LOCAL ANESTHETIC INDUCED CHANGES OF A MEMBRANE-BOUND FLUOROCHROME. A LINK BETWEEN ION UPTAKE AND MEMBRANE STRUCTURE

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1. Introduction

Calcium uptake by mitochondria represents their most rapid and effective energy-linked function. Cytochrome *b* responds to calcium addition in 20 msec [1]; calcium-stimulated respiration exceeds the rate of ADP-stimulated respiration and causes a maximum rate of external hydrogen ion ejection [2,3] and in the absence of a permeant anion, a maximal membrane alkalinization [4]. The affinity of mitochondria for calcium is remarkably high; external calcium concentrations in equilibrium with the endogenous calcium of rat liver mitochondria are of the order of 10^{-8} M, as detected with the calcium indicator, aequorin [1]. Thus, extremely high inside/outside calcium concentration ratios are possible.

The response of the membrane bound pH indicator, bromothymol blue [4], to calcium accumulation in the absence of a permeant anion is greatly stimulated by relatively low concentrations of a local anesthetic, butacaine. The rate of calcium uptake, as reported by the indicator, murexide, has been shown to be enhanced several-fold by butacaine [5,1]. A striking effect of butacaine on the mitochondrial membrane is revealed by a membrane-bound fluorochrome, 8-anilino-1-naphthalene sulfonic acid, ANS, which is insensitive to pH and to oxidation-reduction changes, but shows a sensitive response to changes in the state of the membrane [6,7]. The properties of the membrane-bound fluorochrome and the effects of butacaine and calcium upon the kinetics and stoichiometry of the ANS reaction are described.

2. Materials and methods

ANS fluorescence emission spectra were measured in a specially constructed differential fluorometer (vibrating cuvette type) [8]. This instrument records the difference of fluorescence emission of two suspensions of mitochondria. Thus each trace represents the fluorescence increment on adding the reagents (fig. 1). Alternatively, larger fluorescence changes are recorded in a Hitachi-Perkin-Elmer (Sp-XXX).

More rapid kinetics than those which can be identified by hand-mixing are recorded with a new model of the regenerative flow apparatus which requires no more than approximately 10 mg protein for a series of observations on the reaction with ANS, butacaine and calcium [9]. In this case, fluorescence excitation is applied to the side of a 6 × 22 mm observation chamber, and fluorescence emission is observed normal to its 22 × 22 mm face. The chamber can be filled in approximately 2 msec, and readout is obtainable during the continuous flow interval of about 100 msec, or in the stopped flow interval which immediately ensues. The fluorescence signal from ANS bound to the mitochondria can be obtained from recordings with rise times of 10–20 msec without difficulty. The excitation wavelength has been chosen with due regard to concentration of ANS employed. Thus, in fig. 1, with low ANS concentrations, 405 nm was used while in fig. 3, 436 nm was used.

Rat liver mitochondria are prepared as described in [4]. Butacaine and ANS are obtained from K & K Laboratories, Inc., other chemicals are reagent grade.

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3. Results

3.1. *Spectra*

Fig. 1 shows the emission spectra (390 nm excitation) on a relative energy basis of ANS bound to rat liver mitochondria (20 μM to approximately 1.5 mg mitochondrial protein per ml). The emission peak is at 470 nm, as compared to 520 nm for ANS in water, in curve A. Curve B records a further enhancement of the fluorescence intensity caused by the addition of 50 μM butacaine. Trace C shows that the addition of 100 μM Ca^{2+} causes further fluorescence increase.

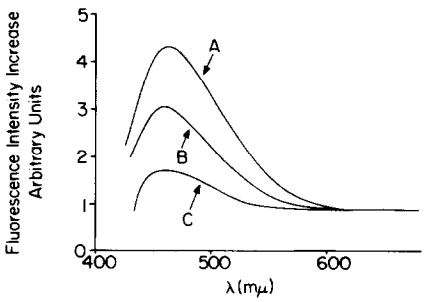

Fig. 1. Fluorescence spectra for rat liver mitochondria in the presence of ANS. In all experiments the concentration of rat liver mitochondria was 1.5 mg protein per ml. 390 nm excitation was used. (A) The fluorescence emission difference spectrum between rat liver mitochondria and rat liver mitochondria supplemented with 22 μM ANS. (B) The fluorescence emission difference spectrum between rat liver mitochondria supplemented with 22 μM ANS versus the same suspension supplemented with 50 μM butacaine. (C) Fluorescence emission difference spectrum between rat liver mitochondria supplemented with 22 μM ANS and 50 μM butacaine versus the same suspension supplemented with 100 μM calcium.

