Leakage of Aldolase from Rat Diaphragm Induced by EDTA (34694)

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Increased enzyme activity in serum is found in several nondisease states such as exercise (7) as well as under pathological conditions (5) including delirium tremens (19). The cellular mechanism of this elevation is obscure but may be related to the control, by divalent cations, of the passive permeability properties of several membrane types. Examples of this mode of regulation have been observed as an increased passage of macromolecules across mammalian intestine (17), the leakage of protein from rat liver slices (10), and the uptake of actinomycin by bacteria (12). Accordingly this study was designed to elucidate cellular enzyme loss from muscle with the intent of demonstrating the role of calcium and magnesium in the maintenance of membrane permeability and the functional integrity of skeletal muscle. The ionic permeability status of the preparation was monitored by assessing the transmembrane ionic gradient for sodium and potassium under the experimental conditions. Disodium dihydrogen ethylenediaminetetraacetate (Na_2H_2EDTA) was the chelating agent used to promote enzyme loss from rat diaphragm muscle *in vitro*. Aldolase was selected for study because it is present in high concentration in muscle; it is believed to be a soluble intracellular enzyme; its leakage from rat diaphragm has been studied under other circumstances (18); and, unlike the metalloenzymes, the assay procedure for aldolase may be undertaken successfully in the presence of chelating agents (9).

Methods. Entire diaphragms of adult, 300–400 g, Sprague-Dawley rats maintained on commercial chow were removed under ether anesthesia and washed in ice-cold, physiologic saline solution. Each diaphragm was cut into segments of approximately 50 mg and placed in fresh, ice-cold saline to remove blood clots from the surface. The segments were blotted, weighed, and placed immediately into the appropriate incubation medium. The basic medium was Tyrode's solution buffered at pH 7.4. Various concentrations of Na_2H_2EDTA, $CaCl_2$, and $MgCl_2$ were added with appropriate reductions in NaCl content to maintain osmolarity within the physiologic range. The pH of the Tyrode's solution was checked after the addition of Na_2H_2 EDTA and adjusted to 7.4, if necessary. The initial volume of the medium was 10 ml. After 5 min of incubation at 37° in a closed system containing an atmosphere of 95% oxygen and 5% carbon dioxide at a shaking rate of approximately 80 strokes/min, 1 ml of fluid was removed and its enzyme content was taken as the value for "zero" time. An equal volume of fluid was removed after 10, 20, and 30 min of additional incubation. Aldolase activity in the medium was determined colorimetrically (15). All samples were analyzed in duplicate and the activity was expressed as IU/liter $\times 10^{-2}$ /mg of wet weight of diaphragm in order to eliminate variation created by the study of segments of different weight. In some experiments ^{14}C-labeled polyethylene glycol (PEG 4000) was present in the incubation medium. The distribution of this extracellular probe solute between tissue water and incubation fluid was determined by liquid scintillation

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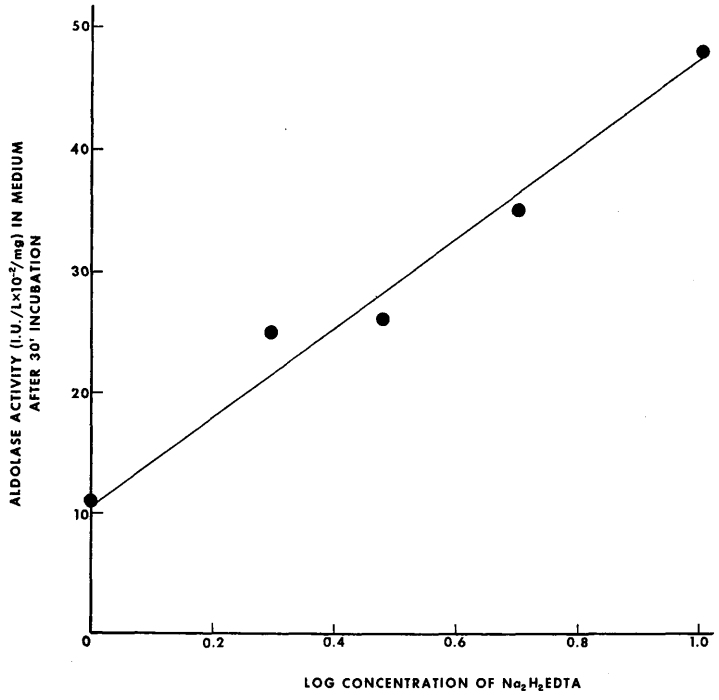

