Addendum. In the article "Membrane Sealing in Frog Skeletal-Muscle Fibers", by De Mello, W. C., which appeared in the April 1973 issue of Proc. Nat. Acad. Sci. USA 70, 982-984, the author listed several cells where sealing of relatively large holes in their surface membrane occurs only in the presence of divalent cations, a phenomenon similar to Heilbrunn's "surface precipitation reaction." Inadvertently, the author failed to cite the paper "Junctional Membrane Permeability, Effects of Divalent Cations", by Oliveira-Castro, G. M. & Loewenstein, W. R. (1971) J. Membrane Biol., 5, 51-77, where similar observations were made on the non-junctional surface membrane of Chironomus salivary-gland cells. It is of interest that some of the results obtained in frog muscle differ from those obtained in Chironomus. In frog muscle Mg++ ions do not promote sealing but Sr++ ions do, whereas in Chironomus magnesium is effective and strontium is not. In addition, phospholipase A does not prevent the calcium-induced sealing in Chironomus, whereas phospholipase C markedly retards sealing in frog muscle.

Correction. In the article "A DNA-Binding Protein Induced by Bacteriophage T7," by Reuben, R. C. & Gefter, M. L., which appeared in the June 1973 issue of Proc. Nat. Acad. Sci. USA 70, 1846-1850, Figs. 3 and 4, p. 1848, were inadvertently transposed by the printer at press time. On page 1846, right-hand column, the section entitled Preparation of Phage Stocks should end with the sentence: "Titers of 3 × 10^10 phage per ml were obtained." A new section should have been inserted as follows: "Preparation of Cells. E. coli B was grown to a cell density of 7.5 × 10^8 cells per ml and infected with T7 am 147 at a multiplicity of 7.18 min after infection, the culture was poured over crushed ice 0.15 M in NaCl, harvested by centrifugation, and stored at −70°. Uninfected cells were prepared in an identical manner except for infection with phage."
Membrane Sealing in Frog Skeletal-Muscle Fibers
(calium/temperature/phospholipase C/proteases)

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ABSTRACT  Self-sealing, not found in frog skeletal muscle fibers immersed in Ringer's solution, can be induced by solutions rich in calcium ion. Strontium replaced calcium on the sealing process, but magnesium did not. The sealing accomplished in high-calcium media was preserved in those fibers reimmersed in normal Ringer's solution. Measurements of the rate of sealing at different temperatures indicated that self-sealing induced by calcium has a high activation energy. Phospholipase C, an enzyme that hydrolyzes membrane phospholipids, produced a marked depression on the rate of sealing. Trypsin or chymotrypsin had no influence on the sealing process.

It is known that when the surface cell membrane is damaged a protective mechanism comes into play insulating the cytoplasm from the external medium. This protection is accomplished by formation of a film that is continuous, at the boundary of the lesion, with the intact cell membrane. Concomitantly with the establishment of this new insulating barrier, the small drop of cytoplasm that usually protrudes through the damaged area coagulates.

This set of processes, described as a "surface precipitation reaction," has been observed in plant cells, in protozoa, in muscle cells of frog, rats, and crabs, and in marine eggs (1). The plug formation in muscles and in marine eggs require Ca++ ions; in a calcium-free medium no surface precipitation reaction occurs (1). Too much calcium, however, also impedes the reaction (1).

Although the establishment of a plug at the injured site certainly prevents the flow of materials between the intra- and extracellular spaces, it remained doubtful whether such a plug represents an electrical insulating barrier. It had been shown, for instance, that the flow of depolarizing current originated by cutting of a skeletal muscle fiber spreads along the fiber, which becomes depolarized for an indefinite period of time (2). Since no specific information was available on this point and—in general—on the effects of microlesions of the surface membrane of skeletal-muscle fibers, I decided to investigate this problem, as well as the influence of some physical and chemical factors on the sealing process. This note summarizes my preliminary observations.

MATERIALS AND METHODS

Sartorius muscles of frogs were used. The muscles were dissected and mounted in a transparent chamber filled with Ringer's solution. In some experiments isotonic calcium (83 mM CaCl2) or strontium solutions (83 mM SrCl2) with 2 mM KCl added were used. Phospholipase C and trypsin were obtained from Sigma Chemical Co. Measurements of membrane potential were made with conventional KCl-filled microelectrodes. Input membrane resistance (V/I) was recorded by implantation of two microelectrodes close together in the same muscle fiber by the technique of Fatt and Katz (3). Injuries were introduced by perforation of the cell membrane with a third glass micropipette (empty); different tip diameters were used. Lesions were produced by displacement of the micropipette in and out of the cell membrane, while the input resistance was continuously monitored. Usually, the inward movement of the micropipette did not exceed 8 μm, to avoid intracellular damage.

