INHIBITION OF EMPTYING OF SKELETAL MUSCLE CELL SEGMENTS BY ADENINE NUCLEOTIDES AND POLYVALENT CATIONS

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ABSTRACT
A correlation had previously been established between actomyosin content of homogenized skeletal muscle cell segments as determined by extraction in strong salt solution and the ability of those segments to empty when extracted with buffered water. In this study, we examined the ability of certain compounds to inhibit the process of emptying. Adenosine triphosphate (ATP) and adenosine diphosphate (ADP), which dissociate actomyosin, inhibited the process of emptying, while adenosine monophosphate (AMP) which does not dissociate actomyosin, did not. We conclude that the formation of actomyosin is a necessary prerequisite for emptying and not just a secondary effect. Polyvalent cations were also found to inhibit emptying. The inhibition was reversible by washing with a solution of NaCl-histidine or with chelating agents, ethylenediaminetetraacetate (EDTA) and ethylene-glycol-bis(β-amino-ethyl ether) tetraacetic acid (EGTA). A factor(s) solubilized from aged muscle functions as an inhibitory agent; the suggestion is made that this factor(s) may be a polyvalent cation.

INTRODUCTION
The sarcolemma of the skeletal muscle cell is comprised of the plasma membrane, an unstructured middle basement layer, and an outer network of collagen fibrils (1). Several early investigators attempted to isolate sarcolemmas, but utilized methods that either destroyed structure or yielded small quantities (2-5). Reasonable yields of intact sarcolemmas were first obtained by Kono and Colowisk (6) from rat muscle by extraction of muscle cell segments with salt solutions of high ionic strength. A modification of this procedure utilizing ATP to disrupt the gel-like extract and make isolation easier was developed by Rosenthal et al. (9) for rat skeletal muscle and applied by Carroll and Sereda (10) in the isolation of the cell membranes of uterine smooth muscle. We developed a method based in large part on the procedure of McCollester (8); however, our method allowed greater variations in the conditions of preparation (11). The major factor which allowed
us to do this was the observation that aging of muscle postmortem induced changes in the muscle which allowed many of the restrictions of preparation to be removed.

However, the methods currently available for producing sarcolemmas give relatively low yields and the preparation requires considerable time. These factors are especially important if it is desirable to use low ionic strengths and pH, and to avoid the period of incubation. To improve the procedure it would be of great value to understand the mechanism by which the contractile proteins become water soluble and thus extractable. When we started our work, the only publications on the mechanism of emptying of muscle cell segments were those of McCollester and Semente (13, 14). These workers concluded that the extraction of the intracellular contents of the muscle cell was due to the removal of a restriction on the water solubility of the myofibrillar proteins, viz., the myofibrillar proteins are ordinarily water soluble but something in the cell protects these proteins from the dissolving action of water, and this must be broken down before the proteins can be extracted. They suggested that it is a "cytoskeleton" which is responsible for this effect and, further, that the cytoskeleton probably consists of the sarcoplasmic reticulum and Z membrane network. It was suggested that enzymic and ionic effects are important in the rupturing of the cytoskeleton, the enzymic process being favored by the incubation and the ionic by homogenization in the presence of Ca++.

Evidence was presented which showed a stabilizing effect of flavin adenine dinucleotide (FAD) on the cytoskeleton.

We undertook to investigate further the mechanism of emptying with the ultimate goal of preparing sarcolemmas of greater purity more rapidly. In an earlier report we established a correlation between the amount of emptying of muscle cell segments and the actomyosin content of the segments (15). In the present report we present work on some studies involving inhibitors of emptying. Results indicate that formation of actomyosin is an absolute prerequisite of emptying and not just a side effect of some other fundamental change.

**MATERIALS AND METHODS**

**Materials**

Domestic chickens of mixed breeds were used. They were maintained on a commercial pelleted diet fed ad lib. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Spermine and spermidine are products of Sigma Chemical Co., St. Louis, Mo. All other reagents were the purest available commercially.

