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A Terbium-160 Probe of the Nicotinic Binding Site of the Acetylcholine Receptor from *Torpedo Californica*

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^{160}Terbium(III) exchange experiments have been performed on the acetylcholine receptor protein isolated form Torpedo californica, *employing a specially designed flow dialysis apparatus constructed in our laboratory. The apparatus is designed to allow continuous monitoring of ^{160}Tb(III) γ-ray emission from the protein compartment of the flow dialysis cell. Nicotinic ligand-induced displacement of ^{160}Tb(III) from the nicotinic binding site of the receptor was monitored as a function of (1) the concentration of nicotinic ligand (*i.e., *acetylcholine chloride) in the 'wash-out' buffer and (2) the nature of the nicotinic ligand in the buffer (*e.g., *acetylcholine chloride, tetraethylammonium bromide and nikethamide). Measured ^{160}Tb(III) exchange half-lives indicate (1) a direct relationship between ^{160}Tb(III) displacement and nicotinic ligand concentration in the 'wash-out' buffer and (2) an enhanced ^{160}Tb(III) displacement for nicotinic agents possessing quaternary ammonium functions (*e.g., *acetylcholine chloride and tetraethylammonium bromide)* versus *neutral ligands (*e.g., *nikethamide).*

Introduction

Trivalent terbium has been employed successfully in numerous investigations of calcium binding sites in biological systems [1, 2]. Terbium is ideally suited for the replacement of calcium since the ionic radius of Tb(III) (1.00 Å) is essentially identical to that of Ca(II) (0.99 Å), and since Tb(III) prefers octahedral coordination to oxygen ligands as does Ca(II). In addition, Tb(III), unlike Ca(II), possesses convenient nuclear, fluorescence and magnetic resonance spectroscopic properties.

The rationale for employing radioactive ^{160}Tb(III) (0.8794 MeV γ-ray emission) as a probe of the nicotinic binding site of the acetylcholine receptor derives from the work of Eldefrawi *et al.* (3) on Ca(II) and Tb(III) binding to the receptor. Eldefrawi found that Tb(III) can substitute for Ca(II) at specific sites on the acetylcholine receptor and that acetylcholine can displace the bound terbium from these sites. Agonists of nicotinic action, such as carbamylcholine, behave similarly to acetylcholine, while antagonists, such as d-tubocurarine, do not induce the receptor to release bound Tb(III). Therefore, Tb(III), by occupying natural Ca(II) binding sites, provides a convenient and specific 'spectroscopic handle' on the binding activity at the nicotinic binding site of the acetylcholine receptor.

In this communication, we report on the ability of various ligands to displace ^{160}Tb(III) bound to the acetylcholine receptor from *Torpedo californica.* Ligand-induced ^{160}Tb(III) displacement was followed in a specially designed flow dialysis apparatus, coupled to a NaI(Tl) γ-ray scintillation spectrometer. A prototype of the flow dialysis/nuclear counting system described here has been used by Nelson *et al.* [4] to study alkaline earth metal ion, transition metal ion and lanthanide ion binding to muscle calcium binding parvalbumin.

Experimental

The acetylcholine receptor was purified from the electric organs of *Torpedo californica* (Pacific Biomarine, Venus, California, U.S.A.), according to the procedure of Eldefrawi *et al.* [5, 6] (*Torpedo californica* is a particularly convenient and appropriate source of the acetylcholine receptor since electric organs from this species are very abundant in cholinergic synapses which bear close resemblance to those found in skeletal muscles from mammalian sources.) Acetycholine chloride and tetraethylammonium bromide were obtained from Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Nikethamide was synthesized from benzene-sulfondiethylamide and nicotinic acid benzene-sulfonate according to the procedure of Oxley *et al.* [7]. Carrier-free $^{160}TbCl_3$