RESULTS AND DISCUSSION

Sartorius muscle fibers can be impaled many times with a microelectrode (0.5-μm tip diameter) without a significant drop in resting potential. Thus, the holes produced by successive punctures must be rapidly sealed. However, when the fibers are punctured by micropipettes with larger tips, an irreversible fall of membrane potential and resistance occurs. Fig. 1 (average from 25 fibers) shows the reduction of resting potential (A) and input resistance (B) of skeletal muscle fibers immersed in Ringer's solution (with 2 mM Ca) that occurred when they were punctured with a micropipette with an outside tip diameter of about 4 μm. As can be seen, no self-sealing follows the damage. Cardiac muscle fibers immersed in saline solution with 2 mM Ca and punctured with similar micropipettes display self-sealing and complete repolarisation in about 25 sec (4). In heart fibers, calcium is essential for the sealing process, since calcium-free solutions (plus EDTA) reduce the rate or suppress completely the sealing process (5, 6). The fact that frog skeletal-muscle fibers did not show self-sealing at 2 mM Ca led me to suspect that the sealing process in this muscle might require a higher calcium concentration. To investigate this possibility, sartorius muscle fibers were immersed in isotonic calcium solution. After 10 min of exposure to high calcium concentration, the resting potential increased from -84 mV (SEM ±1.2), in Ringer's solution to -96 mV (SEM ±0.96) (average from 25 fibers), in agreement with observations reported by Takeuchi (7). The input resistance also increased, as seen in Fig. 2C. As shown in Fig. 2A, the depolarization produced by injury with a 4-μm (tip diameter) micropipette was rapidly reversed when the muscle was kept in this isotonic calcium solution. Fig. 2B and C shows the average sealing curves recorded from 25 fibers damaged with a similar micropipette. Complete sealing was achieved 8-10 sec after lesion. Self-sealing also occurred after injuries large enough to reduce the resting potential by 50 mV or more. Strontium can replace calcium in
this respect, as illustrated in Fig. 3C (average from 20 fibers). No self-sealing was seen, however, when the muscles were immersed in isotonic MgCl₂ solution free of calcium.

Fibers injured in isotonic calcium solution remained sealed when they were transferred back to Ringer’s solution. Measurements of resting potential performed after 2 hr of immersion in normal Ringer’s solution gave a mean value of −84 mV (SEM ±1.05; average from 25 fibers), which is the value obtained before incubation of the muscle in isotonic calcium solution. Although the integrity of the sealed spots is preserved in Ringer’s solution, the rest of the cell membrane did not show self-sealing when damaged, in this medium. This result indicates that immersion in solutions of high calcium concentration does not cause long-lasting changes in the properties of the intact muscle-cell membrane.

The sealed area, easily visualized under a microscope, is extremely labile, since its perforation with a micropipette (0.5 µm in diameter) usually leads to a depolarization of 20% or more.

Even in isotonic calcium solutions, extremely large lesions cannot be sealed. When fibers are cut transversely, for instance, complete and irreversible loss of membrane potential is found.

Measurements of the rate of sealing in isotonic calcium solution at 25° and at 10° demonstrated that at lower temperatures the self-sealing process is largely impaired, or even suppressed. Fig. 3B (average from 20 fibers) shows the depressant action of reduction of the temperature in skeletal muscle fibers immersed in isotonic calcium solution. These results indicate that self-sealing has a high activation energy.

The studies of Heilbrunn (1) have shown that the surface precipitation reaction is also reduced, or even prevented, by cooling of the preparation.

Although the mechanism by which calcium promotes sealing in frog skeletal muscle is not clear, some observations suggest that membrane lipids might be involved in this process. Moreover, calcium induces coalescence in phospholipid films (8). Thin artificial lipid membranes are known to show self-sealing after puncture with large micropipettes (50 µm in diameter) (9). For this reason, I investigated the effect of phospholipase C, an enzyme that hydrolyzes membrane phospholipids (mainly lecithin), on the sealing promoted by solutions of high calcium concentration. Muscles were dissected and kept in Ringer’s solution. Measurements of membrane potential were performed, and then phospholipase C (25 µg/ml) was added in saline solution. A slow and gradual
depolarization starts; when the resting potential was reduced from $-84$ mV (SEM ±1.1) to $-50.9$ mV (SEM ±1.4) [average from 20 fibers—in agreement with other observations (10)], the Ringer's solution was replaced by isotonic calcium solution containing the same concentration of enzyme. There was a slight increase in resting potential [from $-50.9$ to $-58$ mV (SEM ±1.16), probably explained by the fact that high calcium concentration counteracts, in part, phospholipase action on membrane potential (10)]. Fig. 3A (average from 25 fibers) shows the influence of phospholipase C (25 µg/ml) on the rate of sealing of sartorius muscle. The sealing process is largely impaired in those fibers in which the enzyme produced a marked fall in resting potential. In fibers with a resting potential of $-40$ mV or less, small lesions (2 µm in diameter) usually led to complete loss of resting potential, despite the presence of a solution rich in calcium ion. The impairment of the sealing process found in fibers treated with phospholipase C could indicate that integrity of the lipid organization of muscle-surface membrane is essential for sealing to take place.

On the other hand, proteins of the membrane surface do not seem to be as important as lipids in the sealing process, since experiments upon muscles treated with trypsin (200 µg/ml) for 60–70 min showed no change on the self-sealing reaction induced by calcium ion.

The fact that the occurrence of the surface precipitation reaction in skeletal muscle (a) is not necessarily accompanied by "electrical" sealing and (b) requires small amounts of calcium, but (c) is prevented by high calcium concentrations (1) indicates that the process involved in self-sealing induced by calcium solutions is more complex than the surface precipitation reaction, and requires further investigation.

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5. Delesse, J. (1965) in Electrophysiology of the Heart, ed. Tac