**Procedure for Emptying Muscle Cell Segments**

The method used was that of Westort and Hultin (11). The birds were sacrificed by injection of air into the heart. The breast muscle, pectoralis major, was removed as quickly as possible and placed in water at 0°-4°C for 4 hr. 20 g of muscle was then homogenized for 4 sec in 200 ml of a solution of CaCl₂ (specific concentrations used are given in the text) with a Polytron Model BEW cutter (Will Scientific, Inc., Rochester, N. Y.). After straining through cheesecloth, the muscle cell segments were sedimented by a light centrifugation. The segments were resuspended in a solution which was 25 mm with respect to NaCl and 2.5 mm with respect to histidine, pH 7.4, by shaking lightly, and were recentrifuged. This wash with NaCl-histidine was repeated three more times. At this point the muscle cell segments were divided into two portions. One portion was suspended in Tris-buffered water which contained the substance to be tested as an inhibitor of emptying. In the case of the nucleotides, an incubation period of 1 hr in the cold was used. No incubation period was used for the polyvalent cations or the muscle extracts. The other portion of the muscle cell segments served as the control and was held for the same length of time and at the same temperature as the testing samples, except that the inhibitors were not added. Samples of both portions were then extracted in a large volume of water which had been buffered to pH 7.5 with Tris (final concentration about 1 mm). Approximately 400 ml of the extracting solution were used per 10 g of original muscle tissue. This represented a 20-fold increase in the volume of the extracting solution over that of the washing and inhibitor solutions. The emptying phenomenon occurs quickly and is over by the time a sample is ready for microscopic examination. There was no increase in emptying over the period of time ordinarily required to examine the samples, i.e., up to 1 hr. However, the possible effect of longer time periods was not studied. At this time, the empty segments could be distinguished from unemptied or partially emptied ones in the light or phase-contrast microscope. This is illustrated in Fig. 1. An empty cell segment is one that has lost all striations, both cross and longitudinal, and appears transparent, much like an empty cellophane bag open at both ends. It is not pure sarcolemma, nevertheless, and small quantities of...
certain subcellular fractions are usually associated with it (9, 11). The chordlike structure seen protruding from the lower right section of the emptied cell segment is not uncommon in our preparations (12) and has also been observed by other workers (6, 8). It may consist of a strand of endomysial collagen. Counting of empty segments was done as described below.

Cell Counting and Statistical Methods

The percentage of empty cell segments was determined for every sample by counting at least 50 fields in the light microscope. Segments were considered unemptied unless they were completely emptied. The percentage of empty segments was determined by dividing the number of empty segments by the sum of the empty plus the unemptied segments and multiplying by 100. Counts were made without regard to size of the cell segments, since it was observed that there was no correlation between ease of emptying and length of cell segments. Paired differences between treatments were tested for significance by means of the paired t test (16).

RESULTS

In a previous report, a correlation was established between the ability of muscle cell segments to empty when extracted with buffered water and their content of actomyosin as determined after extraction in strong salt solution (15). This does not necessarily mean that formation of actomyosin is the cause of emptying, since some other change could be the prime event in emptying and actomyosin formation could be simply another effect of this primary change. We wished to test the hypothesis that formation of actomyosin is a necessary factor in emptying of muscle cell segments. To do this, we treated muscle cell segments under conditions which would ordinarily result in emptying. However, before emptying, the segments were treated with substances (ATP and ADP) known to dissociate actomyosin in muscle fibrils (17) and to prevent the development of rigor mortis in vivo (18). Rigor mortis is generally considered to occur by the formation of cross-bridges between actin and myosin molecules (19). Results are given in Table I. We also tested the effectiveness of AMP which does not dissociate actomyosin. Both ATP and ADP partially inhibited emptying while AMP did not. The significance of the AMP results was between 0.05 and 0.1, but it was in the reverse direction, viz., AMP tended to enhance emptying.
Inhibition of Emptying by Adenine Nucleotides

Aged chicken muscle was homogenized in 0.5 mm CaCl₂ solution and washed four times in NaCl-histidine solution. The cell segments were treated with solutions of the adenine nucleotides immediately preceding the extraction step and incubated for 1 hr at 0°-4°C. Control samples were suspended in equal volumes of Tris-buffered water and incubated in the same way.

| Experiment | 5 mm ATP | Control | 5 mm ADP | Control | 5 mm AMP | Control |
|------------|----------|---------|----------|---------|----------|---------|
| 1          | 28       | 72      | 20       | 60      | 71       | 69      |
| 2          | 22       | 58      | 28       | 50      | 68       | 62      |
| 3          | 4        | 32      | 18       | 88      | 56       | 54      |
| 4          | 0        | 44      | 16       | 74      | 70       | 55      |
| 5          | 0        | 18      | 30       | 67      | 64       | 60      |
| 6          | 30       | 60      | 40       | 84      |          |         |
| 7          | 34       | 80      |          |         |          |         |
| 8          | 36       | 82      |          |         |          |         |
| 9          | 37       | 72      |          |         |          |         |
| 10         | 20       | 80      |          |         |          |         |

Significance: $P < 0.001 \quad P < 0.01 \quad P > 0.05$

The muscle cell segments were incubated for 1 hr at 0°-4°C in the presence of the nucleotides to allow ample time for diffusion into the cell segments. The low temperature would help to lower the ATPase activity of the segments as well as that of adenylate kinase or adenylate deaminase if they had not been removed during the washings. The latter two enzymes would cause the destruction of ADP and AMP, respectively.

