BBA 75121

CALCIUM EFFECTS IN THE ELECTRICAL EXCITABILITY OF 'SPLIT' FROG SKIN

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(Received October 16th, 1967)

SUMMARY

Electrical excitability, resting potentials, and short circuit currents were found in the epidermis (dermal layer removed) of isolated frog skin. Replacement of the outside Ringer's solution with 10 mM EDTA (calcium-free) Ringer's solution extinguishes the all-or-none response in 2 to 10 sec and simultaneously lowers the skin resistance 5–30 %. Excitability can be restored if the EDTA–Ringer's solution is quickly replaced with a 'calcium-free' solution of relatively low electrical conductivity. EDTA–Ringer's solution on the inside of skin with the dermis removed requires more than 30 min to eliminate excitability. Excitation phenomena in 'calcium-free' solutions are discussed in terms of calcium-dependent leakage paths and membrane-bound calcium.

INTRODUCTION

The discovery of the electrical excitability of isolated frog skin (FINKELSTEIN[1]) has provided another tissue in which excitation phenomena can be studied. The excitation process in frog skin occurs at rather high skin potentials (200–300 mV) and thus appears to be unrelated to any physiological function. However, its existence seems to be more than a curiosity. Excitation may be a consequence of the specialized transport properties of particular tissues, or it may be a general property of all biological membranes which is revealed by proper conditions.

Frog skin is composed of an epidermis containing 1 or 2 outer layers of partially cornified cells (stratum corneum), 3 to 4 intermediate layers (stratum granulosum and stratum spinosum) and a basal layer of cells (stratum germinativum). An underlying dermis or corium contains blood vessels, glands and various fibrillar or cellular connective tissue. Electron microscope studies of amphibian skin (FARQUHAR AND PALADE[2]) have revealed belts of cell membrane fusion which obliterate the intercellular space between adjoining cells of the outermost layer of the stratum corneum and bind these cells into a continuous uninterrupted sheet. These tight junctions at the outside surface very likely close off the interior of the skin from open communication with the outside solution. Water, ions, and other substances may be forced to move through the cells of the outer cornified layer if they are to reach the inside of the skin. Thus tight junctions probably represent high electrical resistance paths

which force an applied current to pass through the cell membranes of the outermost surface of the epidermis. Studies of other epithelia show that calcium maintains the tightness of these junctions which open in the presence of EDTA (SEDAR AND FORTE[3], HAYS, SINGER AND MALAMED[4]). Furthermore, treatment with chelators irreversibly destroys intercellular communication through these junctions (LOEWENSTEIN[5]). Although the essential role of calcium in the excitation of single cells has been recognized for many years, its role in the excitation of frog skin is probably complicated by the calcium requirement of multicellular structures to maintain cellular associations.

Experiments reported in this paper are directed toward a clarification of the role of calcium in the excitation of frog skin. Furthermore, by the introduction of a 'split' skin preparation, evidence is presented that the excitation process resides within the epidermis portion of the frog skin.

MATERIALS AND METHODS

Grass frogs of both sexes were used during all seasons of the year. To prepare split skins, the dermis was removed employing a modified procedure of SKJELKVALE, NIEDER AND HUF[6]. The isolated abdominal skin was placed in Ringer's solution (115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 8 mM Tris, and 2 g per l of glucose (pH 8.0)) for 15–20 min and subsequently laid flat, and pinned to a cork board in a pool of 4 mM $NaHCO_3$ (pH 7) with the dermis side up. The dermis could be peeled off by first tearing its edge with a scalpel, and then enlarging the edges of the tear by scraping. An area larger than 2 cm^2, free of dermis, was easily obtained. Both intact and split skins were rinsed in Ringer's solution and mounted between two Lucite chambers with 1 cm^2 of skin exposed to the experimental solutions bubbled with air on both sides. A Tektronix type 161 pulse generator in series with 100 kΩ was used to apply current pulses to the solution and skin through platinized-silver electrodes. The potential difference across the skin was obtained from 2 calomel electrodes which communicated through 3 M KCl–agar bridges with Ringer's solution filled polyethylene tubes positioned parallel to and against the inside and outside surfaces of the skin. 2 high-input impedance amplifiers, a difference amplifier and a Tektronix 564 storage oscilloscope enabled display of the potential responses. An operational amplifier was used to record the current through the skin. The input to the operational amplifier was connected to the inside platinized-silver electrode providing a virtual ground at this point. The current through the skin was collected at this current electrode and provided an input to the amplifier. The voltage output of the amplifier was thus proportional to this current.

