FLUORESCENT Ca^{+2} ANALOGS IN NERVE: RARE-EARTH IONS

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SUMMARY

To probe the Ca^{+2} ion-nerve membrane interaction, rare-earth ions were used as analogs of Ca^{+2} in the bathing solution of garfish olfactory nerve trunk. We observed the chemically sensitive fluorescence of Eu^{+3} and Tb^{+3} as a measure of rare-earth binding to nerve, both resting and firing, and subjected to treatments with various enzymes, chemical agents, and abnormal salt concentrations. The main results are: 1) rare-earth ions bind to nerve proteins, NADH, and possibly other sites; 2) fractional changes of rare-earth fluorescence synchronous with firing are less than 0.02%; 3) most of the observed binding is a slow, continual uptake of rare-earth ion into the axoplasm, rate-limited by permeation through the axon membrane.

INTRODUCTION

Ca^{+2} ions in the external solution of a nerve stabilize the resting state of the axon membrane and play an essential role in excitability (1). We substituted the fluorescent rare-earth ions, Eu^{3+} and Tb^{3+}, for Ca^{+2}, and observed their characteristically enhanced fluorescence intensities upon chemical binding under varying conditions of nerve firing, solution ion concentrations, enzyme treatments, and chemical agents, as measures of the quantity of rare-earth ions bound to the nerve.

Chlortetracycline has been used previously as a fluorescent probe for Ca^{+2} bound to nerve, with results of ambiguous interpretation (2). Rare-earth ions have been used as fluorescent probes of metal binding sites in the protein transferrin (3).

MATERIALS AND METHODS

The nerve tissue used for this study was the garfish olfactory nerve trunk, which has a uniquely high ratio of axon membrane surface area to volume and to Schwann cell and connective tissue area (4). The dissections were performed as described by Easton (4). All experiments were performed at room temperature. The compositions of the various Ringer solutions used are given in Table 1. The enzymes were obtained commercially and used without further purification.

All the fluorescence emission and excitation spectra (uncorrected) were recorded with a Perkin-Elmer MPF-2a spectrofluorimeter with a spectral resolution of 5 nm. The various spectra of nerve were recorded by pushing a 4 cm length of garfish nerve trunk into a 2 x 2 mm quartz fluorescence cuvette filled with Ringer solution containing the appropriate ions.

A separate apparatus was constructed for experiments examining fluorescence changes. The light from a 900 W Xenon lamp was passed through a water filter, interference filters, and a grating monochromator, yielding an excitation beam of 10 nm spectral bandwidth. A glass plate deflected part of the exciting light directly to a reference photomultiplier, used to monitor the intensity in the Xenon arc. The width of the main beam impinging on the nerve was 0.5 mm after focusing and passage through the quartz side window of the nerve holder. Fluorescent light from the nerve was viewed at 90° through a microscope and interference filters by a photomultiplier mounted above the microscope. The optical set-up was contained completely in a light-tight box during experiments.

The photomultiplier outputs were connected to opposite inputs of a differential amplifier, so that lamp intensity fluctuations would not affect the amplifier output. For the experiments on firing nerves, the amplifier output was filtered to pass .1 Hz to .1 kHz and connected directly to the input of a Computer of Average Transients (CAT) in order to signal average the photomultiplier outputs during several thousand synchronous stimulations of the nerve. For the experiments on resting nerves undergoing changes of bathing solution, the differential amplifier output was DC-coupled with a 12-sec time constant into a strip chart recorder.

Solutions flowed by gravity into the nerve holder and could be switched rapidly without interrupting the flow or disturbing the nerve or optics. The nerve holder itself contained quartz glass windows for passage of the exciting and fluorescent light, provision for the flow of solution into and out of the chamber, plastic supports to prevent nerve sagging, and two pairs of external platinum electrodes for nerve stimulation and recording.

