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Anions and the Contraction of Glycerol-Extracted Muscle Fibers

H. KURT JACOBS[1] AND KARL F. GUTHE

Department of Zoology, The University of Michigan, Ann Arbor, Michigan 48104

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Glycerinated rabbit psoas fibers 5–19 days old were immersed in contracting solutions with and without varying concentrations of different sodium salts. Raising the pH of the solution in small steps identified a threshold pH above which the fibers rapidly developed maximum tension. In solutions with added chloride or acetate (0–0.15 M), threshold pH and maximum tension changed only slightly. In solutions with added nitrate, bromide, or iodide, fibers developed much less tension, according to the series: $CH_3COO^- > Cl^- > NO_3^- > Br^- > I^-$. Addition of excess calcium to the last solutions produced no further tension. After 15 min in NO_3^-, Br^-, or I^- solution, fibers partially regained tension when reimmersed in acetate but not chloride solution. Fibers that had developed tension in chloride solution lost some of it when reimmersed in nitrate, bromide, or iodide solution, according to the same anion series, but they retained more tension than they could develop initially in the latter solution.

There are well-known effects of anions on the twitch tension of fresh muscle (reviewed in Ref. 1), on the calcium uptake of the sarcoplasmic reticulum (2), and on the ATPase activity of the isolated muscle protein myosin (3, 4). The anion series for potentiating muscle tension and for inhibiting calcium uptake by the sarcoplasmic reticulum are identical. The inhibition of myosin ATPase, following quite another series, increases as the anions become more effective disruptors of protein structure. Although glycerol-extracted muscle fibers have been widely used as intermediates between fresh fibers and isolated myosin (reviewed in Ref. 5), little is known of the effects of anions on them. The present paper describes an anion series for the isometric tension of a glycerol-extracted rabbit psoas fiber bundle. The anions do not potentiate tension, and their relative effectiveness as inhibitors follows the series for myosin ATPase.

MATERIALS AND METHODS

Extraction of rabbit psoas muscle following the method of Szent-Györgyi (6) was carried out in 50% glycerol–5 mM histidine at pH 5.8 for 48 hr at 3° with three changes of the extraction medium. The fiber bundles were dissected to smaller bundles after 24 hr. These were stored at −11° and used 5–19 days later. This procedure is less complete than the extraction previously employed by Brown, Carew, and co-workers (7–9), and some excess calcium ions probably remained in our fibers, lowering the threshold pH to the observed values.

To test a fiber bundle (approximately 0.2 mm diameter), it was mounted on an isometric lever system, adjusted to 10-mg tension, and allowed to equilibrate for 15 min with a standard presoak solution (0.05 M maleic acid, 0.025 M HEPES buffer, salt at test concentration) plus 4 mM EDTA at pH 6.4 and 5°. This solution was then replaced by a standard presoak solution without EDTA at pH 6.4 and allowed to warm to 20°. The time interval for this procedure was 12–16 min. This solution was then replaced with a standard medium (0.05 M maleic acid, 0.025 M HEPES, 4 mM $MgCl_2$, 5 mM ATP, salt at test concentration) at pH 5.2 and 20°. Addition of 6 N NaOH in small steps increased the pH until the fiber rapidly developed tension. Above this threshold pH little if any tension developed. No calcium was added to any of the solutions. All solutions were made up in glass-distilled water.

Control tests were run on alternate days or daily with the experimental tests. The solutions for the controls were the standard solution with-

[1] Present address: Department of Physiology, University of Missouri, Columbia, Missouri 65201.

out any added salts. Observed values for tension and threshold pH were used to compile average control values. The fibers were quite consistent in threshold pH (±0.1 pH unit) and in tension development (±0.5 kg/cm²) for the 5- to 19-day period. Daily fluctuations were minimal. The threshold pH values of Fig. 1 and Table I were adjusted to an average control value of pH 5.62. The tension values of Fig. 2 were derived by calculating the kilogram tension per sq. centimeter for each test. An average control value was then determined and a ratio of observed control value to average control value was computed for the individual control tests. This ratio was used to normalize all values to the average control.

