MONOVALENT CATION PENETRATION INTO SYNAPTIC MEMBRANES

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1. Introduction

Although the flux of ions through nerve membranes during electrical activity has been clearly demonstrated by combined electrophysiological and radiotracer techniques [1], the mechanism remains largely obscure. A central issue is membrane control of ionic permeabilities. Recent physiological data has been interpreted in terms of ion translocation through fixed pores controlled by voltage-dependent gates [2]. An alternative mechanism involving the voltage-dependent aggregation of channel forming molecules within the membrane has also been suggested [3]. Specific chemical modification of nerves has been shown to independently alter Na^+ or K^+conductance parameters of voltage clamped crayfish axons [4]. This paper describes a direct spectroscopic method which can detect the presence of certain ions in the hydrophobic interior of synaptic membranes.

Since the intrinsic fluorescence of a suspension of synaptic membranes obtained from bovine gray matter appeared to arise exclusively from tryptophan residues located in the hydrophobic interior of the membrane, it was used as a tool with which to investigate these areas [5]. The abilities of certain ions as well as neutral molecules to quench native fluorescence can be used to estimate their accessibilities to the membrane interior. Chloroform was found to be freely accessible to hydrophobic protein regions of synaptic membranes; and the bimolecular rate constant for the quenching of tryptophan fluorescence by chloroform indicated that these regions were highly fluid [5]. This communication briefly notes the accessibilities of water and iodide ion to hydrophobic protein regions of synaptic membranes and examines several factors which influence membrane penetration by cesium ion.

2. Experimental procedure

Synaptic membranes were prepared as described previously from bovine brain involving the isolation of synaptosomes, their subsequent rupture by freeze-thaw and hypotonic shock, and finally fractionation on a discontinuous sucrose density gradient [5]. After washing twice with water, membranes were thoroughly homogenized in 0.1 M sodium phosphate or Tris buffer with a tight-fitting Teflon and glass homogenizer. The resulting stock suspensions (0.5–1.0 mg protein/ml) were diluted 10-fold with salt solutions and water for spectroscopic measurements. For the solvent isotope effect membranes were washed with the appropriate D_2O-H_2O mixture and subsequently resuspended in the same mixture. Membranes were acetylated in half-saturated sodium acetate with excess acetic anhydride at 0°C [6] and subsequently washed with water and resuspended in 0.1 M buffer. Quenching experiments were performed at room temperature (24 ± 1°C) and constant ionic strength (usually 0.2 M) adjusted with NaCl or KCl. Both the Raleigh and Raman scattering were resolved from the fluorescence emission. A correction was made for the small difference in absorbance between NaCl and NaI solutions at the excitation wavelength (290 nm) since it contributed to the low level of iodide quenching found in the membranes. Solutions of KI and NaI contained 10^{-4} M sodium thiosulfate to prevent I_3^- formation.

Cesium chloride (99.97%) was obtained from Fisher, deuterium oxide (99.7% isotopic purity) from Sigma or Diaprep and L-tryptophan ("Sigma Grade") from Sigma. Other chemicals were of analytical reagent quality.

The apparent accessibilities were determined from

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the slopes of Stern-Volmer plots of the data with the aid of a simple least squares computer program and represent the average of 2–4 independent experiments. Values for quenching constants were within 10% of the mean.

3. Results

The fluorescence intensity of tryptophan in D_2O, F_D, is more than twice the value obtained in H_2O; but the qualitative fluorescence parameters remain unchanged. The data fit a variation of the Stern-Volmer expression [7] for fluorescence quenching (Eqn 1)

$$\frac{F_D}{F} = 1 + K_Q X_H \qquad (1)$$

where F refers to the fluorescence intensity of tryptophan in an isotopic water mixture characterized by the mole fraction of H_2O, X_H, and K_Q refers to the overall quenching constant, which may involve both equilibrium and kinetic terms [8]. The quenching constant determined for tryptophan in synaptic membranes was 16% of the value obtained for the same amino acid (tryptophan) in water (fig.1). The quenching constant was unchanged by overnight incubation at

