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LIPOSOMES

EFFECT OF TEMPERATURE ON THEIR MODE OF ACTION ON SINGLE FROG SKELETAL MUSCLE FIBERS

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Summary

Liposomes containing the fluorescent dye 6-carboxyfluorescein were made from dipalmitoyl phosphatidylcholine and stearylamine. At 4°C the liposomes are adsorbed on the fiber surface and when the temperature is raised to 21°C, their contents are transferred directly into the fibers at a linear rate. Liposomes had little effect on the time course of the maximal twitch tension.

Interactions such as fusion, lipid exchange and endocytosis between liposomes and cells have resulted in the alteration of membrane components, cytoplasmic contents and subcellular organelles of the cells [1—5]. The mode of interaction is dependent on the composition and the content of the liposomes and the type of cell. Whether such interactions also take place between liposomes and muscle cells remains to be determined. The finding of liposome lipid and aqueous contents associated with heart and skeletal muscle tissues after the liposomes are injected into animals and human subjects certainly suggests that some of those interactions do take place [6—9], but the exact mechanism is uncertain. The content of the liposomes could have reached the muscle tissues after leaking out of the liposomes or other cells. Similarly, the liposomes could have disintegrated in the circulation system and reached the muscle tissues as free lipid. We decided to explore the interaction of liposomes with isolated muscle fibers where the conditions are well defined and can be controlled. We report here the use of liposomes composed of

Abbreviation: EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid.

dipalmitoyl phosphatidylcholine and stearylamine to introduce a fluorescent dye into frog skeletal muscle fibers and the effect of temperature on their mode of action. Some preliminary results have been presented [10—11]. Similar experiments on the interaction of liposome-entrapped fluorescent dyes with other cells have been described [12—14].

Liposomes were prepared by sonicating dipalmitoyl phosphatidylcholine and stearylamine (molar ratio, 10:1) in a dye solution under N_2 at 45° C until clarity was obtained. Stearylamine was used to render a positive charge to the liposomes. The liposome suspension was rapidly lowered to room temperatures (20—22° C) and then passed through a Sephadex G-50 column (0.9 × 50 cm) equilibrated with calcium-free Ringer solution to remove untrapped dye. Electron micrographs showed that the liposomes are unilamellar with an average diameter of 335 Å. To check the charge of the liposomes, their electrical mobility in a small capillary tube was observed with a fluorescence microscope. When a constant current was applied across the capillary tube the fluorescent liposomes were observed to migrate towards the negative end, indicating that they are positively charged. The concentration of the liposomes was measured by light scattering. Trapped dye leaked out of the liposomes with a half-time of 4 days at 21° C. Under experimental conditions, no more than 75 μM of free dye was in the liposome suspension.

Single muscle fibers were dissected from the semitendinosus or ileofibularis muscle of the frog, *Rana pipiens*, captured wild in northern California. Connective tissues and nerve endings were removed by first treating the fibers with collagenase (0.5—1 mg/ml) for 30 min and then dissecting away the filamentous material and synapses. The fibers were washed with Ringer solution and observed with a Zeiss Standard microscope equipped with phase-contrast and epi-fluorescence systems. Fluorescence micrographs were taken on Kodak Tri-X film at ASA 1200. The film was developed in Diafine. Fluorescence intensity was measured with a UTD-500 photodiode-amplifier (United Dector, Inc., CA). Excitation and emission wavelengths were 480 and 520 nm, respectively.

The composition of the dye solution was 200 mM recrystallized 6-carboxyfluorescein (Kodak), 0.5 mM EGTA and 10 mM Tris at pH 7.5 and that of the Ringer solution 112 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 3 mM Tris at pH 7.2. Dipalmitoyl phosphatidylcholine was purchased from Calbiochem and stearylamine from Aldrich. Purity of the lipid was checked by thin-layer chromatography and the lipid was used without further purification.