RESULTS

The contraction of a glycerol-extracted fiber bundle depends strongly on the ionic makeup of its reaction medium as well as that of the extraction solution. A typical bundle contracts to near maximum tension upon immersion into a reaction mixture at pH 5.2. This is quickly followed by a complete or nearly complete relaxation. Subsequent raising of the pH in small increments (less than 0.1 pH unit) produces no tension until a threshold value is reached, whereupon a rather rapid increase in tension to near maximum occurs. The present fibers, which increase in tension rapidly to maximum, differ from those (7–9) extracted more exhaustively at a higher pH, which increase in tension by small steps when the pH is raised in small increments above the threshold pH.

The data indicate (Fig. 1, Table I) that the threshold pH depends upon the concentration of the salt added. Control values in the standard solution without added salt are quite consistent and average pH 5.62. The threshold pH is lowered or raised depending upon the added salt and its concentration. For each salt there is an optimum concentration which causes the greatest increase in threshold pH, but the optimum is quite broad. At the higher concentrations of nitrate, bromide, and iodide, the detrimental influence of those ions is shown by complete inhibition of tension development. The fibers, however, become more elastic.

Effects of additional salt on maximum tension are more pronounced. Tension can be enhanced but is usually hindered, depending upon the added anion and upon its concentration (Fig. 2). At high added salt concentrations (0.1–0.25 M), the fibers develop more tension in acetate or chloride than they do in nitrate, bromide, or iodide.

At lower concentrations, the inhibiting ions hinder contraction less than at higher and may even enhance tension.

In order to determine if the initial effect of higher concentrations of the anomalous ions

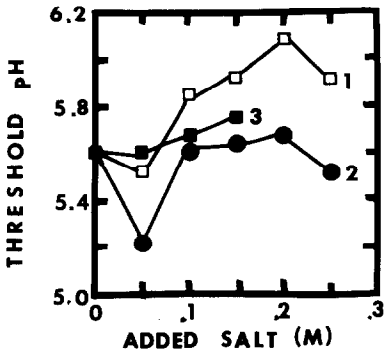

FIG. 1. Threshold pH for contraction in standard solution (0.05 M maleic acid, 0.025 M HEPES buffer, 4 mM $MgCl_2$, 5 mM ATP, 20°) with and without added salt. Curve 1 CH_3COONa, 2 NaCl, 3 $NaNO_3$. (See Table I for NaBr and NaI).

TABLE I

THRESHOLD pH FOR CONTRACTION

Added salt concentration (M)	Nitrate	Bromide	Iodide
0.05	5.59	5.70	5.16
0.10	5.68	5.73	5.46
0.15	5.76	5.88	6.37?
0.20	No tension	No tension	No tension
0.25	No tension	No tension	No tension

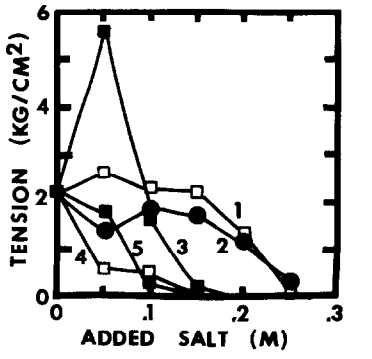

FIG. 2. Tension developed in standard solution with and without added salt. Curve 1 CH_3COONa, 2 NaCl, 3 $NaNO_3$, 4 NaBr, 5 NaI.

TABLE II

PERCENTAGE OF CHLORIDE TENSION MAINTAINED BY FIBERS IN SOLUTIONS WITH 0.15 M ADDED SALT

	Condition A[a]	Condition B[b]
Acetate	124	176
Chloride	100	100
Nitrate	12	86
Bromide	0.2	71
Iodide	0.2	52

[a] Condition A: Fibers developed tension in indicated solutions.

