LOCALIZATION OF PRODUCTS
OF ATP HYDROLYSIS IN MAMMALIAN
SMOOTH MUSCLE CELLS

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ABSTRACT

The sites of lead phosphate precipitation in mouse bladder smooth muscle incubated with adenosine triphosphate and lead nitrate were studied by electron microscopy. The media constituents and incubating conditions were independently varied so that we could determine optimal conditions for histochemical demonstration of ATPase activity in agranular endoplasmic reticulum. Specimens of glutaraldehyde-fixed bladder muscle, frozen, cut into 10–40-μ sections, and incubated for 1 hr at 25°C in medium containing 0.025 M ATP, 0.0025 M lead nitrate, 0.05 M magnesium chloride, and 0.09 M sodium acetate buffer at pH 6.2, exhibited microcrystalline deposits in agranular endoplasmic reticulum and pinocytotic vesicles. Lead salt deposition was also noted in terminal cisternae of sarcoplasmic reticulum in skeletal muscle similarly treated, suggesting that the organelle systems in the two types of muscle cells subserve a common function.

INTRODUCTION

The mammalian visceral smooth muscle cell possesses a sparse agranular endoplasmic reticulum composed of roughly parallel tubules oriented in the long axis of the cell (3). Although the agranular endoplasmic reticulum of striated muscle cells has been considered to play a role in excitation-contraction coupling (2, 6, 9), its relatively small extent and the absence of a demonstrable organization in smooth muscle cells have militated against ascribing to it a similar function in these cells (8). In the present study, the products of hydrolysis of adenosine triphosphate (ATP) were localized to the tubules of agranular endoplasmic reticulum of both smooth muscle cells and striated muscle cells, under the same incubating conditions. This similarity may indicate that the organelle systems in the two cell types have a common function.

MATERIALS AND METHODS

Specimens of mouse urinary bladder smooth muscle

and mouse psoas skeletal muscle, excised as 10-mm × 2-mm strips, were fixed for 10 min at 0°C by immersion in 4% glutaraldehyde in 0.09 M sodium acetate buffer, pH 6.2, and then washed for 1 hr in three changes of the acetate buffer. 10–40-μ frozen sections were cut from these specimens and incubated as free floating sections for 60 min at 25°C in a medium containing 0.025 M ATP, 0.0025 M lead nitrate, and 0.05 M magnesium chloride in 0.09 M sodium acetate buffer adjusted to pH 6.2. We performed this primary experiment three times, using tissue from different animals. For the third run, segments of rat bladder, mouse jejunum, and mouse cardiac muscle were also treated in the same manner in order that we could measure species and tissue specificity.

We performed four types of control experiments, using mouse urinary bladder and mouse skeletal muscle fixed and sectioned as described above. One group of sections was heat inactivated at 60°C prior to incubation; another was incubated in medium lacking ATP; a third was incubated in complete

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medium to which a small quantity of sodium phosphate was added during incubation, and the last was incubated in medium in which the ATP was replaced by 0.09 M Na B glycerol phosphate.

In addition to the primary experiment and its controls, we carried out ancillary experiments to assess, independently, the role of the various incubating conditions and media constituents. Mouse urinary bladder, fixed and sectioned as described above, was utilized in these experiments, each of which was performed twice. The results were compared with those of the primary experiment.

In the first ancillary experiment, the tissue was incubated for the same period of time in the same medium at 37° instead of 25°C. In the second, the incubation was carried out at 25°C, as in the primary experiment, but the media were adjusted to pH 4.5 and 8.0. So that we could evaluate the effect of increased substrate concentration, the medium used in the third ancillary experiment contained 0.005 M ATP, instead of 0.025 M ATP. The media and incubating conditions were otherwise identical with those of the

primary experiment. In the last set of ancillary experiments, the pH, incubating temperature, ATP concentration, and lead nitrate concentration were those of the primary experiment, while the mono and divalent cations were varied. Five different media were used. In one the sodium acetate was replaced with equimolar potassium acetate; in another the magnesium chloride was replaced by equimolar calcium chloride; in the third equimolar calcium chloride was added to magnesium chloride in the presence of potassium acetate; and in the last two sodium acetate and potassium acetate were used, each in the absence of divalent cations.