RESULTS AND DISCUSSION

Properties of split skin

Transport properties of the split-skin preparation (which consists simply of the epidermal layer alone) correspond very favorably with similar properties in the intact skin. When bathed in Ringer's solution on both sides the open circuit potential across the split skin ranged from 2 to 42 mV (mean $\pm$ S.E. $=$ 22.6 $\pm$ 1.8 mV) and the short circuit current ranged from 9 to 150 $\mu A/cm^2$ (mean $\pm$ S.E. $=$ 48.6 $\pm$ 5.4 $\mu A/cm^2$) for 37 split skins. The resistance of split skin ranged from 150 to 600 $\Omega \cdot cm^2$. Further,

each of these skins displayed the typical all-or-none response characteristic of excitation (Fig. 1). The excitable process as well as the source of short circuit current (presumably the sodium pump) occurs in the epidermis without the underlying dermis.

Role of calcium

FINKELSTEIN[7] observed that variations in calcium concentration of the Ringer's solution bathing the inside of isolated frog skin have no immediate effects upon the all-or-none response, but that excitability is sometimes lost following a 30-min exposure to calcium-free Ringer's solution. Effects upon excitability after reducing the calcium concentration in the outside Ringer's solution required 5 to 15 min to develop although changes were apparent in a few seconds. He also reported that half of the skins were excitable with calcium-free Ringer's solution outside. We have confirmed these observations, but we find that excitability occurs very readily with a calcium-free isosmolar sucrose solution* containing a small amount of NaCl (200 mM sucrose, 10 mM NaCl, 8 mM Tris, pH adjusted to 8.0 with H_2SO_4) bathing the outside surface and Ringer's solution on the inside. In addition, all-or-none behavior can be induced in some inexcitable skins by replacing the outside Ringer's solution with the calcium-free sucrose solution.

Effective removal of calcium frequently requires a chelating agent such as EDTA. When this agent is applied to either the inner or outer surface of intact or split frog skin, we find that excitability disappears. The time required for loss of excitability in these two cases was measured as an aid in localizing the calcium-dependent process.

In these experiments, the Ringer's solution was modified by substituting 10 mM EDTA for $CaCl_2$ to obtain a calcium-free EDTA–Ringer's solution. An above threshold

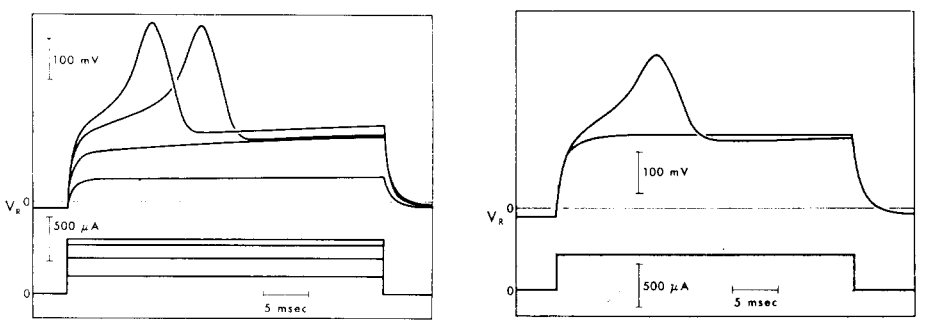

Fig. 1. Electrical excitability of split frog skin (dermis removed). (Upper traces) Potential responses between the outside and inside surfaces of split skin. (Lower traces) Successively applied rectangular pulses of increasing current through the skin. The spike with minimum latency occurs for the highest current pulse. The time interval between successive current pulses is 50 sec. V_R is the resting potential. 1 cm^2 of split skin.

Fig. 2. Effect on excitability of replacing outside Ringer's solution with (calcium-free) EDTA–Ringer's solution. All-or-none response with Ringer's on both sides disappears in 2 sec when outside Ringer's solution is replaced with EDTA–Ringer's solution. In this and subsequent figures, solution replacement was made after the skin was out of its refractory state. Current pulse amplitude is the same for both potential responses. V_R is the resting potential. 1 cm^2 of split skin.

