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A SPIN-LABEL STUDY OF PLASMA MEMBRANES OF ADRENAL CHROMAFFIN CELLS

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Chromaffin-cell membranes were labeled with two nitroxide spin labels, one probing the interior of the membrane and one probing the interfacial region. Both spin labels indicate that the membrane undergoes a phase transition at about 26°C. An Arrhenius plot of acetylcholinesterase activity exhibits a discontinuity at 26°C, consistent with the existence of a phase transition at that temperature. Acetylcholine, which stimulates chromaffin cells to secrete catecholamines, and hexamethonium, a cholinergic blocker, do not affect the rotational correlation times of the spin labels. These results argue that cholinergic stimulation does not affect the fluidity of the chromaffin-cell membrane.

The chromaffin cells of the adrenal medulla secrete catecholamines (epinephrine and norepinephrine) in response to stress. The immediate stimulus triggering secretion is acetylcholine released by the splanchnic nerve. Acetylcholine causes catecholamine storage vesicles, the chromaffin granules, to migrate to the cell periphery, fuse with the plasma membrane, and release their contents into the extracellular space by exocytosis [1,2]. This 'stimulus-secretion coupling' requires extracellular calcium [2–4]. Acetylcholine thus initiates a number of events including changes in the permeability of the plasma membrane and fusion between the chromaffin-granule and plasma membranes.

It has been suggested that hormone binding may cause gross structural changes in plasma membranes [5]. In particular, Schneeweiss et al. [6] reported that acetylcholine increases the fluidity of chromaffin-cell plasma membranes and that hexamethonium, a cholinergic blocker, inhibits this effect. The implication is that changes in membrane fluidity mediate stimulus-secretion coupling. The above results were obtained using fluorescence depolarization to measure membrane fluidity. We report here results of a study using nitroxide spin labels to probe the chromaffin-cell plasma membrane.

Materials and Methods

Isolation of plasma membrane. Bovine adrenal glands were acquired from a local slaughterhouse. After separation of the cortex, medullae from about 25 glands were placed in approx. 150 ml of 0.3 M sucrose/10 mM Tris-HCl (pH 7.4) and minced using a Tekmar SDT Tissumizer. This was further homogenized in a Potter-Elvehjem tissue grinder equipped with a motor-driven teflon pestle. The homogenate was centrifuged at 750 × *g* for 10 min at 4°C. The supernatant was saved; the pellet was resuspended in the same buffer and centrifuged again. The two supernatants were combined and centrifuged at 27000 × *g* for 20 min at 4°C. The pellet was resuspended in 65 ml of 0.5 M

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Abbreviations: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl) ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; 2N14, 2-dodecyl-2,5,5-trimethyloxazolidine-*N*-oxyl; 7N14, 2-heptyl-2-hexyl-5,5-dimethyloxazolidine-*N*-oxyl; τ_{rel}, relative rotational correlation time.

sucrose/10 mM Tris-HCl (pH 7.4). Aliquots (20 ml) were layered over 14 ml of 1.4 M sucrose/10 mM Tris-HCl(pH 7.4) and centrifuged at 82 500 × *g* (25 000 rev./min in a Beckman SW27 rotor) for 30 min at 8°C. The interface was collected and diluted to 50 ml with 10 mM Tris-HCl (pH 7.4). Aliquots (24 ml) were layered over 10 ml of 8% Ficoll (w/v) in 0.3 M sucrose/10 mM Tris-HCl (pH 7.4) and centrifuged at 82 500 × *g* for 90 min at 8°C. The interface, which contained purified plasma membranes, was collected and used immediately. All experiments were completed within 15 h of the cattle being slaughtered.