[b] Condition B: Fibers developed tension in 0.15 M added chloride and were then transferred to indicated solutions.

was reversible, fibers were immersed in the acetate test solution after they had developed tension and maintained it for 15 min in the other solutions. The fibers recovered 39.5% of their control tension in 3 min, nearly all in the first minute. No recovery occurred in chloride. Apparently the effects of nitrate, bromide, and iodide on tension can be overcome by acetate but not by chloride. Nor can calcium ions reverse these effects. After some complete trials in the detrimental solutions, excess Ca^{2+} was added. It did not change the tension of fibers in these solutions although it increased the tension of fibers in control, chloride, or acetate solutions.

Table II shows the effects of immersion in other test solutions after a bundle had been presoaked and had developed maximum tension in chloride. The same series of decreasing tension for the anions at added concentration of 0.15 M is apparent as in the separate tests of Fig. 2, but more tension is maintained in the detrimental solutions than the fibers develop initially when placed in them.

DISCUSSION

The present experiments were designed to explore the characteristics of the contractile machinery and not to investigate either the anion potentiation of the twitch tension of live muscle or the role of calcium as the activator of contraction.

The results show that anions hinder rather than potentiate tension in glycerol fibers: maximum tensions follow the series $CH_3COO^- > Cl^- > NO_3^- > Br^- > I^-$. Twitch tensions developed in live muscle follow the series $I^- > NO_3^- > Br^- > Cl^-$, and anions do not affect the capacity of the active state to produce tension (1). Inhibition of calcium uptake by the sarcoplasmic reticulum follows the same order of anions (2) as twitch potentiations, which is now generally interpreted as the result of lowering calcium uptake, thus prolonging the active state. That glycerination quickly destroys the functional reticulum is often assumed, and the quite different effect of ions on glycerol fibers tends to strengthen that assumption. The failure of excess calcium to affect tension in inhibited glycerol fibers also implies that the inhibition is not caused by interference with calcium mobilization. Although we cannot exclude the possibility that surviving fragments of sarcoplasmic reticulum are active, our results seem more readily interpretable as effects on the contractile machinery.

Although calcium ion is generally accepted as the activator of contraction, its mechanism of action is indirect. Calcium ions bind to troponin (10, 11), affecting tropomyosin and then actin (12) so that the actin and myosin filaments can link and slide. We assume that our fibers contain a constant amount of calcium readily available to the contractile machinery, but that contraction occurs only after increasing pH converts inactive proteins to a state permitting development of tension. The threshold pH is lower for more tighly bound anions at a fixed salt concentration, and higher for any given anion as the concentration increases. Brown and Carew (7–9) found that threshold pH depended much more strongly on sodium maleate concentration than we find for the present anions, but their fibers were extracted more exhaustively and at a higher pH than ours. They are clearly in a different initial state, as indicated by the graded pH-dependent change in tension for their fibers in contrast to the abrupt change from zero to maximal tension in ours.

The change in state might be simply a

change in protein charge. The isoelectric points of muscle proteins are near 5, and increasing pH would be expected to charge the proteins negatively. If a given negative charge is required for contraction, it might also be attained by anion binding. The presence of more tightly bound anions at a fixed salt concentration would enable the proteins to reach the charge level at a lower pH. For any given anion, increasing concentration would increase electrolyte screening and raise the threshold pH. The abrupt rise in tension with pH implies a cooperative effect, with the charging of one group facilitating the charging of others. Unusual even for cooperative effects are the rapidity of the development of tension (a minute or two) and the rise from zero to maximal tension in 0.1 pH unit or less. The response recalls the abrupt change in the acid-titration curve of hemoglobin near pH 4 (13). If the present effect is analogous, increasing pH would release previously restrained carboxyl groups. Either increasing pH or structure-disrupting ions might produce local changes in contractile protein structure to facilitate the release of such groups and thereby increase the negativity. Hoeve and co-workers (14, 15) also noted abrupt tension changes in glycerol fibers as they increased in water content, but interpreted them as conformation changes leading to an order–disorder change in arrangement of the protein molecules.