The segments were incubated with the nucleotides at a concentration of 5 mm. A 20-fold dilution accompanied the treatment for emptying, resulting in a final concentration of the nucleotides of 0.25 mm during the extraction. Either this concentration of ATP and ADP is sufficient to partially inhibit emptying, or there is insufficient time during the extraction for bound nucleotides to be removed.

In early attempts to determine if there were substances in the muscle which inhibited emptying, we found that there was a heat-stable, dialyzable material in the supernatant fraction of the muscle homogenate which completely inhibited emptying. Since CaCl₂ had been added to the homogenizing medium, we tested to see if this substrate and other multivalent cations could prevent the muscle cell segments from emptying. Results are given in Table II. NaCl and KCl were also studied and did not inhibit emptying. This indicates that neither monovalent cation nor Cl⁻ is an effective inhibitory agent. Samples treated with cations were not incubated. The suspension of cell segments was centrifuged and the supernatant fraction was discarded before resuspension in the extracting medium. All multivalent cations tested were inhibitory. Mn⁴⁺ was especially potent. Spermine and spermidine are polycationic substances normally found in muscle. The levels of spermine and spermidine which were found in these experiments to produce 50% inhibition of emptying were 4-10 times lower than those levels previously found to inhibit Ca²⁺-activated actomyosin ATPase by 50%, and are of the same order of magnitude as those found in muscle tissue (20).

The effect obtained with CaCl₂ was completely reversible. If the muscle cell segments which had been treated with CaCl₂ were rewarshed with the NaCl-histidine solutions and then extracted with Tris-buffered water, the degree of emptying was equal to that found in samples not treated with Ca²⁺.

We interpret these results to show that the CaCl₂ added during homogenization is capable of inhibiting emptying, whether there be natural inhibitors present in the muscle or not. One of the reasons for washing with the NaCl-histidine is possibly to displace bound Ca²⁺ by an ion.
TABLE II

Inhibition of Emptying by Polyvalent Cations

Aged chicken muscle was homogenized in 50 mM CaCl₂ solution and washed three times in NaCl-histidine solution. The cell segments were treated with solutions of the cations immediately preceding the extraction step. All cations were used as the chloride salts and were buffered to pH 7.5 with Tris-Cl. Results represent the average of at least two experiments.

| Cation   | Final concentration (mM) | Empty cells |
|----------|------------------------|------------|
| Ca⁺⁺     | 5                      | 0          |
|          | 1                      | 27         |
|          | 0.5                    | 53         |
|          | 0.1                    | 79         |
| Mg⁺⁺     | 5                      | 14         |
|          | 1                      | 35         |
|          | 0.5                    | 71         |
|          | 0.1                    | 80         |
| Mn⁺⁺     | 1                      | 0          |
|          | 0.5                    | 0          |
|          | 0.1                    | 30         |
|          | 0.05                   | 44         |
| Sr⁺⁺     | 1                      | 24         |
| Spermine*| 1                      | 0          |
|          | 0.1                    | 9          |
|          | 0.05                   | 37         |
|          | 0.01                   | 57         |
| Spermidine*| 10                     | 0          |
|          | 1                      | 0          |
|          | 0.5                    | 14         |
|          | 0.1                    | 20         |
|          | 0.01                   | 32         |
| Na⁺      | 50                     | 69         |
|          | 1                      | 83         |
| K⁺       | 50                     | 71         |
|          | 1                      | 89         |
| Control (25 mM NaCl-2.5 mM histidine) | 75 |

* The average control value for the series of experiments with spermine and spermidine was 38%.

exchange phenomenon. Thus, if muscle has been homogenized in CaCl₂ solution, washing the segments with water instead of NaCl-histidine will produce no emptying (12).

If the Ca⁺⁺ added in the homogenizing medium inhibits emptying, the addition of chelating agents to the wash solution might remove this Ca⁺⁺ and reverse the inhibition. The effectiveness of EDTA and EGTA was evaluated by a single wash at a concentration of 10 mM with a sample brought to the point of emptying and then treated with divalent cations. Results are shown in Table III. EDTA was more effective than EGTA in reversing the inhibition of emptying by the cations. It is clear that chelators can reverse the inhibitory effect of divalent cations.