Fig.1 compares the uptake of fluorescence by two single fibers bathed at different temperatures in a suspension of liposomes containing the fluorescent dye 6-carboxyfluorescein. The intensity of the fiber bathed at 4° C (curve B, Fig.1) followed an S-shaped curve reaching a plateau. In contrast, the intensity of the fiber bathed at 21° C (curve A, Fig.1), instead of reaching a plateau, began to increase linearly. We were unable to determine whether the intensity would reach a plateau during longer incubation times because of local damage to the fibers from repeated washing.

A fluorescence micrograph (Fig.2B) taken when curve B of Fig.1 reached

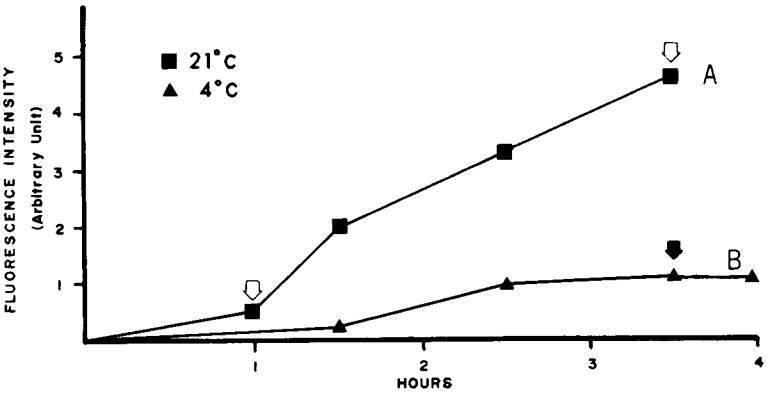

Fig.1. Effect of temperature on the fluorescence intensity of single frog skeletal muscle fibers bathed in a suspension containing the fluorescent dye 6-carboxyfluorescein. (A) 21°C, fiber diameter 65 μm, liposome concentration 1.3 mg lipid/ml. (B) 4°C, fiber diameter 50 μm, liposome concentration 1.1 mg lipid/ml. Note that at 21°C the intensity did not reach a plateau. The arrows (⬇ and ⇩) indicate the times when fluorescence micrographs were taken which are shown in Figs. 2 and 3, respectively. Liposome composition: dipalmitoyl phosphatidylcholine and stearylamine at a molar ratio of 10:1.