FIG. 1. Relationship between the leakage of aldolase and log concentration of Na_2H_2EDTA in mmoles/liter.

counting of tissue and bath fluid samples. Extracellular space determinations were calculated in the conventional manner from these measurements. Tissue sodium and potassium were determined after dry ashing by standard flame photometric procedures.

Results. Aldolase leakage from diaphragm induced by EDTA. Increasing concentrations of Na_2H_2EDTA ranging from 1 to 10 mmoles/liter led to a progressive increase in the levels of aldolase activity in the medium at 10, 20, and 30 min of observation. There was a linear relationship between the aldolase activity in the medium and the log of the concentration of the EDTA as shown for the 30-min period of incubation (Fig. 1).

Five rats were sacrificed by skull fracture in order to test whether ether potentiated the effect of EDTA. The leakage of enzyme into the medium from tissues of animals anesthetized with ether was not statistically different ($p < 0.05$) from that observed with tissues of animal that did not receive ether.

Effects of calcium and magnesium on aldolase leakage induced by EDTA. The effects of 10 m*M* $MgCl_2$ and 10 m*M* $CaCl_2$ on the progressive leakage of enzyme induced by 5 m*M* Na_2H_2EDTA are shown in Fig. 2. At 30

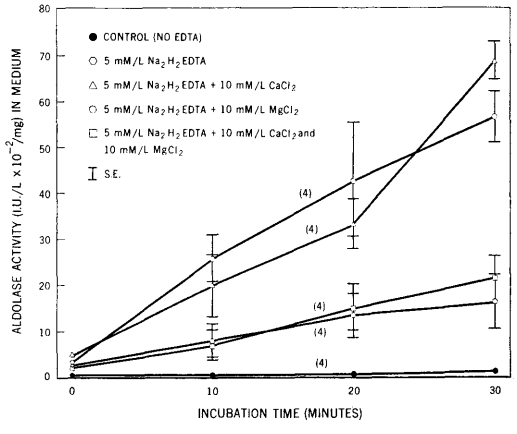

FIG. 2. Effects of calcium and magnesium upon the leakage of aldolase by 5 m*M* Na_2H_2 EDTA. Magnesium and calcium were deleted from all Tyrode's solutions except that which was subsequently added before tissue incubation. Sodium was substituted in millimoles equivalent to magnesium and calcium that were deleted. The numbers in parentheses represent the number of observations.

min of incubation addition of the magnesium salt had reduced the loss of aldolase by approximately 70%, the addition of the calcium salt had increased the loss of aldolase by approximately 25%. The differences between the aldolase leakage into the medium induced by 5 m*M* Na_2H_2EDTA and the aldolase leakage induced by 5 m*M* Na_2H_2EDTA containing Ca^{2+} and Mg^{2+} in paired tissues from experiments of the same day are shown in Fig. 3. The horizontal axis labeled 0 represents the aldolase activity in the medium after 30 min of incubation in the presence of EDTA without added Ca^{2+} or Mg^{2+}. Bars are placed above the concentrations of Ca^{2+} and Mg^{2+} indicated at the bottom of Fig. 3. A bar above the horizontal axis (potentiation) indicates that more aldolase appeared in the medium than in its paired EDTA control; a bar below this line (inhibition) indicates that less aldolase was lost from the tissue than in its paired EDTA control. All of the paired differences were significantly different from zero difference at the $p < 0.01$ level. There was potentiation of aldolase leakage into the medium induced by 5 m*M* Na_2H_2EDTA when $CaCl_2$ was present in concentrations of 2.5, 10, 15, and 20 m*M*, whereas inhibition of the leakage was obtained by 5 m*M* $CaCl_2$. The results obtained with $MgCl_2$ were dissimilar from those with $CaCl_2$; that is, inhibition of aldolase leakage was attained with $MgCl_2$ up to 15 mmoles/liter. The concentration of $MgCl_2$ which induced maximum inhibition was 10 m*M*, whereas 20 m*M* $MgCl_2$ appeared to potentiate the enzyme leakage. When mixtures of $CaCl_2$ and $MgCl_2$ were present along with a 5 m*M* concentration of chelating agent, inhibition of aldolase leakage was found at all concentrations of the combinations of salts which were studied. The combination which induced maximum inhibition in