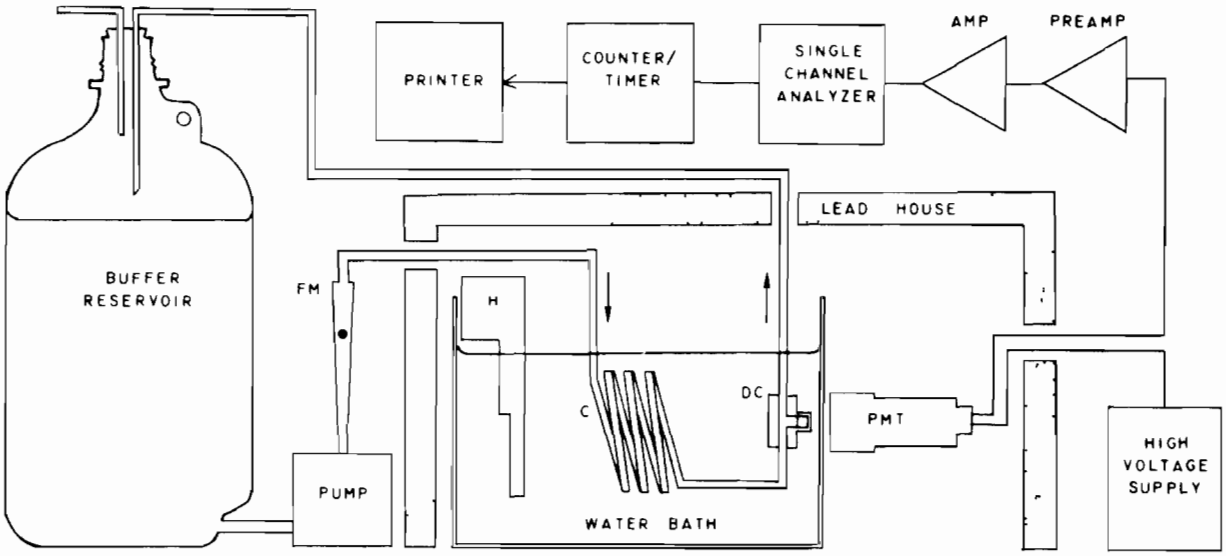

Fig 1 The flow dialysis system The acetylcholine receptor protein, labelled with terbium-160, was placed in the 1 cm^3 chamber of the dialysis cell (DC) Buffer, usually containing a nicotinic agent, was pumped through the buffer side of the dialysis cell at a rate of 150 ml/min The 'washout' of terbium-160 was followed with a NaI (T1) scintillation detector, optically coupled to a photomultiplier tube (PMT) (FM = flow meter, H = heater, C = coil of tubing)

was obtained from New England Nuclear (Cambridge, Massachusetts, U S A)

Nuclear γ-ray scintillation spectroscopy, employing ^{160}Tb(III)-labelled acetylcholine receptor protein, was performed in a two compartment (1 0 ml sample volume) flow dialysis apparatus constructed in our laboratory A schematic diagram of the equipment is shown in Fig 1 The flow cell is positioned 5 cm in front of the γ-ray scintillation crystal, in order to facilitate the continuous monitoring of terbium(III)-160 release from previously labelled receptor Receptor protein was routinely labelled by adding 50 μl 99 9% carrier-free $^{160}TbCl_3$ to 3 ml of approximately 0 5 μM protein in 10 mM TRIS buffer, pH = 7 4 (total activity = 17 μCi) Following overnight incubation at 4 °C, the terbium-receptor mixture was exhaustively dialyzed against 10 mM TRIS buffer, pH = 7,4, to remove any unbound terbium A typical kinetic experiment involved placing ^{160}Tb(III)-labelled receptor, dissolved in 10 mM TRIS buffer (pH = 7 4), in the protein side of the flow dialysis cell Buffer, containing 100 mM ligand (*e g*, acetylcholine, tetraethylammonium bromide or nikethamide), in the buffer side of the flow cell, separated from the protein compartment by a semipermeable cellulose membrane (pore size = 23 Å), was then rapidly replaced via an inlet port at the bottom of the apparatus and an outlet port at the top A constant flow rate of 150 ml/min was maintained during these 'wash-out' experiments with a peristaltic pump

Nicotinic ligand-induced ^{160}Tb(III) exchange was followed by monitoring the γ-ray decay of terbium (III)-160 (half-life = $t_{1/2}$ = 72 3 days) remaining in the protein compartment with a 3 in X 3 in cylindrical Harshaw NaI (T1) scintillation crystal detector, optically coupled to a photomultiplier tube The output of the photomultiplier tube was amplified using an Ortec No 113 preamplifier and then further amplified and shaped with an Elscint CAV-3 linear amplifier In order to minimize the effect of background radiation and to effectively integrate the Tb-(III)-160 0 879 MeV γ-ray peak, a single-channel analyzer (Ortec 420-A) was employed to select pulses corresponding to 0 879 ± 0 50 MeV Logic pulses from the single channel analyzer were routed to an Ortec 776 counter/timer Data accumulated for dwell times of 2000 sec were recorded using an Ortec 77a line printer Exchange half-lives were obtained using a least squares decay curve analysis program implemented on a PDP 11/70 computer

The carbon-13 nuclear magnetic resonance spectrum of nikethamide was obtained on a Bruker SXP 22/100 NMR spectrometer operating at 22 6 MHz Broad band noise decoupling was 12 watts modulated over 2 KHz, centered on the ^{1}H spectrum Chemical shifts are reported with respect to tetramethylsilane (TMS) as a reference

Results and Discussion

Figure 2 presents the results of acetylcholine-induced Tb(III)-160 exchange from the receptor protein as a function of acetylcholine concentration Exchange half-lives at 0 001 M, 0 05 M, 0 10 M and