In all experiments, the medium, lacking ATP but containing the appropriate cations and 0.005 M lead nitrate, was brought to 37°C and adjusted to pH 8.0 with 1.0 N sodium hydroxide or potassium hydroxide. The precipitate which formed was removed by filtration, the desired pH was attained by addition of 1.0 N hydrochloric acid, and the ATP was added to complete the medium. No further precipitate formed on addition of ATP, and the final lead nitrate
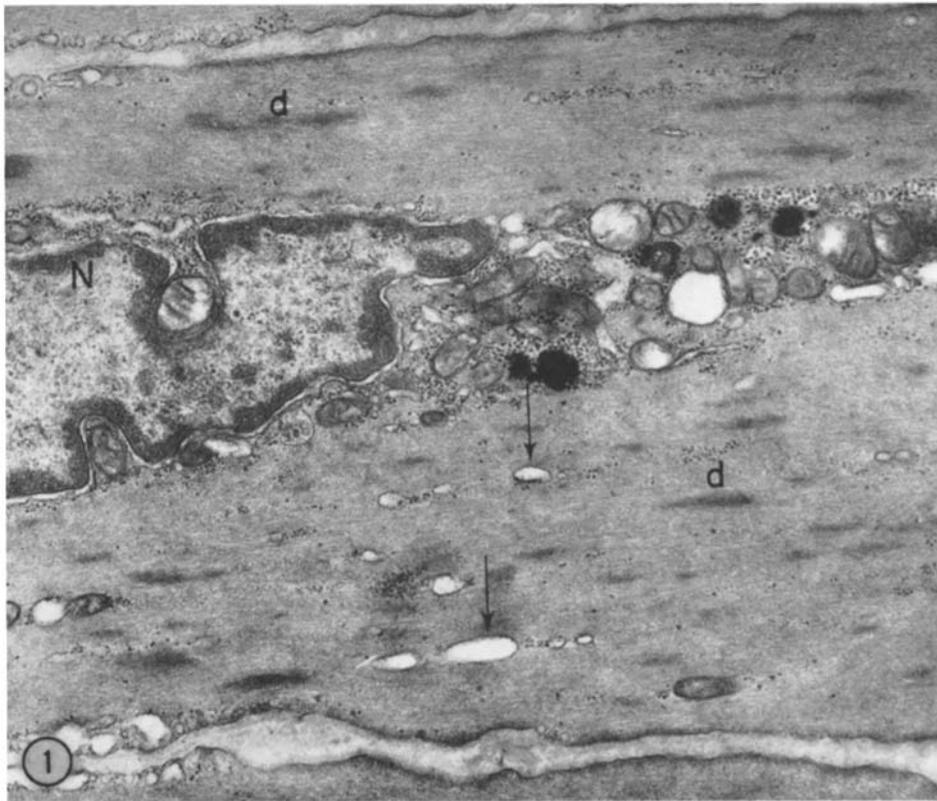