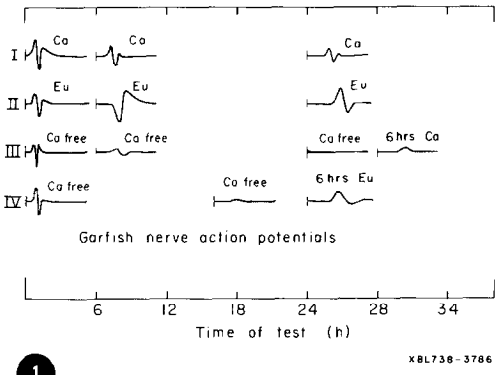
RESULTS

Rare-earths as Physiological Substitutes for Ca^{+2}. Previous work indicates that several polyvalent cations (La^{+3}, Ba^{+2}, Ni^{+2}, Co^{+2}, Cd^{+2}) can substitute for Ca^{+2} in the Ringer solution and still maintain nerve excitability (5-7). These polyvalent cations have a physiological effect somewhat similar to that of high [Ca^{+2}] (8). Because of the fairly non-specific nature of successful Ca^{+2} replacements and the chemical homology of the lanthanides, one would anticipate that any of the trivalent rare-earth ions would successfully substitute for Ca^{+2}. This substitution was tested on garfish olfactory nerve by bathing each of several nerves in solutions containing either one type of polyvalent ion or none at all. The ions used were Ca^{+2}, Ni^{+2}, Mn^{+2}, and Eu^{+3}. The excitability of the nerves was checked frequently over a period of hours by monitoring the relative persistence of

the compound action potential (Fig. 1). The results for Ca-, Eu-, and Ca-free-Ringers indicate that: 1) Garfish nerves maintain excitability in Ca- and Eu-Ringers longer than in Ca-free-Ringer; 2) bathing a nerve in Ca- or Eu-Ringer previously made inexcitable by Ca-free-Ringer restores some excitability. On the basis of such evidence, we proceeded with the use of Eu^{+3} and a homologous rare-earth ion, Tb^{+3}, as physiological substitutes for Ca^{+2}.

Rare-earth Fluorescence Spectra in Nerve. The fluorescence intensity of most of the emission bands of both Eu and Tb aqueous solutions increases upon chemical binding of the ions to strong ligands (9). Comparison of the emission spectra of aqueous $EuCl_3$ vs. Eu-EDTA Cl_3 (Fig. 2), shows that upon Eu^{+3} complexation, all emission bands are greatly enhanced, but the relative intensities of the 590 nm and 614 nm peaks become reversed. This reversal can be taken as a definite indication that at least some Eu^{+3} in a sample is chemically bound to ligands. The fluorescence spectrum of nerve bathed at 0°C for 24 hr in Eu-Ringer (Fig. 3) also shows this intensity reversal, indicating that a considerable portion of the Eu associated with the nerve was chemically bound. The background fluorescence is probably from oxidized flavins (10).

The fluorescence excitation spectrum of Eu-nerve was obtained point-by-point by measuring the height of the Eu^{+3} 614 nm peak above the background as a function of excitation wavelength. The resulting (uncorrected) spectrum shows a broad band in the 320-380 nm region not present in the aqueous Eu excitation spectrum (Fig. 3). This band must result from energy transfer from an organic species near the bound Eu^{+3} which absorbs in this wavelength region, probably NADH. The excitation spectrum of nerve bathed in Tb-Ringer using the emission line at 543 nm (Fig. 4) shows a strong band in the 290-300 nm region not present in the aqueous Tb^{+3} excitation spectrum. This band indicates energy transfer from, and probable Tb^{+3} binding to, proteins. Since Eu^{+3} and Tb^{+3} are similar in chemistry, we conclude that both probably are bound to NADH and proteins. In vitro 1:3 mixtures of Eu^{+3} and