TABLE I Summary of Cholinergic Ligand-Induced ^{160}Tb(III) Displacement from the *Torpedo Californica* Acetylcholine Receptor

Ligand	Background ^{160}Tb(III) Exchange Half-Life	^{160}Tb(III) Exchange Half-Life in the Presence of 100 m*M* Ligand
Acetylcholine	274 hr	72 hr
Tetraethylammonium Bromide	262 hr	197 hr
Nikethamide	265 hr	340 hr

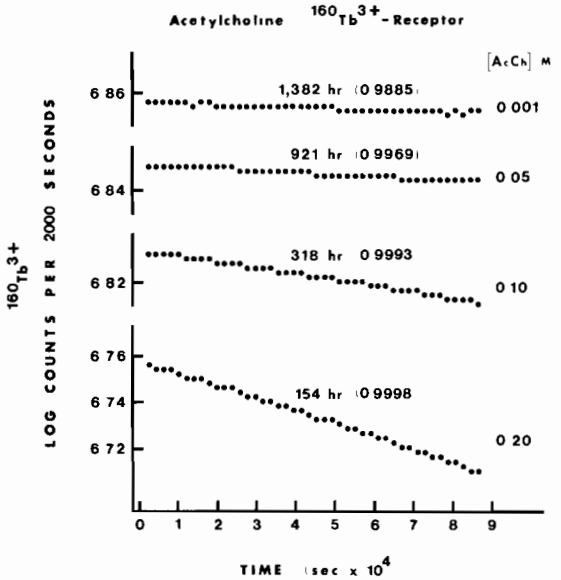

Fig 2 Time–activity spectra for the exchange of acetylcholine receptor-bound terbium(III)-160, induced by varying concentrations of acetylcholine Each point corresponds to 2000 sec of data acquisition The buffer employed was 10 m*M* TRIS (pH = 7 4) Protein concentration = 0 1 mg/ml Half-life values for each exchange experiment, along with correlation coefficients, are given above each spectrum

0 20 *M* acetylcholine are 1 382 hr (correlation coefficient = 0 9885), 921 hr (0 9969), 318 hr (0 9993) and 154 hr (0 9998), respectively As might be expected, there is an inverse relation between the concentration of the nicotinic ligand and the terbium-(III)-160 exchange half-life Figure 2 also reveals the high precision of the measurements being made (*i e*, a 154 hr half-life can be measured in a 20 hour period of time with a correlation coefficient of 0 9998) This illustrates the usefulness of this new flow dialysis/γ-counting technique in general kinetic studies of small molecule and ion binding to macromolecules

Table I presents comparative terbium(III)-160 exchange half-lives for three ligands (Half-lives obtained in this set of experiments are not directly comparable to the half-lives presented in Fig 2, since the $^{160}TbCl_3$ used in these experiments was not 'carrier-free' and a different receptor concentration was employed) For each of the experiments a fresh sample of Tb(III)-160-labelled receptor (0 5 mg/ml) was used, and in each experiment terbium γ-ray emissions were counted for at least 24 hours while 10 m*M* TRIS buffer (pH = 7 4), without any nicotinic ligand, was passed through the dialysis chamber Following acquisition of this 'background' spectrum, ligand was added to the TRIS buffer to the 100 m*M* level The conclusions drawn from the data presented in Table I are the following (i) the kinetic system is well behaved, yielding reproducible results (*e g*, all three background spectra, obtained from three separate terbium-labelled receptor samples, yield the same half-life within 5%), (ii) acetylcholine, a naturally occurring neurotransmitter substance, not surprisingly, induces the most rapid displacement of receptor-bound terbium ($t_{1/2}$ = 72 hr), (iii) tetraethylammonium bromide (TEA), a quaternary ammonium ligand known to block the action of acetylcholine upon postsynaptic receptors [8], induces significant displacement of terbium-160 bound to the receptor ($t_{1/2}$ = 197 hr), and (iv) nikethamide, a relatively strong nicotinic agent [9], produced a terbium exchange rate significantly slower than that observed for either acetylcholine chloride or tetraethylammonium bromide, with a terbium-160 exchange half-life of 340 hours, even longer than the average exchange half-life associated with the passage of nicotinic ligand-free TRIS buffer through the dialysis cell ($t_{1/2}$ = 267 hr)

The tetraethylammonium bromide result is significant, since we would expect any interaction between the TEA anion and the receptor protein to occur at the anionic region of the nicotinic binding site [10] Our results, indicating significant TEA-induced terbium-160 displacement, confirm that TEA does, in fact, bind to the nicotinic binding site of the receptor and strongly suggests that the site of terbium-160 binding is at or very near the 'anionic region' of the binding site

The unexpectedly slow rate of terbium-160 exchange in the presence of nikethamide can be