FIGURE 1   Electron micrograph of smooth muscle cell in specimen of mouse urinary bladder incubated at 25°C in ATP-free medium. No lead salt deposition is noted. Nucleus (N), interfilamentous dense areas (d), agranular endoplasmic reticulum (arrows). Lead citrate stained. × 15,000.
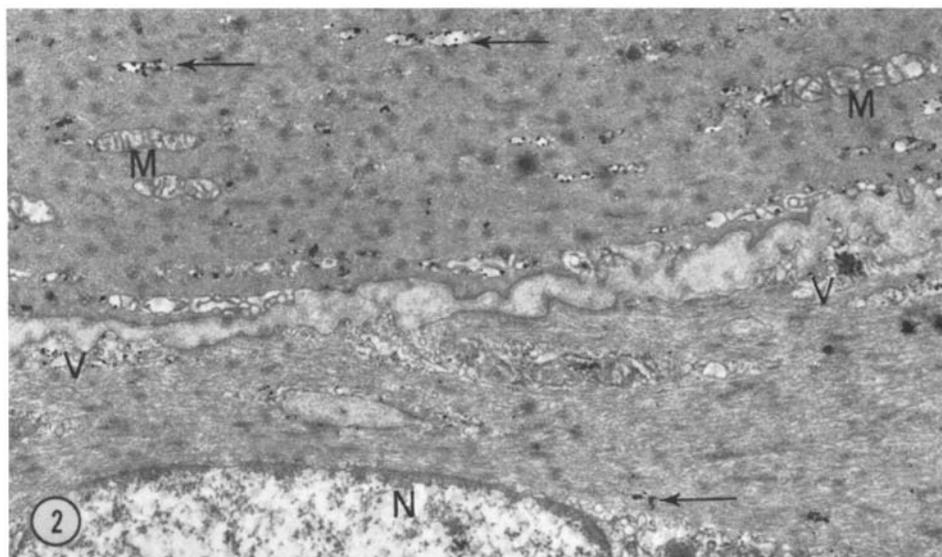
FIGURE 2   Electron micrograph of smooth muscle cells in mouse urinary bladder incubated in medium at 25°C containing 0.0025 M ATP, 0.025 M lead nitrate, and 0.05 Mg^{++} in sodium acetate buffer at pH 6.2 Lead deposits are present in the longitudinally oriented tubules of agranular endoplasmic reticulum (arrows) and in the pinocytotic vesicles (V) at the plasma membrane. The nucleus (N), mitochondria (M), and extracellular spaces are free of precipitates. Lead citrate stained. × 8,000.
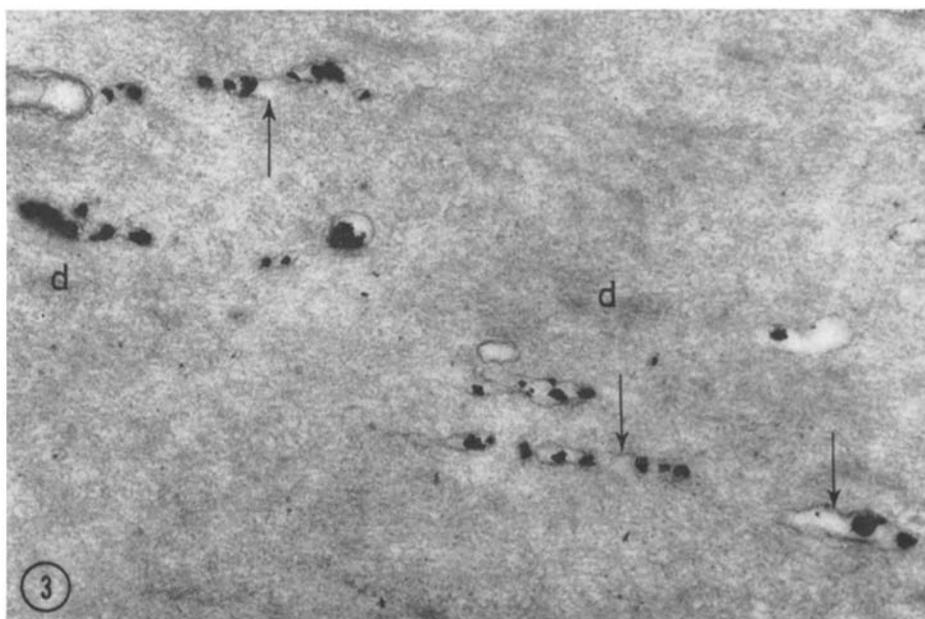