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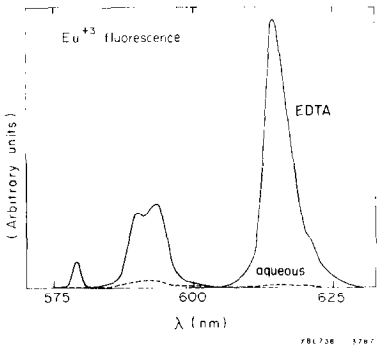

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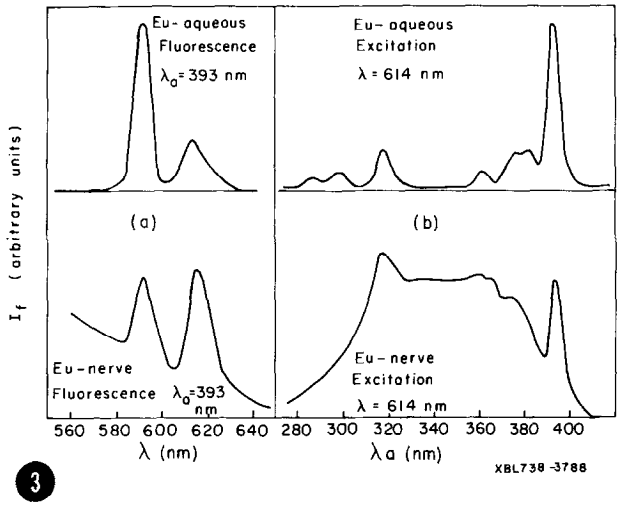

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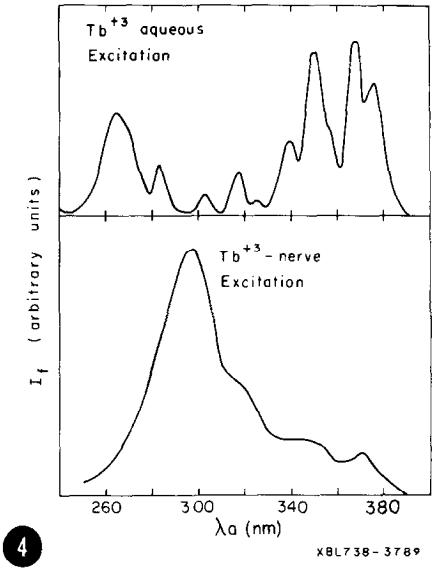

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Fig. 1. Each trace is the action potential of the nerves bathed in the indicated solutions and elicited at the times (in hr) shown at the beginning of the trace, The duration of each trace is 500 ms. For example, the three traces on line IV show the action potential of a nerve: (1) at t=0 hr, immediately after placement in Ca-free-Ringer; (2) at t=16 hr, still in Ca-free-Ringer; and (3) at t=24 hr, after bathing in Eu-Ringer for the last 6 hr.

Fig. 2. Fluorescence emission spectra of $EuCl_3$ (25 mM) aqueous solution (dashed line) vs. Eu (25 mM) - EDTA (50 mM) solution (solid line). Resolution = 2.0 nm, λ=614 nm, λ_a=393 nm.

Fig. 3. (a) Fluorescence emission spectra of $EuCl_3$ aqueous solution vs. Eu-nerve. λ_a=393 nm. Resolution = 5.0 nm. (b) Fluorescence excitation spectra (uncorrected) of $EuCl_3$ aqueous solution vs. Eu-nerve. λ=614 nm. Resolution = 5.0 nm.

Fig. 4. Fluorescence excitation spectra (uncorrected) of $TbCl_3$ aqueous solution vs. Tb-nerve. λ=543 nm. Resolution = 5.0 nm.

NADH at pH 7.8 were found to exhibit a fluorescence emission characteristic of bound Eu^{+3} and an excitation spectrum indicating energy transfer from NADH to Eu^{+3}.