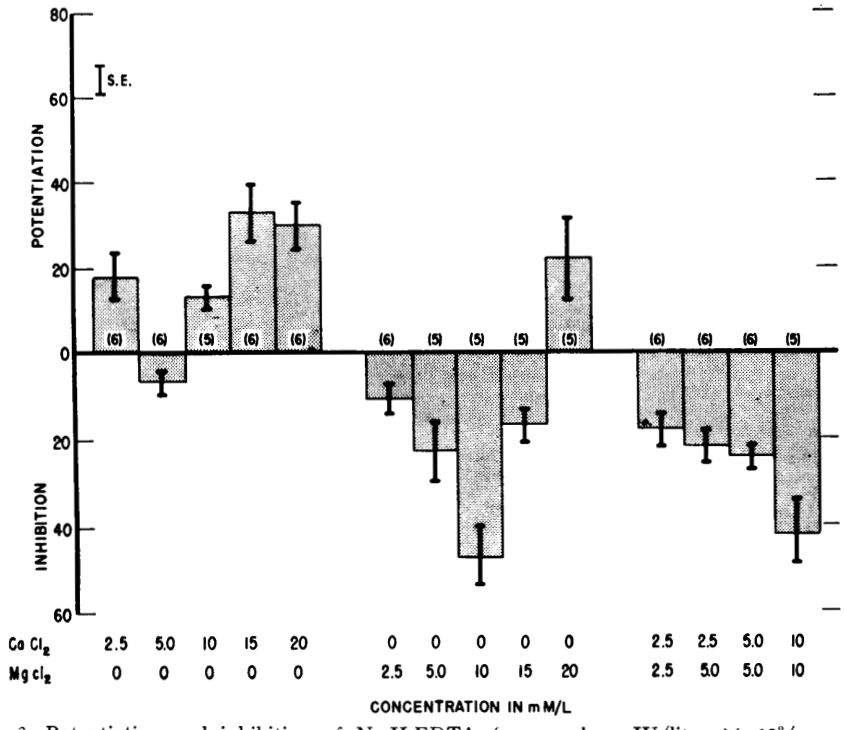

FIG. 3. Potentiation and inhibition of Na_2H_2EDTA (expressed as IU/liter $\times$ 10^2/mg of wet wt of tissue) effected by Mg^{2+} and Ca^{2+}. Each bar represents the difference between the leakage of aldolase induced by 5 m*M* Na_2H_2EDTA and the leakage of aldolase induced by 5 m*M* Na_2H_2 EDTA, in which the divalent cations were added to paired tissues in experiments done on the same day. The mean aldolase for the 15 control experiments was 1.56 $\pm$ 0.5; and for the 15 EDTA experiments, 64.0 $\pm$ 3.0 IU/liter $\times$ 10^{-2}/mg of wet weight of tissue.

this group was 10 mmoles/liter of each salt. This yielded a degree of inhibition approximately equal to that induced by 10 mmoles/liter of $MgCl_2$.