FIGURE 3   Higher magnification of lead salt precipitate in agranular endoplasmic reticulum (arrows) of smooth muscle cell from mouse urinary bladder treated as described in Fig. 2. The myofilaments surrounding the elongate tubules in this intact cell are free of the products of hydrolysis of ATP. Dense areas (d). Lead citrate stained. × 40,000.
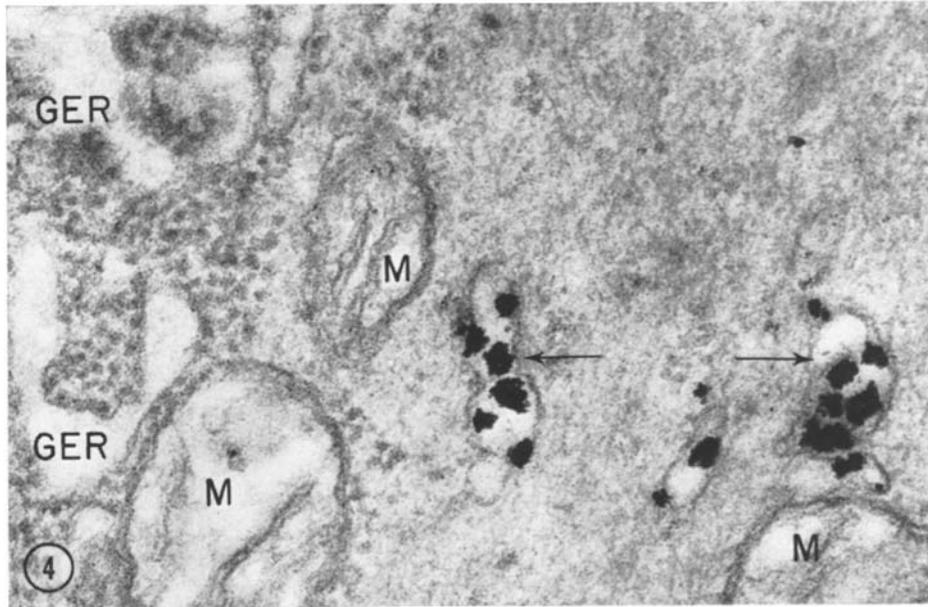
FIGURE 4 An area near the nucleus of a smooth muscle cell in mouse urinary bladder incubated as described in Fig. 2. The granular endoplasmic reticulum (GER) and mitochondria (M) are free of precipitates, while well defined lead salt deposits are present in the tubules of agranular endoplasmic reticulum (arrows). Lead citrate stained. × 80,000.

FIGURE 5 Electron micrograph of mouse skeletal muscle cell from specimen incubated at 25°C in medium containing 0.025 M ATP, 0.0025 M lead nitrate, and 0.05 M Mg^{++} in sodium acetate buffer at pH 6.2. Lead deposits are present in sarcoplasmic reticulum, most prominently in the terminal cisterns (arrows). Lead citrate stained. × 26,500.

TABLE I

Localization of ATP Hydrolysis Products in Smooth Muscle

Effects of temperature

pH 6.2   ATP 0.025 M   Lead nitrate 0.0025 M   Mg 0.05 M

Temp (Centigrade)	Filaments	Pinocytotic vesicles	AER	Nucleus	Mitochondria
37°	−	+	±	+	−
25°	+*	+	+	−	−

* Inconsistent; limited to fragmented cells.

TABLE II

Localization of ATP Hydrolysis Products in Smooth Muscle

Effects of pH

Temp 25° C   ATP 0.025 M   Lead nitrate 0.0025 M   Mg 0.5 M

pH	Filaments	Pinocytotic vesicles	AER	Nucleus	Mitochondria
4.5	−	±	+	−	−
6.2	+*	+	+	−	−
8.0	−	+	−	−	−

* Inconsistent; limited to fragmented cells.

TABLE III

Localization of ATP Hydrolysis Products in Smooth Muscle

Effects of ATP concentration

Temp 25° C   pH 6.2   Lead nitrate 0.0025 M   Mg 0.05 M

ATP(M)	Filaments	Pinocytotic vesicles	AER	Nucleus	Mitochondria
0.005	−	+	+	+	+
0.025	+*	+	+	−	−

* Inconsistent; limited to fragmented cells.