Electron spin resonance. For spin labeling, plasma membranes were diluted to 34 ml with Brooks solution (135 mM NaCl/10.7 mM KCl/10 mM glucose/5.7 mM Hepes (pH 7.4)). These were pelleted by centrifugation at 48 000 × *g* for 20 min at 8°C and resuspended in 4 ml of Brooks solution. The spin label (36.7 nmol of 2N14 or 39.7 nmol of 7N14) was placed in the bottom of a 6 × 50 mm test tube and solvent removed by evacuation. Then, 90 μl of the membrane suspension was added along with 11 μl 25 mM $CaCl_2$, 11 μl 0.25 mM neostigmine and 11 μl H_2O. For samples containing acetylcholine, 11 μl 5 mM acetylcholine was added instead of H_2O. Samples containing hexamethonium received 5 mM hexamethonium in the neostigmine solution. Final concentrations were 2.2 mM $CaCl_2$, 22 μM neostigmine, 0.45 mM acetylcholine and 0.45 mM hexamethonium. The spin-labeled membrane suspension was drawn up into a 1 mm inner diameter glass capillary and placed inside a 3 mm inner diameter quartz tube. Spectra were recorded at 8.98 GHz at 5 mW of incident microwave power in a Varian E-109E spectrometer equipped with an E-238 TM cavity and an E-257 gas flow temperature controller. Sample temperature was monitored with a copper-constantan thermocouple placed adjacent to the capillary in the quartz sample tube.

Relative rotational correlation times (τ_{rel}) were determined from the relative heights and widths of the spin-label hyperfine lines using the following equation [7]:

$$\tau_{rel} = 6.5 \cdot 10^{-10} \cdot W_0\left(\sqrt{h_0/h_{-1}} - 1\right)$$

W_0, h_0 and h_{-1} are the first-derivative peak-to-peak mid-field line width, mid-field line height, and high-field line height, respectively. The high-field line height was taken as twice the height of the upward deflection of the first-derivative spectrum, because the downward deflection is distorted, particularly in the case of 2N14, by motional anisotropy and partitioning of the nitroxide reporter group into the aqueous phase [8]. The above equation gives relative rather than absolute rotational correlation times because $6.5 \cdot 10^{-10}$ is an estimated value [9]. The actual value depends upon the microwave frequency, the anisotropic *g* values, and the hyperfine tensors for the particular spin label in a crystal form. The latter parameters have not been determined for 2N14 and 7N14.

Assays. Acetylcholinesterase activity was assayed as described by Steck and Kant [10] using acetylthiocholine as substrate [11]. Protein was assayed using Biuret reagent calibrated against bovine serum albumin [12].

Materials. Spin labels were synthesized by a modification of the technique of Keana et al. [13] and purified to homogeneity on a silicic acid column. Acetylcholine chloride, hexamethonium bromide, acetylthiocholine chloride, neostigmine bromide, and 5,5′-dithio(2-nitrobenzoic acid) were from Sigma Chemical Co., St. Louis, MO. Ficoll 400, purchased from Pharmacia, was dialyzed before use.

Results and Discussion

Schneeweiss et al. [6] monitored the fluidity of chromaffin-cell plasma membranes by measuring the depolarization of 1,6-diphenylhexatriene fluorescence. They observed an increase in the fluidity of plasma membranes upon addition of acetylcholine or nicotine and reversal of this effect when hexamethonium, a cholinergic agonist, was added. Luly and Shinitzky [5] reported that insulin decreases the fluidity of the liver cell plasma membrane. This too was deduced from 1,6-diphenylhexatriene fluorescence depolarization.

Hare et al. [14] demonstrated that microviscosities measured by fluorescence depolarization do not always agree with those determined by spin labeling. Discrepancies arise because the probes themselves may perturb the membrane and they

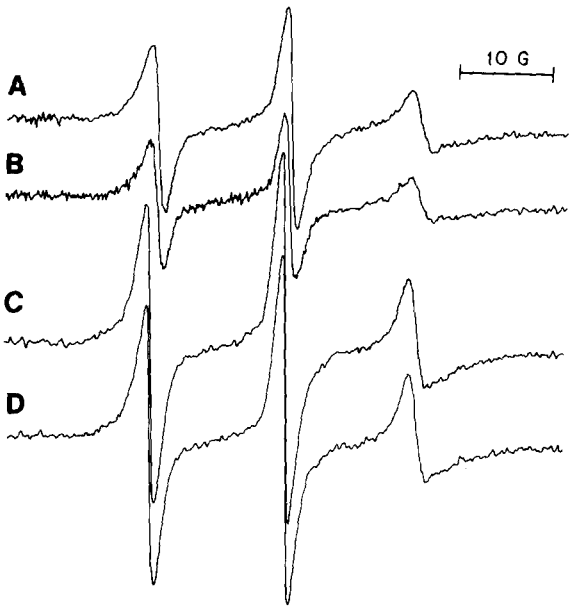

Fig. 1. ESR spectra of chromaffin-cell membranes labeled with 2N14. Membranes (122 μg protein in 90 μl) were mixed with 37 pmol 2N14, 11 μl 25 mM $CaCl_2$, 11 μl 0.25 mM neostigmine, and 11 μl of either H_2O or 5 mM acetylcholine. Spectra were recorded under the following conditions: (A) 23°C; (B) 23°C with acetylcholine; (C) 33°C; (D) 33°C with acetylcholine.