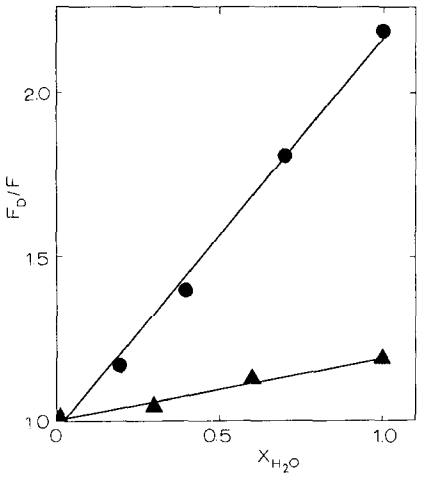

Fig.1. H_2O quenching of aqueous tryptophan (●—●) and synaptic membrane (▲—▲) fluorescence in H_2O - D_2O mixtures according to Eqn (1).

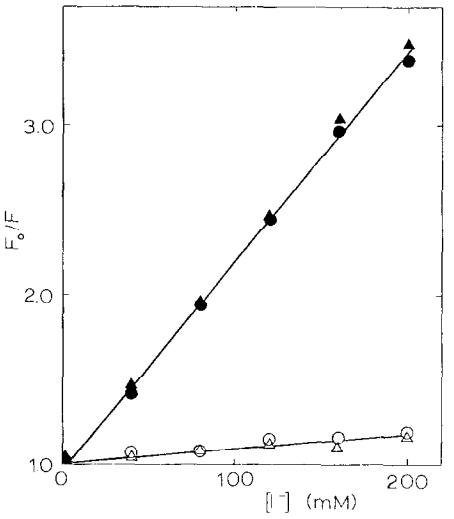

Fig.2. Iodide ion quenching of aqueous tryptophan fluorescence by KI (▲—▲) and by NaI (●—●); and of synaptic membrane fluorescence by KI (△—△) and by NaI (○—○) at pH 7.5. The ionic strength was maintained at 0.2 M with NaCl.

4°C or the addition of 10 mM $CaCl_2$; it was also independent of a 4-fold decrease in the concentration of membranes as predicted by Eqn. 1.

Iodine ion is an efficient quencher of indole fluorescence and has been used to distinguish between buried and exposed tryptophan residues in proteins [9]. The value of the quenching constant for synaptic membranes was 7% of the value obtained for the amino acid in water [fig.2]. The quenching abilities of sodium iodide and potassium iodide were equal and independent of membrane concentration.

Cesium ion is also a quencher of indole fluorescence [10; but as shown in fig.3 the level of tryptophan quenching by cesium ion in synaptic membranes at pH 7.5 was within the limit of detectability for quenching by cesium ion (5% of the value for the amino acid in water). The presence of 10^{-3} M EDTA, extensive reduction with excess sodium borohydride or reaction with *p*-mercuribenzoate did not significantly increase the quenching. As the pH was raised the fluorescence intensity of the membranes decreased and simultaneously the level of quenching by cesium ion increased. At pH 11 the quenching constant became 15% of the value for the amino acid in water (fig.3).

Upon acetylation of the membrane with acetic anhydride the fluorescence characteristics remain

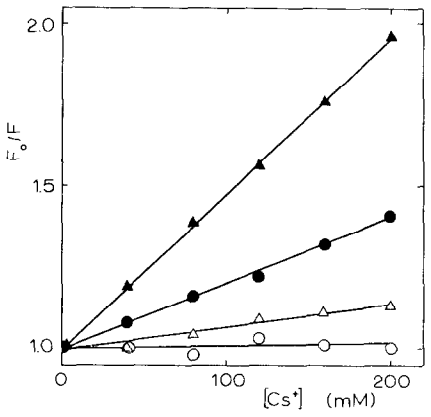

Fig.3. Cesium ion quenching of aqueous tryptophan fluorescence (●—●) and of synaptic membrane fluorescence (○—○) at pH 7.5; and of tryptophan (▲—▲) and membrane (△—△) fluorescence at pH 11.0. The ionic strength was maintained at 0.2 M with NaCl.

virtually unchanged while the level of quenching by cesium was increased to 26% of the value for the amino acid in water (fig.4). No increase in quenching was observed for a non-acetylated control in which only acetic anhydride was omitted. The value of the quenching constant for the acetylated membranes was not affected by the presence of 10 mM $CaCl_2$ or increasing the ionic strength from 0.2 M to 0.4 M.