3.2. *Titration with ANS*

Fig. 2A indicates a titration of 13 μM ANS with rat liver mitochondria (405 nm excitation, 520 nm measurement). When the trace reaches the plateau. 3 mg of protein per ml have been added (~ 4 nmoles ANS per mg protein). The titration is repeated after 200 μM butacaine is added. The profile has roughly a two-fold steeper slope, and reaches a plateau at about 6 nmoles ANS per mg protein. Fig. 2B illustrates the titration of 1 mg/ml of rat liver mitochondria with ANS. Here no clearly defined end point is obtained in the presence or absence of butacaine. One interpretation is that fig. 2A indicates the titration of a small number of high affinity binding sites out of a total of many more indicated by fig. 2B. Fig. 2C illustrates the titration of 1.0 mg/ml of rat liver mitochondria and 13 μM ANS with butacaine. A sharp end point at 200 nmoles of butacaine per mg protein is obtained. The polarization (P) of the ANS fluorescence of 0.29 does not change significantly when butacaine or Ca^{2+} are added.

Titrations at such low concentrations of ANS are preferable since high concentrations of ANS (~ 100 μM) together with butacaine (~ 200 μM) are inhibitory to energy-linked functions such as Ca^{2+} uptake.

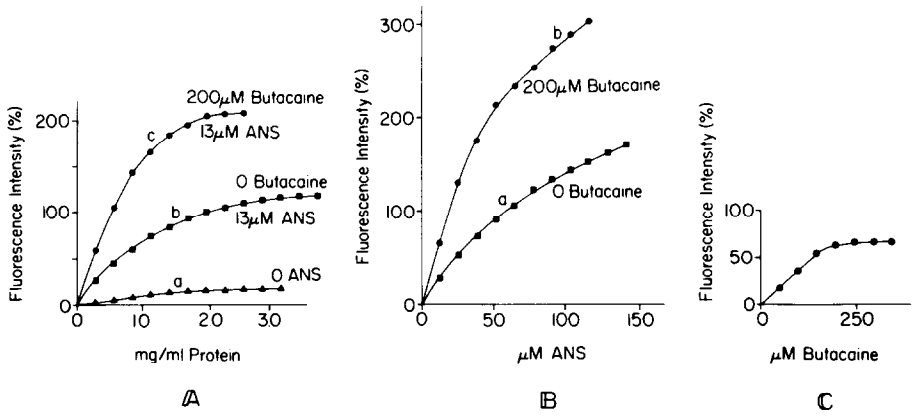

Fig. 2. (A) Titration of ANS with rat liver mitochondria. (B) rat liver mitochondria titrated with ANS. (C) rat liver mitochondria supplemented with ANS titrated with butacaine. In part A: curve labelled a indicates no ANS added, b, 13 μM ANS, and c, 13 μM ANS plus 200 μM butacaine. In part B: 1.0 mg mitochondria protein per ml; a, zero butacaine, b, 200 μM butacaine. In part C: 1.0 mg mitochondrial protein, 13 μM ANS.

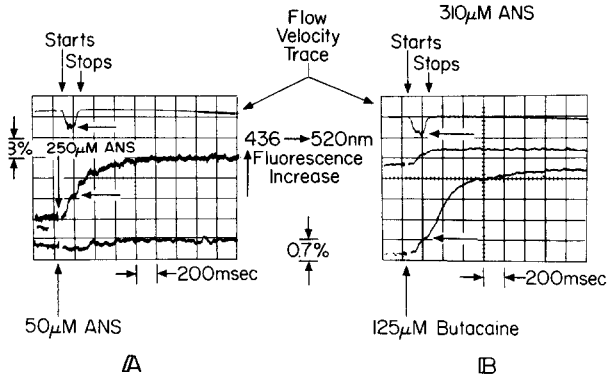

Fig. 3. Kinetics of the enhanced fluorescence in rat liver mitochondria, 0.25 mg protein per ml. (A) Upon addition of 50 μM or 250 μM ANS and (B) 125 μM butacaine in the presence of 310 μM ANS.

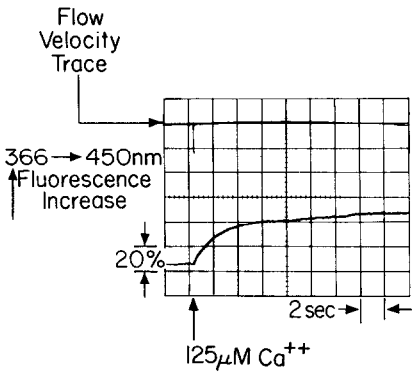

Fig. 4. Kinetics of the enhancement of the fluorescence intensity of 40 μM ANS in rat liver mitochondria, 0.5 mg protein per ml supplemented with 5 mM succinate, 2 μM rotenone and 60 μM butacaine.