TABLE IV

Localization of ATP Hydrolysis Products in Smooth Muscle

Effects of ions

Temp 25°C   pH 6.2   Lead nitrate 0.0025 M   ATP 0.025 M

Ions	Filaments	Pinocytotic vesicles	AER	Nucleus	Mitochondria
Mg, Na	+*	+	+	−	−
Mg, K	−	+	+	−	−
Ca, Na	−	−	−	−	−
Ca, Mg, K	−	+	+	+	+
K	−	−	−	−	−
Na	−	−	−	−	−

* Inconsistent; limited to fragmented cells.

concentration in the medium was approximately 0.0025 M. The use of 0.0025 M lead nitrate initially did not prevent precipitation during adjustment of pH and resulted in lower lead ion concentration in the medium. Media at pH 6.0 and pH 8.0, which were not previously made alkaline and filtered, became cloudy upon addition of ATP, upon adjustment of pH after addition of ATP, or during the period of precipitation. Attempts to raise the pH to 9 prior to addition of ATP resulted in precipitation of virtually all lead, as measured by the absence of a precipitate after addition of sodium phosphate to the filtrate.

After 1 hr of incubation, the tissues were washed for 30 min in three changes of 0.09 M sodium acetate buffer, pH 6.2, at 0°C, and then treated with 2% osmium tetroxide in distilled water. The specimens were dehydrated by passage through a series of alcohols and embedded in Epon 812. Sections, unstained or stained with lead citrate (10), were examined with an RCA EMU 2E electron microscope.

RESULTS

After incubation for 60 min at 25°C in medium containing 0.025 M ATP, 0.0025 M lead nitrate, 0.05 M magnesium chloride, and 0.09 M sodium acetate at pH 6.2, a precipitate was present within pinocytotic vesicles and agranular endoplasmic reticulum profiles in all types of smooth muscle studied (Figs. 1–4). A precipitate was also observed in the terminal cisterns of sarcoplasmic reticulum of skeletal muscle and in cardiac muscle incubated under like conditions in this medium (Fig. 5).

In the control experiments, incubation of heat-inactivated tissue in the medium resulted in occasional randomly placed precipitates in both types of muscle. Similar sparse precipitation was observed after incubation in ATP-free medium, while a dispersed heavy precipitate showing no preferential localization to cells or to particular areas in cells was present after incubation in complete medium to which sodium phosphate was added during the incubation period. The substitution of β-glycerol phosphate for ATP resulted in scattered randomly placed precipitates throughout the cell.

In the experiment in which the tissue was incubated in the primary medium at 37°, a precipitate was seen in all nuclei, predominantly near nucleoli, as well as in pinocytotic vesicles and scattered smooth endoplasmic reticulum profiles
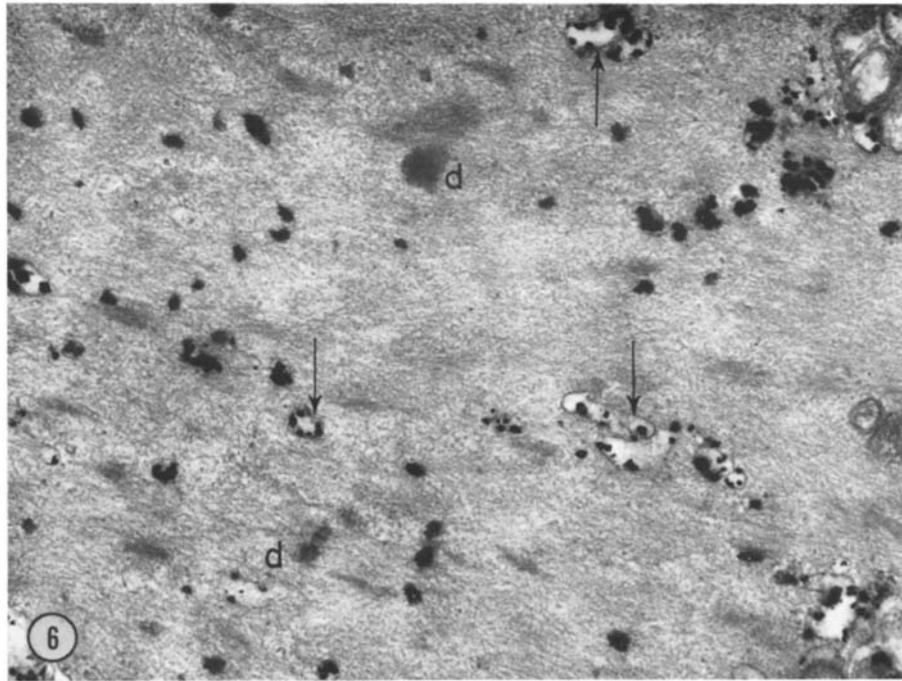
FIGURE 6   Electron micrograph of portion of fragmented smooth muscle cell from mouse intestine incubated at 25°C in medium containing 0.025 M ATP, 0.0025 M lead nitrate, and 0.05 M Mg⁺⁺ in sodium acetate buffer at pH 6.2. Lead salt precipitates are present in tubules of agranular endoplasmic reticulum (arrows) and some dense areas (d) among myofilaments. The precipitates in the endoplasmic reticulum are in the form of large, sharply bordered particles, while those in dense areas are composed of small particles dispersed among myofilaments. Lead citrate stained. × 30,000.