Tissue levels of sodium and potassium together with diaphragm water content and extracellular space, as measured by polethylene glycol, are shown in Table I. There is no significant alteration induced by EDTA in either extracellular space or water content of the tissue. For the purpose of calculating intracellular concentrations of these ions in the fresh tissue an extracellular space of 12% for the *in vivo* muscle has been assumed from the literature (4). The concentration of sodium and potassium in the cell water was estimated to be 44.0 and 117 meq/liter, respectively, in the feshly dissected tissue. These levels rose to 71.9 meq/liter for Na^+ and diminished to 63.0 meq/liter for K^+ upon incubation of the diaphragm segment *in vitro*. There was a further increase in sodium to 86.9 meq/liter and a decrease in potassium to 41.4 meq/liter when 5 m*M* EDTA was present in the incubation medium. These alterations in tissue sodium and potassium from the *in vivo* levels are all significant at the $p < 0.01$ level with the exception of the effect of EDTA on tissue sodium which is significant at the $p < 0.05$ level.

Discussion. The results of the present study indicate that exposure of rat diaphragm *in vitro* to EDTA leads to increased activity of aldolase in the medium. The rate of increase appears to be dependent upon the concentration of EDTA and the duration of exposure. The progressive increase in enzyme activity in the medium presumably represented leakage of enzyme from the cytoplasm. EDTA has been shown to alter permeability of cell membranes (10, 12). Cassidy and Tidball (3) have reported that EDTA increases aqueous permeability of intestinal epithelium and produces alteration of the ultrastructure of the tissue. They also found the calcium and magnesium contents of the epithelium to decrease by 47 and 27%, respectively, and were able to restore normal permeability by rinsing with isotonic $CaCl_2$ and $MgCl_2$. Restoration of normal permeability and of the divalent cation contents of the tissue led to a return of the normal ultrastructural appearance. Other permeability changes brought about by EDTA also have been attributed to the chelation of divalent cation (16, 17).

The present data are consistent with this view in that the addition of magnesium ion to the system in a concentration equal to, or two and three times as great as, that of EDTA, inhibited much of the enzyme leakage induced by the chelating agent. However, a magnesium ion concentration four times that of EDTA failed to inhibit the EDTA-induced leakage and indeed even potentiated it (Fig. 3). The effects of the addition of

TABLE I.[a,b]

Condition	(mmoles/kg of wet wt) Sodium	Potassium	% H_2O	% Extracellular space (PEG 4000)
Freshly excised muscle	40.8 ± 2.2	76.3 ± 1.5	73.9 ± 0.6	12.0[d]
Muscle incubated in Tyrode's solution for 30 min	64.5 ± 3.0[c]	47.4 ± 5.2	80.7 ± 0.5	12.3 ± 1.2
Muscle incubated in Tyrode's solution with 5 m*M* EDTA for 30 min	72.9 ± 3.5[c]	30.4 ± 2.0	80.9 ± 0.7	10.0 ± 1.9

[a] Values shown are the means ± standard errors for 8–10 experiments/group. For estimates of intracellular Na^+ and K^+ concentrations, see text.

[b] The values for tissue sodium and potassium are all significantly different from each other at the 99% confidence level with the exception of the values indicated by superscript *c* which are significantly different from each other at the 95% confidence level.

[d] An *in vivo* extracellular space of 12% for diaphragm muscle has been listed for purposes of comparison.

calcium ion to the system, were different. Only the 5 m*M* $CaCl_2$ inhibited the enzyme leakage; other concentrations of $CaCl_2$ failed to inhibit, and indeed, seemed to enhance the EDTA-induced enzyme leakage. These data suggest that EDTA treatment of muscle is somewhat different from that reported for intestinal epithelium: the data suggest a specific toxic effect of chelated calcium and magnesium on muscle tissue. It has been noted that chelate breakdown is not essential for a toxic action since certain metals exert their effects on enzymic processes in the chelated form (13). The EDTA-induced alteration in intestinal absorption appeared to depend on changes that were primarily structural; furthermore, several divalent cations seemed to be equally effective and interchangeable in reversing the EDTA effect (6). By contrast, the data of the present study, in spite of observations over an equally wide range of cation concentration, demonstrate qualitative differences between the magnesium and calcium roles in influencing the EDTA effect on the rat diaphragm.