The anions markedly influence maximal tension. We suggest that nitrate, bromide, and iodide affect at least two processes. Fibers that develop tension in chloride presumably retain active actin-myosin links when chloride is replaced by nitrate, bromide, or iodide. Nevertheless, the tension drops, indicating that already active links can exert less tension in these solutions.

When the fibers are initially activated in solutions of the inhibiting ions, they develop much less tension than they retain after activation in chloride. The failure of excess calcium to increase the tension of these inhibited fibers implies that the anions do more than simply change a calcium-binding constant. Apparently the anions not only hinder tension but also prevent some actin-myosin links from becoming active. Replacement by acetate, but not chloride, increases tension by partially reversing one or both effects. Because live muscles contain more organic anion than chloride, acetate may be a more suitable substitute for the natural environment of the fibrils. Other studies of ion effects [most recently (4)] have used acetate as well as tetramethylammonium and cesium ions because they bind less readily to charged regions of proteins. The methyl group of the acetate ion may also facilitate its entry into a hydrophobic pocket of a protein.

That the same series of anions inhibits tension and destroys protein structure may only reflect the dependence of both phenomena on anion binding or may suggest that some disruption precedes contraction. ATP apparently has some influence. Laki and Bowen (16) found that glycerol fibers became more elastic in the absence of ATP and shortened as iodide bound to the proteins (17). Our fibers in NaI in the presence of ATP also become more elastic, but they develop and maintain less tension. Perhaps iodination of a fiber allows it to shorten but interferes with tension development, or different iodide–protein complexes may form in the absence of K^+ or the presence of ATP.

We conclude that these anions probably act on the contractile machinery, but that much further work is needed before their mechanism of action can be explained. They might affect specific binding sites with or without conformation changes, or they might act by altering general properties of whole filaments, as suggested by Elliott (18).

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REFERENCES

1. SANDOW, A., *Pharmacol. Rev.* **17**, 265 (1965).
2. EBASHI, S., OTSUKA, M., AND ENDO, M., *Excerpta Med. Intern. Congr. Ser.* **48**, 899 (1962).
3. WARREN, J. C., STOWRING, L., AND MORALES, M. F., *J. Biol. Chem.* **241**, 309 (1966).
4. SEIDEL, J. C., *J. Biol. Chem.* **244**, 1142 (1969).

5. HASSELBACH, W., AND WEBER, A., *Pharmacol. Rev.* **7,** 97 (1955).
6. SZENT-GYÖRGYI, A., *Biol. Bull.* **96,** 140 (1949).
7. CAREW, E. B., PhD Dissertation, The University of Michigan (1963).
8. CAREW, E. B., AND BROWN, D. E. S., *Federation Proc.* **23,** 420 (Abstr. 1911) (1964).
9. BROWN, D. E. S., CAREW, E. B., AND BAUER, R. S., *Arch. Biochem. Biophys.* **100,** 45 (1963).
10. EBASHI, S., EBASHI, F., AND KODAMA, A., *J. Biochem. Tokyo* **62,** 137 (1967).
11. FUCHS, F., AND BRIGGS, F. N., *J. Gen. Physiol.* **51,** 655 (1968).
12. TONOMURA, Y., WATANABE, S., AND MORALES, M., *Biochemistry* **8,** 2171 (1969).
13. STEINHARDT, J., AND HIREMATH, C. B., *J. Biol. Chem.* **242,** 1294 (1967).
14. HOEVE, C. A. J., AND WILLIS, Y. A., *Biochemistry* **2,** 279 (1963).
15. HOEVE, C. A. J., WILLIS, Y. A., AND MARTIN, D. J., *Biochemistry* **2,** 282 (1963).
16. LAKI, K., AND BOWEN, W. J., *Biochem. Biophys. Acta* **16,** 301 (1955).
17. BOWEN, W. J., AND LAKI, K., *Am. J. Physiol.* **185,** 92 (1956).
18. ELLIOTT, G. F., *J. Gen. Physiol.* **60,** No. 6, Part 2, 171 (1967).