* The calcium contamination in this solution was found to be about 4 μM by atomic absorption spectrophotometry.

current pulse was applied to the skin so that an all-or-none potential response was obtained with Ringer's solution on both sides of the skin (Fig. 2). Keeping the applied current pulse amplitude constant, the outside Ringer's solution was replaced by EDTA–Ringer's solution and the all-or-none response disappeared in 2–10 sec (mean value $\pm$S.E. $= 5.0 \pm 0.6$ sec for 26 skins). The loss of the all-or-none response is complete and is probably not due to an increase in threshold since increasing stimulus intensity up to 3 times threshold produces only a graded overshoot response. A drop in subthreshold skin resistance of 5–30% (mean value $\pm$S.E. $= 14.5 \pm 0.6$% for 16 skins) also occurs (not shown in the figure) in the interval in which excitability is lost. Return of Ringer's solution (containing calcium) to the outside surface after the EDTA replacement restores the all-or-none response in less than a minute. This reversibility can be demonstrated repeatedly in the same skin provided the EDTA–Ringer's solution is not allowed to remain outside for extended periods. The current threshold, however, rises after each EDTA treatment. In contrast to the rapid action of EDTA in the outside solution, replacement of inside Ringer's solution by EDTA–Ringer's solution outside requires more than 45 min for elimination of excitability in skin with a dermis and more than 30 min for skin without a dermis. From these results three points are apparent: (1) The action of EDTA confirms the crucial role of calcium in the excitation process. (2) The large difference between the time of action of EDTA on the outside from that on the inside suggests that the excitable process occurs near or at the outward-facing epidermal surface. This may explain why skins which are shedding the outside surface have a low resistance and are frequently inexcitable. (3) Intercellular communicability through tight junctions was reported to be *irreversibly* disrupted by the action of chelators (LOEWENSTEIN[5]). Since the loss of excitability is *reversible* after calcium removal, intercellular communicability through tight junctions apparently is not a necessary requirement for excitability.

The observed decrease in skin resistance accompanying elimination of the all-or-none response after calcium removal suggests the existence of calcium-dependent shunt paths in the skin. These may involve (1) pathways through the tight junctions between the outermost cells and/or (2) pathways through the outermost cell membranes. It is not possible to distinguish between these two possibilities by indiscriminately removing calcium. Nevertheless, in either or both cases if a shunting of the excitable pathways occurs, it should be possible to increase the resistance of the shunt by using media of low conductivity and thereby restore excitability.

A sequence of outside solution substitutions was performed in order to test this hypothesis (Fig. 3, top). Maintaining the current pulse amplitude constant for all recorded skin potential responses, the following was observed. (1) The all-or-none response, (a), was recorded with Ringer's solution on both sides of the skin. (2) The outside Ringer's solution was replaced with EDTA–Ringer's solution and the all-or-none response was eliminated, (b), in 5 sec. (3) The EDTA–Ringer's solution (outside) was then replaced 30 sec after its introduction by the calcium-free isosmolar sucrose solution. The all-or-none response reappeared, (c), in 1 min. (4) The sucrose solution was finally replaced 2 min after its introduction by a sucrose solution of the same composition but with 1 mM EDTA in addition. The all-or-none response disappeared, (d); the response was a graded overshoot with no latency. (This overshoot response to sucrose *plus* EDTA can occur with concentration of EDTA as low as 100 μM.)

These results are obtained when mannitol is used instead of sucrose. In addition, this sequence has been conducted with the terminal substitution being calcium-free, EDTA-free Ringer's solution* instead of the sucrose solution with EDTA (Fig. 3, bottom). In this case, the excitability observed in the sucrose solution (without EDTA)