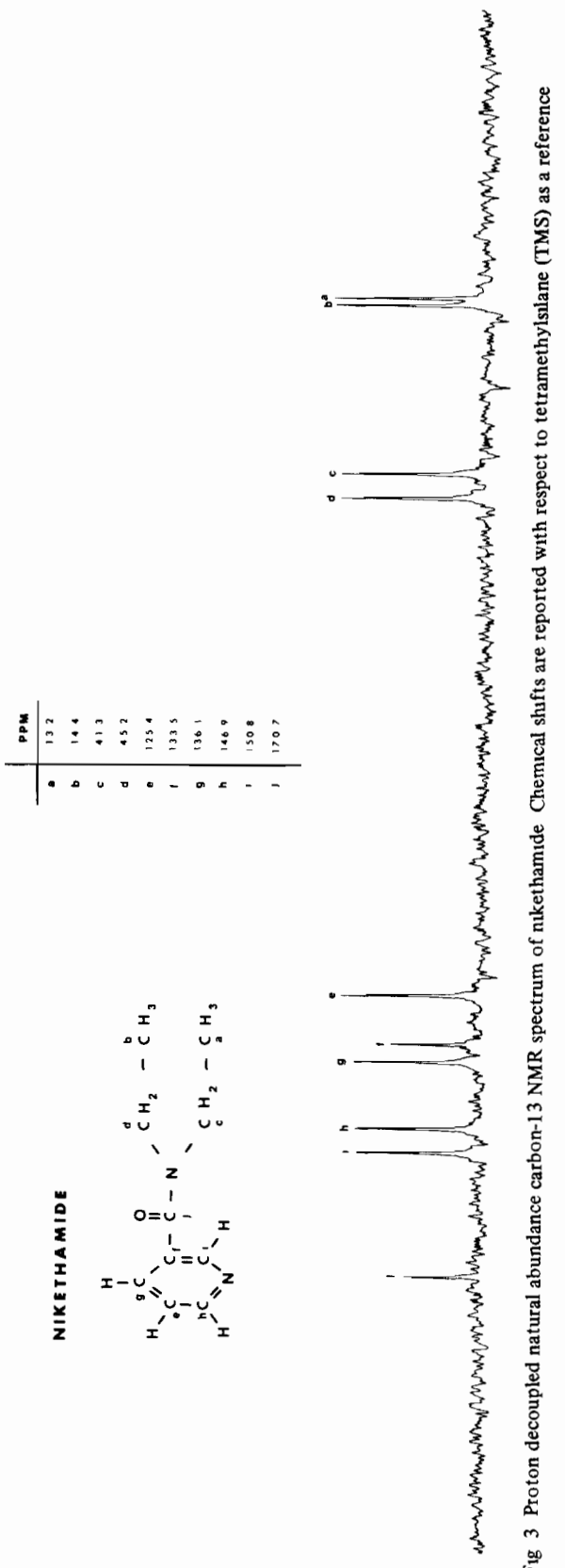

Fig 3 Proton decoupled natural abundance carbon-13 NMR spectrum of nikethamide Chemical shifts are reported with respect to tetramethylsilane (TMS) as a reference

attributed to the fact that nikethamide, unlike acetylcholine and TEA, does not possess a quaternary nitrogen atom bearing a full positive charge In fact, carbon-13 NMR clearly reveals that the bond between the carbonyl carbon atom and the adjacent nitrogen has substantial double bond character Figure 3 presents the natural abundance spectrum of nikethamide, following synthesis of the ligand in our laboratory The upfield region of the spectrum reveals that both methyl carbons 'a' and 'b' and methylene carbons 'c' and 'd' are magnetically nonequivalent This result provides firm support for the existence of partial double bond character for the adjacent carbonyl carbon–nitrogen bond The existence of two resonance signals for the two methyl and two methylene carbons further implies that there is no free rotation about the carbonyl carbon–nitrogen bond and that the two methyls, along with the two methylene groups, form a plane with the carbonyl function Based on this interpretation, resonance signal 'd' is assigned to the methylene carbon closest to the carbonyl oxygen (*i e.*, the electronegative oxygen would withdraw more electron density from the closer methylene carbon and this 'electronic de-shielding' effect would result in a downfield shift of the resonance signal 'b' to the methyl carbon closer to the carbonyl oxygen Since the two methylene carbons are closer to the carbonyl function than the two methyl carbons, the resonance pair 'c'/'d' is 'split' to a greater extent than the 'a'/'b' pair

Our interpretation of the nikethamide ^{160}Tb-(III) exchange data supports the hypothesis that while nikethamide binds to the nicotinic binding site of the receptor, the electrostatic attractive forces between the anionic region of this site (where, presumably, the terbiums bind) and the nicotinic ligand are comparatively weak Terbium displacement is not promoted by nikethamide binding, and in fact may be retarded by the ligand 'overlaying' the terbium binding site

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