3.3. *Kinetic studies*

Fig. 3A illustrates the reaction of low and high concentrations of ANS with rat liver mitochondria (0.25 mg protein/ml). Rapid mixing of 50 μM ANS gives a small fluorescence increase which proceeds halfway to its plateau value in approximately 200 msec. At 250 μM ANS, the 100 msec interval of continuous flow (time after mixing, 5 msec) shows a fluorescence increase corresponding to 30% of the maximum value. (This is indicated by the plateau of the fluorescence trace (see arrows) synchronized with the interval of continuous flow.) Thereafter, the trace rises exponentially to the equilibrium point; the half-time for the phase after the flow has stopped is 180 msec.

The 30% progress of the reaction at 5 msec and the 180 to 200 msec concentration-independent slower portion of the reaction are inconsistent with a one-step reaction. For this reason, it is concluded that a two-step reaction occurs, the first step of which may be regarded as essentially complete at 5 msec at 250 μM ANS and is presumably a second-order step with a rate constant in excess of 10^6 M^{-1} sec^{-1}. The other, slower phase, with a half-time of 180–200 msec is probably due to a first order transformation consequent to the faster binding of ANS at sites of low fluorescence efficiency. Experiments with successive additions of ANS show the fast and slow phases of the reaction suggesting that fast binding precedes in each case the slow reaction of ANS with the membranes.

The reaction with butacaine is illustrated in fig. 3B in which mitochondria supplemented with 310 μM ANS are mixed with 125 μM butacaine in the flow apparatus. At the 5 msec time after mixing, 10% of the total change has occurred as indicated by the small plateau in the trace (arrows). Thereafter the half-time for the fluorescence increase is 200 msec. The trace directly above illustrates a second discharge of butacaine and shows that the fluorescence increase is largely saturated by the first 125 μM addition in accordance with the previous fig. 2.

Butacaine does not affect the initial phase of the reaction with ANS, the increased fluorescence (see fig. 1) occurs as a part of the slow phase of the ANS reaction.

3.4. *The reaction with calcium*

Fig. 4 shows that the further fluorescence increase caused by the calcium reaction (see fig. 1) is considerably slower than the preceding reactions, and has a half-time of 2.1 sec for a calcium concentration of 125 μM. Expanded time scale recordings of this phenomenon show no fast initial step. This reaction is in part inhibited by uncoupling agents.

4. Discussion

The shift of the emission peak of ANS to shorter wavelengths (cf. fig. 1) is characteristic of a change in ANS environment from one of high to low dielectric

constant. A more precise measure of the micro-environment of the chromophore is the spectroscopically derived "Z-value" [10]. Although the spectral shift observed when ANS binds to mitochondrial membranes corresponds to a dielectric constant of 35 (80% ethanol) [11]; this is probably an accurate measure of the polarity of the immediate environment of the dye. Butacaine and Ca^{2+} sufficiently alter the ANS environment to cause a further fluorescence increase but cause negligible change of the fluorescence depolarization.

When rat liver mitochondria are titrated with a fixed amount of ANS over 100 nmoles of ANS/mg, protein is required to give a maximal fluorescence. In the presence of butacain the fluorescence increases are larger but are generally parallel to those in the absence of butacaine.

In fig. 2A, high affinity binding sites are titrated, while all sites are eventually titrated in the procedure of fig. 2B. A butacaine-induced increase of the ANS binding sites is suggested by fig. 2A, although the increase of binding sites is smaller (corresponding to 4 to 6 nmoles of ANS per mg protein) than the fluorescence increase (100 to 200%).

Alternatively, a transition in the state of the membrane may be induced by butacaine, leading to an increased hydrophobicity, or to relative movements of ANS and its environment. A motion of water molecules out of the ANS environment, or electrostatic or other forces which would cause a greater penetration of ANS molecules into a phase boundary, or cause the phase boundary to move towards the ANS molecules, might account for the observed fluorescence increase. The idea that membrane transitions involve water movements that render the membrane environment more hydrophobic is consistent with theories of energy conservation in which alterations of water accessibility cause hydrolysis of intermediates of oxidative phosphorylation. In summary, the greater fluorescence of the butacaine-supplemented membrane is indicative of a generalized increase of membrane hydrophobicity, regardless of the detailed nature of the effect.

The observation that calcium accumulation and the formation of a pH gradient are considerably accelerated by concentrations of butacaine similar to those effective in altering the ANS environment suggests that the kinetics of action of the calcium carrier are increased as the hydrophobicity of its environment is decreased [1,5,12].

Whatever may be the structural nature of the butacaine effects, the kinetic data indicate that the phenomenon occurs in approximately 200 msec, in a concentration-independent reaction. This is presumably the time required to "open" the membrane to increase the number of ANS binding sites, or to otherwise alter the state of the membrane as described above. It is of interest that the half-time for the slow phase of the ANS reaction is approximately the same value, suggesting that ANS itself may induce changes of the membrane. However, it is the butacaine and not the ANS that alters the rate of the calcium accumulation and membrane alkalinization. The rate at which calcium alters the ANS fluorescence is much slower than that caused by butacaine and is presumably related to the membrane alkalinization which involves pH-induced changes of the membrane structure.

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