(Table I). When the primary medium was adjusted to pH 8.0, incubation at 25°C resulted in precipitates in pinocytotic vesicles only (Table II). With the medium at pH 4.5, the agranular endoplasmic reticulum was the site of densest precipitation, although pinocytotic vesicles also contained deposits of lead salts (Table II). Tissue incubated in medium in which the ATP concentration was reduced to 0.005 M exhibited precipitates in mitochondria and nuclei, as well as in pinocytotic vesicles and agranular endoplasmic reticulum (Table III).

With incubating conditions and media constituents at the values of the primary experiment, the substitution of equimolar potassium acetate for sodium acetate yielded results which, except for the absence of any precipitates on myofilaments, were similar to those of the primary experiment. Substitution of equimolar calcium chloride for magnesium chloride eliminated all precipitation, while addition of 0.05 M calcium chloride to the medium

containing 0.05 M magnesium chloride and 0.09 M potassium acetate resulted in deposition in nuclei and mitochondria, as well as in endoplasmic reticulum and pinocytotic vesicles. No precipitates were noted in tissue incubated in media from which the divalent cation was omitted (Table IV).

In two of the five specimens of smooth muscle incubated in the primary medium, a precipitate was observed in the dense areas among myofilaments in an occasional cell whose plasma membrane had been disrupted during preparation (Fig. 6). Such precipitates were not seen in most cells in these two specimens, nor were they present in the many disrupted cells in the remaining three specimens. In addition, instead of the microcrystals deposited at other sites, this precipitate was finely particulate.

DISCUSSION

The demonstration of products of hydrolysis of ATP in the sarcoplasmic reticulum of striated

muscle cells (1, 13, 14) has been considered further evidence for the participation of this organelle in excitation-contraction coupling. A similar role would be suggested for agranular endoplasmic reticulum in smooth muscle by the demonstration of ATPase activity at this site, although this function may be unlikely in view of the sparsity of the organelle and its longitudinal disposition in the cell. A previous study of ATPase localization in smooth muscle cells by Rostgaard and Barrnett (12) revealed lead phosphate deposition in pinocytotic vesicles only, and the present report confirms this finding under conditions of incubation similar to those utilized in the previous work. Deposition of lead salts in agranular endoplasmic reticulum did occur, however, when several changes were made in the constitution of the medium and in the incubating conditions.

These modifications of the Wachstein-Meisel protocol for ATPase demonstration (16) were predicated on the hypothesis that the ATPase in smooth muscle has relatively low activity, requiring deviations from the standard histochemical technique to more closely approximate optimal conditions for enzyme activity. Since, in this laboratory, attempts at isolation of the agranular endoplasmic reticulum of smooth muscle and characterization of an ATPase at this site were not successful, the adjustments in pH of the medium and the cationic constituents were made by trial-and-error experimentation. In order to reduce enzyme inactivation by heat, we maintained the medium used in the primary experiment at a lower temperature than that suggested by Wachstein and Meisel. We employed increased substrate levels to insure against rate-limitation by this factor.

