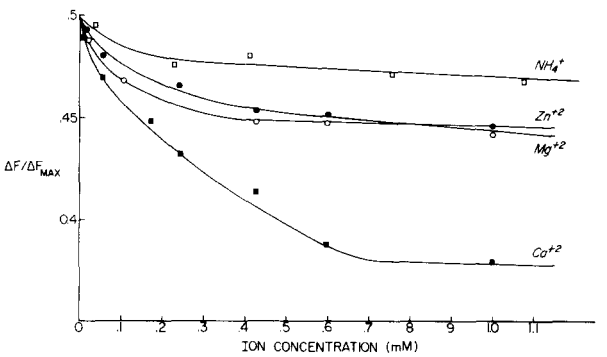

Fig. 4. Displacement of bound terbium. 100 μg vesicle protein are incubated with 12 μM $TbCl_3$ in 2 ml of 2 mM Pipes, 50 mM NaCl buffer (pH 6.8). The relative fluorescence was measured after addition of NH_4^+ (□———□), Zn^{2+} (●———●), Mg^{2+} (○———○) or Ca^{2+} (■———■). Experiments were run as in Fig. 1.

Discussion

Terbium exhibits saturable binding to axonal membrane vesicles. The binding appears to be biphasic with high- and low-affinity components exhibiting different sensitivities to Na^+ and Ca^{2+}. While the high-affinity sites are competitively inhibited by Ca^{2+} and not inhibited by Na^+, the low-affinity component can be blocked by both ions. The selectivity observed between Mg^{2+} and Ca^{2+} in displacing Tb^{3+} suggests that these axonal sites are physiologically relevant [18]. In Tb^{3+} binding studies using erythrocyte membranes this ion selectivity was not observed [19].

$^{45}Ca^{2+}$ binding to lobster axonal membranes has recently been measured by Baumgold [20] who reports that uptake as well as binding of Ca^{2+} can occur with these vesicles. The association of $^{45}Ca^{2+}$ with the vesicles exhibits ion specificity in high salt medium (412 mM), as more Ca^{2+} associates in NaCl than in KCl media. The author interprets this as being caused by altered rates of uptake in the presence of NaCl or KCl. Whether these Ca^{2+} sites reported by Baumgold [20] are related to the Tb^{3+} binding sites reported here is uncertain. Terbium fluorescence does not increase with time and the bound Tb^{3+} is completely accessible to displacement by EDTA (data not shown), suggesting that, similarly to other systems, uptake of Tb^{3+} is not occurring in the axonal membrane vesicles [11].

The advantage of using Tb^{3+} as a calcium probe in axonal membranes is that it focuses on a particular subset of Ca^{2+} binding sites. Only those sites on or close to protein will fluoresce when excited at 280 nm, as energy transfer efficiency decreases by the sixth power of the distance between the fluorophores [17]. The biphasic nature of Tb^{3+} binding may represent either (a) heterogeneity of protein binding sites, including nonspecific negative surface membrane sites, or (b) phospholipid binding sites in proximity to protein, as Tb^{3+} may bind to the phosphate groups of membrane phospholipids. The nature of these binding sites is presently being investigated with proteases and phospholipases.

It is hoped that by using Tb^{3+} as a fluorescence probe many of the putative roles of Ca^{2+} in excitable membrane function can be elucidated. For example, Blaustein and Goldman [7] have shown that Ca^{2+} inhibits the conduction blocking action of procaine in lobster circumesophageal axons. We are currently investigating the interaction of Tb^{3+} and local anesthetics with axonal vesicles to characterize further the role of Ca^{2+} in local anesthetic function and other calcium-mediated phenomena in peripheral nerve.

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References

1 Keppens, S., Vandenheede, J.R. and DeWulf, H. (1977) Biochim. Biophys. Acta 496, 448—457
2 Ebashi, S. (1976) Annu. Rev. Physiol. 38, 293—309
3 Katz, B. and Miledi, R. (1965) Proc. R. Soc. (London) Ser. B 161, 496—503
4 Meech, R.W. (1978) Annu. Rev. Biophys. Bioeng. 7, 1—18
5 Chang, H.W. and Neumann, E. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3364—3368
6 Rübsamen, H., Hess, G.P., Eldefrawi, A.T. and Eldefrawi, M.E. (1976) Biochem. Biophys. Res. Commun. 68, 56—63
7 Blaustein, M.P. and Goldman, D.E. (1966) J. Gen. Physiol. 49, 1043—1066
8 Martin, R.B. and Richardson, F.S. (1979) Q. Rev. Biophys. 12, 181—209
9 Mikkelsen, R.B. (1976) in Biological Membranes (Chapman, D. and Wallach, D.F.H., eds.), pp. 153—190, Academic Press, New York
10 Luk, C.K. (1971) Biochemistry 10, 2838—2843
11 Weiss, G.B. (1974) Annu. Rev. Pharmacol. 14, 343—354
12 Takata, M., Pickard, W.F., Lettvin, J.Y. and Moore, J.W. (1976) J. Gen. Physiol. 50, 461—470
13 Denburg, J.L. (1973) Biochim. Biophys. Acta 298, 967—972
14 Marquis, J.K., Hilt, D.C. and Mautner, H.G. (1977) Biochem. Biophys. Res. Commun. 78, 476—482
15 Chester, J., Lentz, T.L., Marquis, J.K. and Mautner, H.G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3542—3546
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
17 Förster, T. (1959) Disc. Faraday Soc. 27, 7—17
18 Williams, R.J.P. (1976) in Calcium in Biological Systems (Duncan, C.J., ed.), Symp. XXX, Soc. Exp. Biol., pp. 1—18, Cambridge University Press
19 Mikkelsen, R.B. and Wallach, D.F.H. (1974) Biochim. Biophys. Acta 363, 211—218
20 Baumgold, J. (1979) Biochim. Biophys. Acta 558, 149—165