Transient Changes of Eu-Nerve Fluorescence. Fractional transient changes of Eu^{+3} (λ=614 nm) or Tb^{+3} (λ=543 nm) fluorescence, synchronous with the passage of an action potential, were less than 0.02%, the noise limit. This result was obtained irrespective of (1) excitation wavelength or (2) the time the nerve had been bathed in Eu-Ringer before the start of the experiment, from a few minutes to a day. Similarly, transient changes of intrinsic fluorescence from proteins, NADH, and oxidized flavins, both for total and polarized fluorescence, were less than 0.01%. Each of these experiments was tried on at least three garfish nerves.

Fluorescence of Eu-Resting Nerve. The λ=614 nm Eu^{+3} fluorescence of a resting nerve was monitored as a function of time. The bathing solution flowing through the nerve holder could be switched between Ca-Ringer and Eu-Ringer at any time. The Eu^{+3} fluorescence, presumably proportional to Eu^{+3} uptake, rose at a fairly linear rate, at least for the first 7 hr. Switching the solution back to Ca-Ringer caused no decrease in fluorescence; i.e., the Eu^{+3} uptake is irreversible. With Eu-Ringers containing only 0.01 mM Eu^{+3}, the rate of uptake was less, but still linear, even at early times. Differences in the rate of uptake between a resting nerve and the same nerve stimulated at the rate of 1 pulse/sec were immeasurably small. The time required for uptake of the membrane-impermeable dye, trypan blue, was measured to be <1 hr, suggesting that the gradual Eu^{+3} uptake was not limited by diffusion into the extracellular spaces between the axons.

Since a high $[K^+]$-induced depolarization might change the Eu^{+3} binding state, we observed Eu^{+3} fluorescence as the solutions were switched between low and high $[K^+]$ Eu-Ringer. A small change was observed, but it might result from a simultaneously observed light scattering change.

Eu-Ringer solutions containing either of two proteases, trypsin (1 mg/ml)

Table 1. Compositions of various garfish Ringer solutions used

(Concentrations in mM/liter)

Ringer name / Salt	Ca	Ca-free	Eu	Tb	High[K^+]Eu	Low Eu
NaCl	204	204	204	204	3.5	204
KCl	3.5	3.5	3.5	3.5	204	3.5
$CaCl_2$	3.5	-	-	-	-	-
$EuCl_3$	-	-	0.5	-	0.5	0.05
$TbCl_3$	-	-	-	0.5	-	-
Glucose	24	24	24	24	24	24
pH (Hepes Buffer)	6.9	6.9	6.7	6.7	6.7	6.9

or pronase (1 mg/ml), or the enzyme neuraminidase (0.1 mg/ml) affected Eu binding to nerve by less than 0.5%. However, treatment by either phospholipase-c (0.2 mg/ml) or dimethylsulfoxide (which increases membrane permeability) led to a 2- to 4-fold increase in the rate of Eu uptake. The dimethylsulfoxide effect was reversible; the phospholipase-c effect was irreversible.

DISCUSSION

The results of this work are: (1) continual, slow uptake of Eu^{+3} by nerve; (2) enhancement of the rate of uptake after membrane alteration by phospholipase-c and dimethylsulfoxide; and (3) spectroscopic evidence of rare-earth binding to protein and NADH in the nerve. These are all consistent with the interpretation that the bulk of Eu^{+3} fluorescence results from Eu binding in the axoplasm, possibly to the mitochondria and as organic complex precipitates, rate limited by permeation through the axon membrane. Ca is known to accumulate in mitochondria (11). The small fraction of Eu possibly bound to the axon membrane may undergo binding changes during nerve stimulation, but the background of axoplasmic Eu^{+3} and intrinsic fluorescence interfered with optical detection of these changes, if any.

The technique of replacement of Ca^{+2} with a fluorescent rare-earth analog might be especially suited to probing Ca^{+2} binding sites in internally perfused squid axons, where the axoplasmic composition can be controlled, in systems in which Ca involvement is greater, as in muscle, and in more homogeneous *in vitro* biochemical systems.

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