As a measure of the effects of each of these changes in the protocol, a set of ancillary experiments was performed in which one factor was varied while the others were held constant at the values of the primary experiment. While these data do not aid in a comparison of the findings with those obtained with the unmodified procedure of Wachstein and Meisel, they do suggest that the adjustments in pH, temperature, and cationic constituents resulted in a closer approximation of optimal conditions for histochemical demonstration of the enzyme. The fivefold increase in substrate levels, on the other hand, did not appear to enhance activity at the site under study. Reaction products deposited in mitochondria and nuclei when the muscle was incubated in the medium

containing the lower substrate concentration, however, were eliminated. This finding and the similar deposition of lead salts with medium containing both magnesium and calcium ions were not further explored.

Histochemical localization of an enzyme whose presence has not been demonstrated by isolation and chemical assay must be viewed with suspicion in the light of the relative insensitivity of the method and the propensity to produce artifacts. In evaluating the technique as applied to hepatocyte ATPase, Novikoff and coworkers (7) noted marked enzyme inactivation with the usual lead concentration, but commented that this level was optimal for histochemical demonstration because it resulted in quantitative trapping of freed phosphate. The studies of Moses and Beaver (5) and Rosenthal (11) on renal tubular ATPase also indicated that the lead concentration necessary for histochemical demonstration nearly completely inactivated the enzyme and, at the same time, could produce significant nonenzymatic hydrolysis of the substrate. These latter investigators also reported that reduction of lead levels to 0.0005 M or less, while preserving enzyme activity, resulted in deposition of lead phosphate at loci distant from the sites of enzyme-catalyzed liberation of phosphate. The level of lead employed in the present experiment was well above that observed to yield this spurious localization, but the degree of enzyme inactivation or of lead-mediated substrate hydrolysis was not ascertained by an independent chemical assay. However, the results of the four control experiments indicate that the lead salt deposits observed in the modified protocol mark the sites of enzymatic hydrolysis of ATP. Inactivation of the enzyme by heat or elimination of the substrate prevented lead deposition, while addition of phosphate ions during incubation produced randomly distributed precipitates without preferential deposition in agranular endoplasmic reticulum. The inability to catalyze the hydrolysis of sodium glycerol phosphate is evidence that the enzyme is not a nonspecific acid phosphatase.

Incubation of striated muscle in the medium resulted in lead salt deposits in sarcoplasmic reticulum. Although ATPase activity does not necessarily indicate a role in excitation-contraction coupling, the demonstration of the enzyme under the same conditions in agranular endoplasmic reticulum of smooth muscle and sarcoplasmic reticulum of striated muscle supports the possibility

that the organelles in the two cell types have a similar function. The inability to demonstrate this activity in smooth muscle with standard techniques which can be used with striated muscle may be related to lower enzyme activity rather than qualitative differences.

Since smooth muscle cells are much smaller than striated muscle cells and lack a sarcomeric array, the individual smooth muscle cell might be thought of as a basic contractile unit analogous to the sarcomere of striated muscle. Without the transverse organization of myofilaments of the sarcomere, however, and with contraction proceeding in a wavelike fashion from end to end of the cell (4), excitation-contraction coupling in the smooth muscle cell may require a less extensive apparatus without transverse elements.

The inconsistent finding of lead phosphate deposits in dense areas among myofilaments might also be tentatively considered an indication of ATPase activity at this site. These precipitates, however, were composed of fine particles rather than clearly defined microcrystals and were not present in cells in which the plasma membrane was intact. Both these factors cast doubt on a proposed enzymatic site. However, the finding parallels the localization of ATPase activity to the A band of striated muscle (15), also enhanced by fragmentation of cells, and, taken together with the previously reported observation of increase in length and number of dense areas during contraction (3), suggests that these areas may play a role in contraction similar to that of the A band.

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