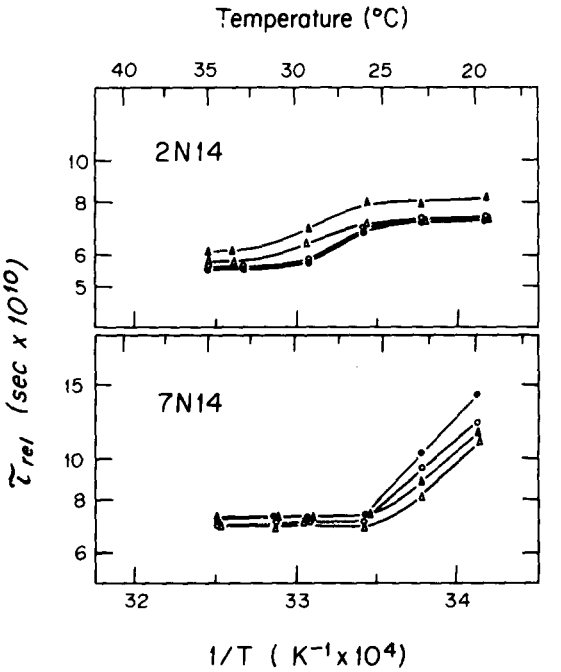

Fig. 3. Arrhenius plots of relative rotational correlation times. Spectra were recorded as shown in Figs. 1 and 2 with the following additions: (○) None; (●) 0.45 mM acetylcholine; (△) 0.45 mM hexamethonium; (▲) 0.45 mM acetylcholine and 0.45 mM hexamethonium. τ_{rel} was calculated as described in the text.

may partition differently between separated phases in the membrane. Kleinfeld et al. [15] reported that rates of translational diffusion of membrane proteins and lipid do not correlate with micro-

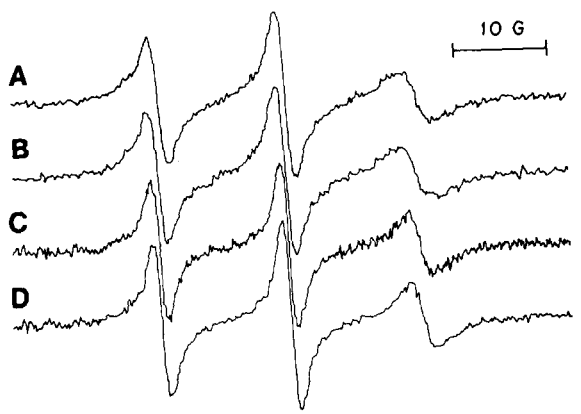

Fig. 2. ESR spectra of chromaffin-cell membranes labeled with 7N14. Membranes (116 μg protein in 90 μl) were mixed with 40 pmol 7N14, 11 μl 25 mM $CaCl_2$, 11 μl 0.25 mM neostigmine, and 11 μl of either H_2O or 5 mM acetylcholine. Spectra were recorded under the following conditions: (A) 23°C; (B) 23°C with acetylcholine; (C) 34.5°C; (D) 34.5°C with acetylcholine.

viscosities determined by fluorescence depolarization. Finally, in fluorescence depolarization studies, it is always possible that differences are caused by changes in the probe's fluorescence lifetime rather than by changes in its rotational mobility.

Because of the aforementioned problems, we felt it necessary to study the fluidity of the chromaffin-cell plasma membrane using electron spin resonance instead of fluorescence depolarization. Chromaffin-cell membranes were prepared by a rapid procedure to minimize deterioration. The purified plasma membranes had a high acetylcholinesterase activity (0.86 μmol/min per mg protein at 25°C). Others have reported specific activities of 0.35 [16], 0.23 [17] and 0.43 [6] μmol/min per mg protein.