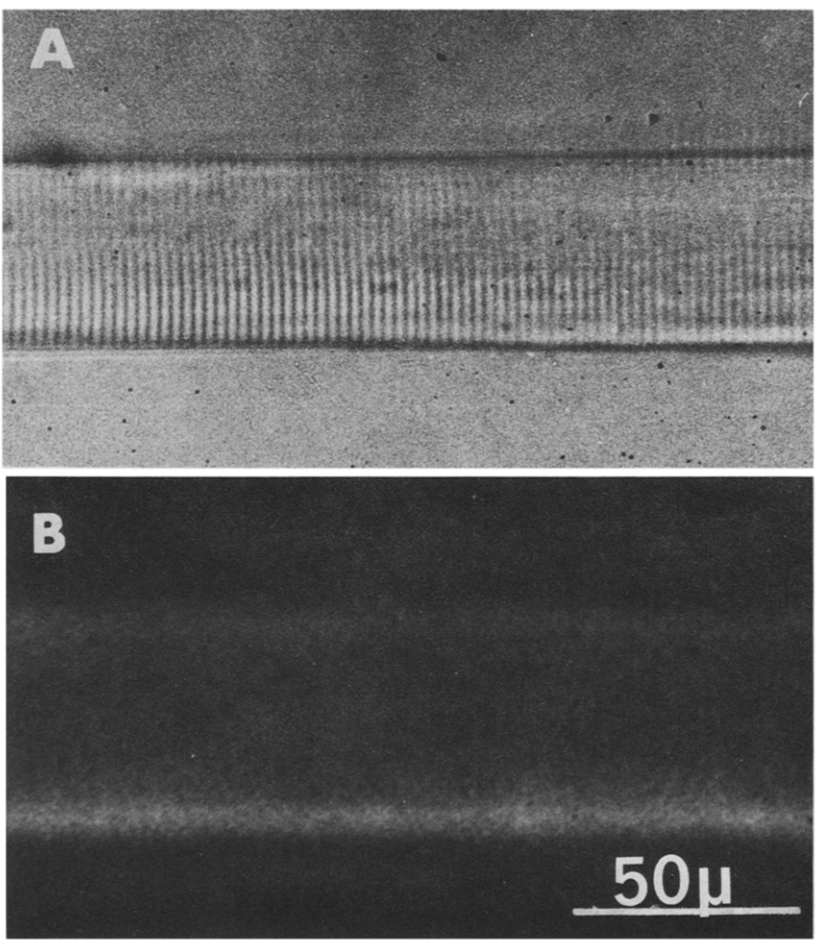

Fig.2. Phase-contrast (A) and fluorescence (B) micrographs of the fiber described in Fig.1, curve B. The micrograph was taken at the time indicated by the black arrow in Fig.1.

the plateau region shows that the observed intensity is due to the adsorption of liposomes on the surface of the fiber, giving the fiber two bright edges. The existence of an intensity plateau suggests that the surface of the fiber is saturated with liposomes. Fluorescence micrographs taken during the course of incubation at 21° C revealed a possible reason for the continuing increase of fluorescence without reaching a plateau. Fig.3A, taken during the early period of incubation, shows that the early part of curve A of Fig.1 is due predominately to the adsorption of liposomes on the fiber surface. Fig.3B, taken during the latter part of the incubation, shows that the linear part of curve A of Fig.1 is due predominately to an additional fluorescence coming from the myoplasm of the fiber.

To determine whether the fluorescence intensity arising from the myoplasm of the fiber was that which increases linearly, we separated the uptake of fluorescence due to adsorption of liposomes on the fiber surface from that of the myoplasm in the following way. Since the myoplasm became fluorescent at 21 but not at 4° C, we first incubated the fiber at 4° C in a suspension of liposomes until the surface of the fiber was saturated with liposomes, then we quickly raised the temperature to 21° C. Fig.4 shows that the fluorescence intensity indeed increased at a linear rate after the temperature change. Liposomes adsorbed on the fiber surface contributed little to this fluorescence increase for two reasons. Firstly, because of concentration quenching, the intensity of the liposomes is independent of the internal dye concentration in the range between 100 and 200 mM [15]. The liposomes used here contained 200 mM dye and after 4 days at 21° C half of the dye

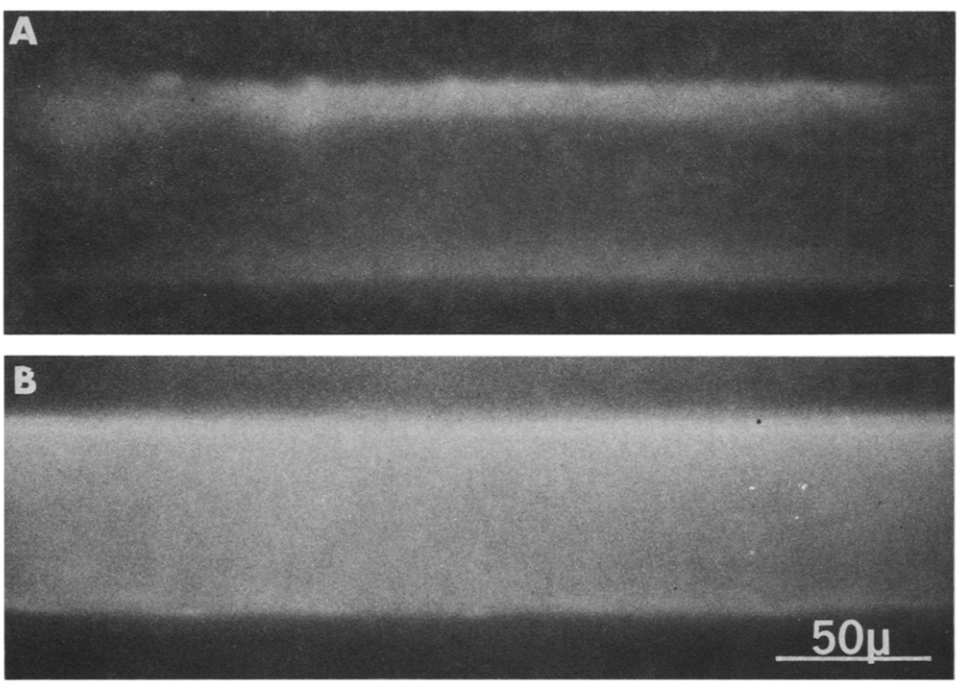

Fig.3. Fluorescence micrographs of the fiber described in Fig.1, curve A. The micrographs were taken at the time indicated by the white arrows in Fig.1. (A) 1 h and (B) 3.5 h.