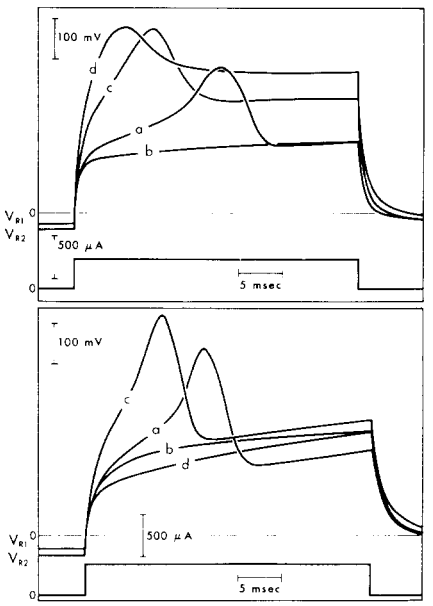

Fig. 3. (Top) Potential responses for a sequence of outside solution replacements. The current pulse amplitude is constant for all the potential responses. (a) All-or-none response with Ringer's solution on both sides. (b) Disappearance of all-or-none response 5 sec after replacement of outside Ringer's solution with EDTA–Ringer's solution. (c) Return of all-or-none response 1 min after replacing EDTA–Ringer's solution with low conducting isosmolar sucrose solution. (d) Overshoot response 1 min after replacing sucrose solution with a sucrose solution containing 1 mM EDTA. Variation of the current pulse amplitude showed that this response is graded with no latency. (Bottom) Same sequence as top (a–c). (d) Response 1 min after replacing sucrose solution with calcium-free Ringer's solution. Only a graded overshoot response is elicited upon increasing the current pulse amplitude. V_{R1} is the resting potential for Ringer's solution, EDTA–Ringer's solution and calcium-free Ringer's solution; V_{R2} is the resting potential for both sucrose solutions. 1 cm^2 of intact skin.

disappears. When the subthreshold skin resistance is monitored (not shown in the figure) during this substitution, it falls to a value below its initial value in Ringer's solution at the beginning of the sequence. Finally, the possibility that these results merely reflect fortuitous differences in the traces of calcium in the calcium-free sucrose and calcium-free Ringer's solutions must be accounted for. To exclude this interpretation, the trace calcium level in the calcium-free Ringer's solution was brought up to (4 μM) and beyond (as high as 500 μM) the trace calcium level in the sucrose solution. The results were not changed; the skin was not excitable in calcium-free Ringer's solution, but excitable in the calcium-free sucrose solution.

It is important to emphasize that these experiments do not bear on the actual

* The calcium contamination in this solution was found to be about 1.5 μM by atomic absorption spectrophotometry.

location of any calcium-dependent shunt that may exist. The results are equally consistent with a shunt pathway residing at cellular junctions or within cell membranes*. However, even with this ambiguity, the opening of simple shunt paths does not explain fully the following results of the above experiment: Excitability occurs in the sucrose solution but disappears in a sucrose solution with added EDTA ($\geqq$ 100 μM). This would follow if the skin were capable of re-supplying calcium when the EDTA is removed. However, this interpretation implies that excitability should return in the sequence terminating with calcium-free Ringer's solution. This did not occur (Fig. 3, bottom, trace d). Furthermore, if calcium merely served to reduce shunt pathways, then the excitable response should reappear in the calcium-free Ringer's solution but at an increased current threshold. This did not occur either. Although it is not possible from this data to determine whether calcium-dependent shunts exist in the tight junctions or in the outermost membranes, it appears that the effect of calcium removal upon excitability is probably complex.

An alternative interpretation is to consider the possibility that calcium is bound to the membrane and participates directly in the excitation process. Following EDTA treatment, excitation does not return in calcium-free Ringer's solution (high ionic strength) because the trace amounts of calcium present are insufficient to maintain an adequate level of bound calcium. In the calcium-free sucrose solution (low ionic strength) calcium is bound more firmly to the membrane so that excitation will occur even though the calcium level in solution is low.

In summary, we have not been successful in interpreting all of the results solely in terms of calcium-dependent shunts. This does not imply that these shunts do not exist, but it does suggest that other factors are operative. These may involve more subtle membrane phenomena which require membrane-bound calcium. The differences in excitability observed in these experiments would then be attributed at least in part to differences in the levels of bound calcium.

ACKNOWLEDGEMENTS

We thank Dr. K. S. COLE and Dr. J. G. FORTE for their interest and suggestions during this investigation. We also wish to thank Dr. H. SANUI for his measurement of the calcium contamination in our calcium-free solutions. H.M.F. is a predoctoral fellow in the Biophysics Group and was supported by National Institutes of Health Grant (5-T1GM829 06,1967). R.I.M. was supported by National Science Foundation (GB1928).

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* There is evidence for a leakage role for calcium in squid giant axons (ADELMAN AND TAYLOR[8], LECAR *et al.*[9]).