To determine if there are natural inhibitors of emptying present in the muscle cell, we homogenized muscle in water and collected the supernatant fraction of the homogenate by centrifugation. The supernatant fraction was tested in the usual way to see if it inhibited emptying, viz., it

TABLE III

The Effect of Chelating Agents on Emptying after the Addition of Divalent Cations

Aged chicken muscle was homogenized in 50 mM CaCl₂ solution and washed three times with NaCl-histidine. The cell segments were then treated with a solution of the chloride salt of the divalent cations at the concentrations indicated in the Table. After centrifugation the segments were resuspended in a 10 mM solution of either EDTA or EGTA. This was followed by extraction with Tris-buffered water in the usual way. The control was not subjected to the washings with divalent cation or chelator. The results represent the average of two determinations.

| Treatment                              | Empty cells | 10 mM EDTA | 10 mM EGTA |
|----------------------------------------|-------------|------------|------------|
| Control (25 mM NaCl-2.5 mM histidine)  | 75          |            |            |
| Ca⁺⁺, 5 mM                             | 83          | 0          |
|                                          |             | 1 mM       | 84         |
| Mg⁺⁺, 5 mM                             | 81          |            | 29         |
|                                          |             | 1 mM       | 88         |
| Mn⁺⁺, 1 mM                             | 70          |            | 14         |
|                                          |             | 0.5 mM     | 77         |
|                                          |             | 32         |
| Control                                | 86          |            |            |
was added to washed cell segments from aged muscle just before extraction with Tris-buffered water. Results of typical experiments are given in Table IV. The supernatant fraction from the homogenate of fresh muscle had no inhibitory effect; however, that from muscle aged for 10 hr at 0°-4°C partially inhibited emptying. Boiling the fraction had no effect on the inhibition, but dialysis eliminated it. We conclude that there is a natural inhibitor in muscle and that it is released on aging. That it is present in rather small amounts is indicated by the fact that 10 hr of aging are required to obtain enough to partially inhibit emptying. The level of polyvalent cations in muscle is high enough (20-22) to be the source of this inhibitor. The release of Ca++ with time from the terminal sacs of the sarcoplasmic reticulum has been demonstrated (23).

**DISCUSSION**

We have shown in this report that certain substances are capable of inhibiting the emptying of skeletal muscle cell segments previously treated to induce emptying. ATP and ADP were found to be effective agents while AMP was not. This is consistent with our hypothesis (15) that the formation of the actomyosin complex is a necessary condition to produce emptying. The formation of the actomyosin complex is accomplished by removing ATP and ADP (18). During the aging of muscle the content of ATP decreases, as does that of ADP (19, 24-26); under these conditions, the formation of actomyosin takes place (19). Huxley (27) has obtained data consistent with the hypothesis that actin and myosin are not linked in live, relaxed muscle but are in rigor. Chicken breast muscle aged under our conditions is most likely in the state of rigor (28) and, therefore, its ATP and ADP would be low. We have suggested (15) that the function of adding Ca++ to the homogenizing medium is to stimulate myosin ATPase (21) which would serve to convert ATP to ADP which, in turn, would be subject to the catalytic action of adenylate kinase. It is significant that the presence of CaCl₂ is mandatory only for fresh muscle, although with its use there is a slight enhancement of emptying in aged muscle as well (12). We suggest that the addition of ATP and ADP functions to dissociate the actomyosin complex. AMP, which does not dissociate actomyosin in vitro, does not inhibit emptying.

A hint of the possible inhibitory role of Ca++ is provided by the work of Straub (29) and Szent-Györgyi (30), who found that divalent cations precipitated contractile proteins. The concentrations required are low in the absence of high concentrations of monovalent chlorides. Presumably one of the functions of the NaCl-histidine washes is the removal of the polyvalent cations by an ion exchange phenomenon. The number of times that the segments are washed with the NaCl-histidine determines how well the cell segments will empty (12), which is consistent with this suggestion. Ca++ plays a rather unique role in the process of emptying since it is necessary to add it in the early stages (at least with fresh muscle), but it must be removed before the extraction step; it is, therefore, both an activator and an inhibitor of the process.

Our results indicate that natural inhibitors of emptying are formed or released on aging of muscle. It is possible that these inhibitors are polyvalent cations, since these would tend to be released on aging (23), whereas ATP and ADP would decrease with time. However, one must consider the possibility that other unknown inhibitors are produced by the muscle.

The data presented in this report support the position that the solubility characteristics of the contractile proteins are related to emptying of

**Table IV**

| Emptying inhibitor                      | Empty cells |
|-----------------------------------------|-------------|
| Water homogenate supernatant, aged muscle | %           |
| No treatment                            | 18          |
| Boiled                                  | 22          |
| Dialyzed                                | 51          |
| Water homogenate supernatant, fresh muscle | %           |
| No treatment                            | 74          |
| Control                                 | 65          |
muscle cell segments. Compounds known to dissociate or insolubilize actomyosin inhibit the emptying process. It is more likely, for example, that ATP and ADP function as inhibitors by dissociating actomyosin rather than by restoring a cytoskeleton. The data do not rule out the possibility, nevertheless, that breakdown of a cytoskeleton is also involved in emptying and that both cytoskeletal breakdown and actomyosin complex formation are required to obtain emptying of muscle cell segments.

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