Reger (14) has studied the fine structure of frog toe muscle treated with EDTA. He found an increase of more than 1000% in T tubule widths and hypothesized that this phenomenon might reflect calcium and water loss by the tissue. In this study we did not detect any alteration in muscle tissue space accessible to polyethylene glycol (PEG 4000); nor was any change in water content detectable following incubation in buffered EDTA saline. We did find alterations in sodium and potassium ionic concentration gradients consistent with either (a) a change in pore size or charge distribution (8), or (b) reduced enzymatic activity resulting in an alteration in sodium pumping rate (1). These observations on intracellular ion content of muscle treated with EDTA are also in accord with previous studies which indicated a decrease in resting potential and membrane resistance concomitant with a loss of electrical excitability (1).

Of the components of the cell membrane that might be affected by magnesium depletion and not by calcium depletion, Mg-dependent ATPase warrants consideration. The influence of increments of magnesium ion and of a low concentration of calcium ion in a system containing 5 m*M* EDTA (Fig. 4) resemble that observed with mag-

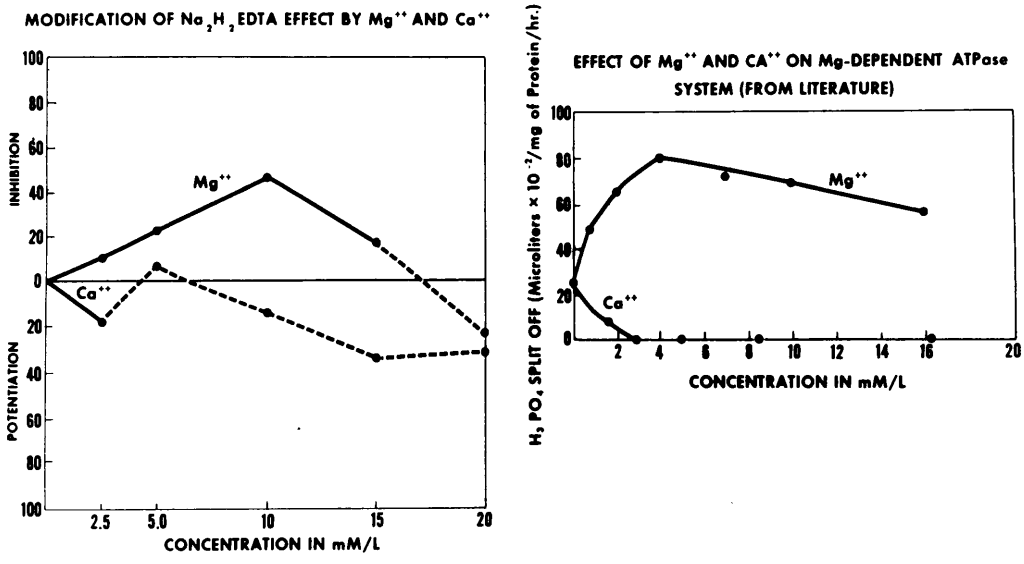

FIG. 4. Comparison of the effects of Mg^{2+} and Ca^{2+} on Na_2H_2EDTA-induced leakage of aldolase observed in the present study with the effects of these ions on Mg-dependent ATPase activity as reported by Kielley and Meyerhof (11). Inhibition and potentiation of leakage expressed as IU of aldolase/liter $\times$ 10^{-2}/mg of tissue.

nesium-dependent ATPase (11). No significant influence of divalent cations added to the system without EDTA was observed in preliminary experiments. This result is qualitatively consistent with the view that EDTA leads to enzyme leakage by its effect on membrane ATPase, since Ca^{2+} ion and higher concentration of Mg^{2+} ion inhibit magnesium-dependent ATPase activity. Even the optimal concentration of magesium ion, however, reduced the EDTA effect by only 70% (Fig. 2), but did not inhibit it completely. Accordingly, it may be inferred that an effect of EDTA on a component of the muscle membrane other than ATPase may also have been involved. The inhibition of enzyme leakage by combinations of calcium and magnesium ions in several concentrations and by 5 m*M* $CaCl_2$ but not by other concentrations, suggests the possibility that the role of magnesium ion may be in maintaining normal ATPase activity of the membrane, while the role of calcium ion appears to be different and perhaps related to the structural integrity of the membrane.

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