Two nitroxide spin labels, 2N14 and 7N14, were used to monitor changes in membrane fluidity. 2N14 probes the part of the membrane close to the polar region while 7N14 monitors a region about half-way into the phospholipid interior [8].

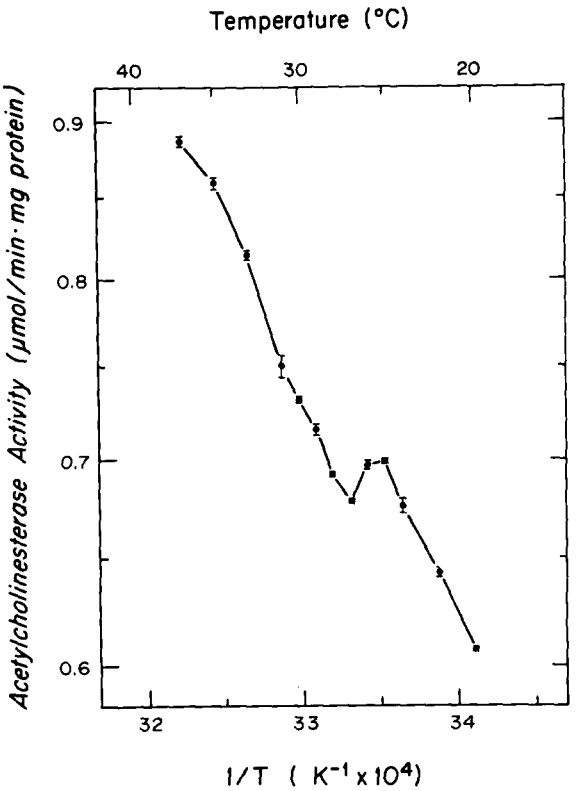

Fig. 4. Arrhenius plot of acetylcholinesterase activity. Membranes (106 μg protein in 50 μl) were mixed with 2.7 ml 100 mM sodium phosphate buffer, pH 7.5 and acetylcholinesterase activity assayed as described in Methods. Each plotted value is the average (± standard devation) of four assays.

Figs. 1 and 2 show spectra of 2N14 and 7N14 in chromaffin-cell plasma membranes. From a comparison of the spectra, it is apparent that temperature affects the motion of the spin labels whereas acetylcholine does not. Arrhenius plots of relative rotational correlation times confirm this (Fig. 3). A decrease in τ_{rel} indicates an increase in the motional freedom of the spin label and a corresponding increase in the fluidity of the chromaffin-cell membrane. The Arrhenius plots for both spin labels change slope at about 26°C, indicating that a phase transition in the membrane may occur at that temperature.

Acetylcholinesterase activity also exhibits a break at 26°C (Fig. 4). Since acetylcholinesterase is a specific marker for the plasma membrane, the correlation suggests both that the discontinuity in acetylcholinesterase activity is caused by a phase transition and that the spin labels are measuring a transition in the plasma membrane rather than in some contaminant. In contrast to the results of Schneeweiss et al. [6], we find no effect of acetylcholine or hexamethonium (Fig. 3). To be certain that acetylcholine was not degraded during the course of the experiment, neostigmine was added to inhibit cholinesterase activity.

In our studies, membranes were suspended in the same medium as that used by Schneeweiss et al. [6]. Our preparation procedure differs, but our membranes were prepared and tested more rapidly. If acetylcholine binding causes a physiologically important change in membrane fluidity, it should have been detectable using electron spin resonance. Moreover, for the acetylcholine receptor to facilitate exocytosis of the catecholamine storage vesicles through an effect on membrane fluidity, the receptor would have to be near the site of exocytosis. While this might be imagined in the chromaffin cell, it is hardly plausible in the related postganglionic sympathetic neuron. In these cells, acetylcholine receptors are located in the ganglion while exocytosis occurs in the target organ. Given these considerations and our observations that acetylcholine does not affect spin label rotational mobilities, we feel there is no reason to believe that acetylcholine-dependent membrane fluidity changes are important in stimulus-secretion coupling.

Acknowledgement

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