still remained inside the liposomes. Secondly, the numerical aperture of the water immersion objective used in the intensity measurement was 0.65, the ratio of the fiber surface area to the area of the myoplasm of the optical section located at the fiber center would be approx. $1/R$ where R is the radius of the fiber in μm. The radius of the fiber of Fig.4 was 35 μm. Thus, the additional fluorescence came from an area of myoplasm 35 times larger than the area on which the liposomes are adsorbed.

Fibers treated with liposomes still gave propagated twitches and potassium contracture. Liposomes had little effect on the time course of the maximal twitch tension.

To determine whether the uptake of the dye at 21° C by the fiber was due to the dye leaking out of the liposomes and, consequently, either diffusing passively or endocytosed into the fiber, the following experiment was performed. Fibers were incubated at 21° C in a suspension of empty liposomes and 0.5 mM free dye. Under these conditions, the fibers were found not to be fluorescent. Therefore, free dye in the presence of empty liposomes does not enter the fibers.

We conclude from the results presented here that positively-charged liposomes composed of dipalmitoyl phosphatidylcholine and stearylamine are adsorbed on the surface of frog skeletal muscle fibers at low temperatures. At room temperatures, in addition to the adsorption on the surface of the fibers, the liposomes delivered their aqueous contents directly into the myo-

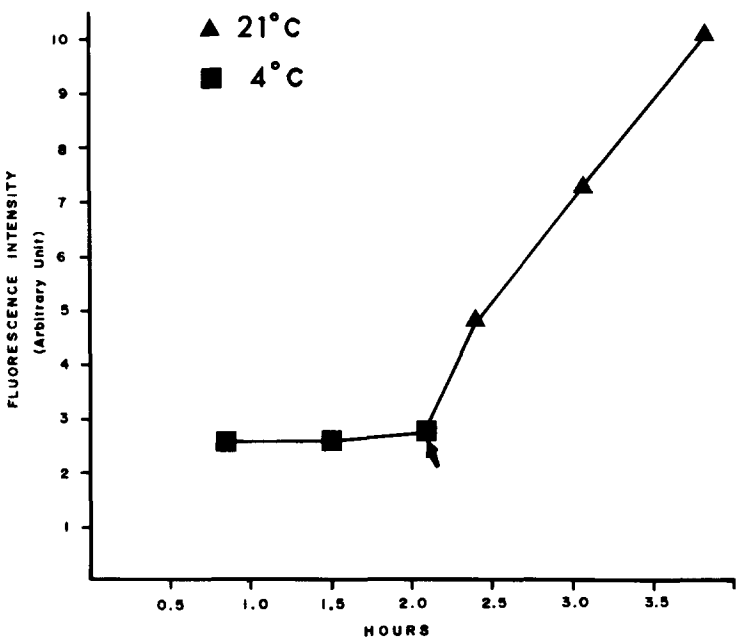

Fig.4. Effect of a fast temperature change on the fluorescence intensity of a single frog skeletal muscle fiber bathed in a suspension of liposomes containing the fluorescent dye 6-carboxyfluorescein. The arrow indicates the time when the liposome suspension was changed to another one at 21° C. The temperature change was completed within 30 s. Fiber diameter, 70 μm; liposome concentration, 1.5 mg lipid/ml; liposome composition, dipalmitoyl phosphatidylcholine and stearylamine at a molar ratio of 10:1.

plasm of the fibers without contacting the bathing medium. When the liposomes are saturated on the fiber surface, they deliver their contents at a linear rate into the myoplasm. These liposomes may be useful in delivering other materials into living muscle fibers without affecting excitability.

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