4. Discussion

The fluorescence quenchers used in this study act at short range; thus, their abilities to quench tryptophan fluorescence in synaptic membranes relative to their abilities to quench the amino acid fluorescence in an appropriate aqueous solution is a measure of the accessibility of the quencher to the membrane region where the tryptophan is located. Apparent accessibility is defined as K_Q(membrane)/K_Q(aqueous). The collisional nature of the quenching by chloroform [11] and iodide ion [9] has been demonstrated; the observation that the quenching by cesium ion was more efficient in methanol than water (data not shown) shown) suggested that the process was dependent on viscosity and thus also diffusion-limited. Water interacts with the excited singlet state of indole through exiplex formation [12] and electron ejection into the solvent [13]. These processes may be responsible for the observed solvent isotope effect [12]. The apparent accessibilities obtained from quenching data could differ from the true values for several reasons. In the case of diffusion-limited quenching, K_Q will be directly proportional to the lifetime of the singlet state; however tryptophan lifetimes in proteins have been found to vary within a narrow range; 2–4 ns [14]. The quenching technique can distinguish between the situation involving a large increase in accessibility of a few tryptophan residues and a smaller increase in the accessibility of all residues. All membrane trytophan residues appeared to be equally affected by the quenchers used in this study.

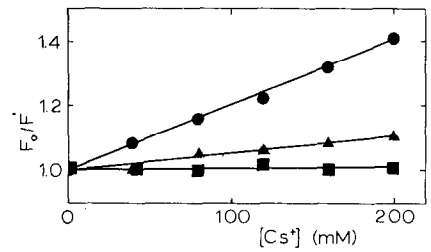

Fig.4. Cesium ion quenching of the fluorescence of aqueous tryptophan (●—●), acetylated synaptic membranes (▲—▲), and non-acetylated control membranes (■—■) at pH 7.5 and ionic strength 0.2 M.

Iodide ion is an efficient quencher of exposed tryptophan residues [9,10]. The low level of quenching of synaptic membrane trytophan fluorescence by iodide ion supports the notion that these residues are buried in the hydrophobic interiors of the membrane.

The solvent isotope effect suggested that water was significantly accessible to hydrophobic protein regions of synaptic membranes. A careful study of solvent effects on the magnetic resonance spectra of nitroxide spin labels located at various positions within lipid bilayer regions of membranes has revealed that water penetrates to at least the C-2 position (adjacent to the phospholipid ester carbonyl) but only infrequently as far as C-9 [15]. Changes in the extrinsic fluorescence parameters of dye-treated axons could be explained by the influx of water into certain membrane regions during excitation [16].

Hydrophobic protein regions of synaptic membranes are quite fluid [5] and partly hydrated; they could conceivably serve as sites for ion translocation. The quenching data clearly demonstrated that the

membrane presents barriers against its penetration by both monovalent anions and cations.

Some insight as to the chemical nature of these barriers against cesium ion might have been gained from chemical modification of the membranes. The two factors which increased the accessibility of cesium ion to hydrophobic protein regions of synaptic membranes altered the charge distribution at the aqueous-membrane interface. Increasing pH both neutralized positive charges and created negative charges. Acetylation, which probably occurred on both lipid and protein amino groups, only neutralized positive charges but produced a comparable increase in cesium ion penetration. This observation suggested that the interaction between membrane amino and carboxyl moieties might have some functional significance for the control of cationic permeabilities.

The inability of other cations to reduce the level of fluorescence quenching by cesium in acetylated membranes emphasized the point that quenching experiments do not measure binding but rather dynamic accessibility, which is relatively insensitive to ionic strength effects.

Acknowledgements

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