THE EFFECT OF GLUTARALDEHYDE AND FORMALDEHYDE ON THE CALCIUM PUMP OF THE SARCOPLASMIC RETICULUM

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It has been shown with biochemical methods that calcium is stored in the sarcoplasmic reticulum (SR) of muscle, and that the process of storage is associated with nucleosidetriphosphatase activity (ATPase) (1). This ATPase is stimulated by Ca^{++} and is referred to as extra ATPase as opposed to the basic ATPase that, although always present, is not related to Ca^{++} uptake. Cytochemical studies have provided complementary observations with respect to the *in situ* localization in the SR of both ATPase activity (2–10) and Ca^{++} deposition (11–13). The agreement of these biochemical and cytochemical observations is striking, and it is tempting to conclude that the cytochemically localized ATPase activity represents the site of the Ca-pump proper, especially since such a localization would also be quite agreeable with the proposed site of impulse–contraction–coupling (ICC), although, significantly, the latter seems to be confined to certain portions of the SR (terminal cisternae, reference 14) that together with portions of the sarcolemma form functional units (couplings, reference 15). Moreover, there is good evidence that muscle contraction is elicited by the release of Ca^{++} (16).

Cytochemical investigations on enzymes depend on an often precarious balance between structural preservation of tissues and cells on one hand and functional preservation of enzyme activity on the other. The choice of concentration and type of fixative may best be derived from data obtained with these fixatives as regards their influence on the isolated in vitro system. Schulze obtained such data, but in his study only the total SR ATPase was considered (17). In the SR, as has already been mentioned, the Ca-pump proper (extra ATPase) is always associated with an ATPase (basic ATPase) unrelated to Ca^{++} uptake. Therefore, if one is to attempt a correlation between the cytochemically observed SR ATPase and the physiologically significant Ca-pump ATPase, then both the extra and basic ATPases must also be separated cytochemically. In the present paper, and as a first step toward this end, an inquiry was made into the in vitro influences of both formaldehyde and glutaraldehyde on the basic and extra ATPases, as well as on the ^{45}Ca uptake. In addition, since ATP prevents the inhibitory effect of N-methyl-maleimide (NEM) and other substances on the Ca-pump (18), experiments were performed to demonstrate whether ATP also protects the extra ATPase and the Ca-pump against the inhibiting effects of the aldehydes.

MATERIALS AND METHODS

The vesicles of the SR of rabbit skeletal muscle were isolated, cleaned, and fractionated according to Hasselbach and Makinose (19). The protein, estimated by the Kjeldahl method, was kept at 0.1 mg protein per milliliter during ATP splitting and Ca^{++} uptake. The vesicles were pretreated in a solution containing 0.048 M (final concentration) phosphate buffer at pH 7.5 with either glutaraldehyde (G-151, biological grade, Fisher Scientific Company, Pittsburgh, Pa.) or formaldehyde (Merck, Darmstadt, Germany); (methanol and acid less than 0.03%). After addition of the aldehydes, the solutions were immediately centrifuged at 40,000 rpm for 60 min or left in the refrigerator (0–4°C) for 1–2 hr prior to centrifugation. The pellets were then homogenized, suspended in 0.1 M KCl, and incubated at room temperature with constant stirring. The incubation solution (pH 7.0) contained the following: 20 mM histidine, 5 mM ATP (disodium ATP from P-L Biochemicals, Inc., Milwaukee, Wis.), 5 mM $MgCl_2$, 5 mM K-oxalate, 50 mM KCl, 0.5 mM EGTA. Vesicles were added at 0 time (0.1 mg protein per milliliter), and Ca^{++} (final concentration, 0.5 mM) was added 6 min later. ATPase activity and Ca^{++} uptake were determined as previously described (19).

As a deviation from the above procedure, the protective effect of ATP on the Ca^{++} uptake of the vesicles was tested as follows. The vesicles were suspended in a solution (pH 7) containing 0.02 mM phosphate buffer, 5 mM magnesium, 5 mM K-oxalate, and 40 mM KCl. Aldehyde, vesicles, ATP, and $^{45}Ca^{++}$ were added in sequence as follows: (*a*) control, vesicles, 5 mM ATP, and 0.1 mM $^{45}Ca^{++}$ after 5 min; (*b*) aldehyde, vesicles, and 5 mM ATP after 5 min, 0.1 mM $^{45}Ca^{++}$ after another 5 min; (*c*) 5 mM ATP, vesicles, and aldehyde after 5 min, 0.1 mM $^{45}Ca^{++}$ after another 5 min.

RESULTS AND DISCUSSION

The two aldehydes exert an inhibitory effect on both the extra ATPase and Ca^{++} uptake. With 2.5 mM glutaraldehyde or 50 mM formaldehyde, there is neither an appreciable extra-ATPase activity (Fig. 1) nor Ca^{++} uptake (Fig. 2). When the concentrations of both aldehydes are lowered to 1.2 mM and 10 mM, respectively, considerable ATPase activity (Figs. 3–4) and Ca^{++} uptake (Fig. 2) can be observed. The time versus concentration dependence of the inhibitory effect of glutaraldehyde can be followed in Fig. 3. Glutaraldehyde is approximately 20 times more effective than formaldehyde in the suppression of extra ATPase activity and Ca^{++} uptake (see Figs. 1, 2). Total inhibition of the basic ATPase requires approximately four times the concentration of glutaraldehyde necessary for the elimination of the extra ATPase (Fig. 4). When the two aldehydes are at concentrations that completely inhibit the extra ATPase (2.5 mM and 50 mM, respectively), the protective effect of ATP is approximately five times greater for formaldehyde than for glutaraldehyde (Fig. 1). When ATP was added to the vesicles prior to the addition of the aldehydes, the Ca^{++} uptake was nearly unimpaired as compared to the reverse sequence.

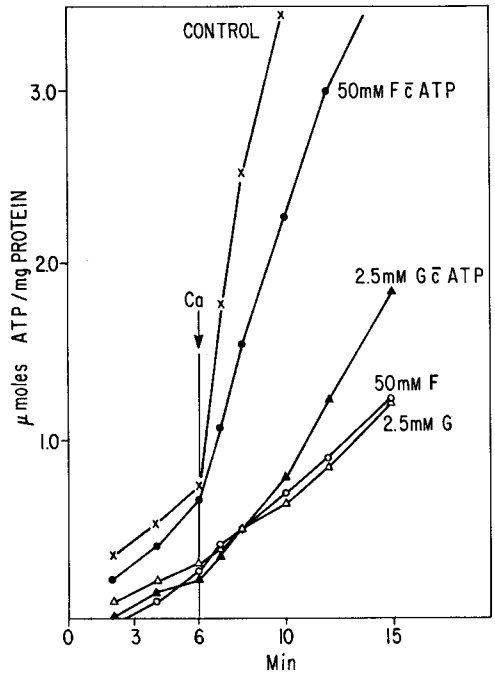

FIGURE 1 Phosphate release after preincubation (1 hr) of SR vesicles with glutaraldehyde (-△-, -▲-) and formaldehyde (-○-, -●-), with and without ATP (-●-, -▲-; -○-, -△-). Control (-×-): Phosphate release by grana that had not been preincubated with either aldehyde. The arrow indicates the time at which 0.5 mM $CaCl_2$ (final concentration) was added.

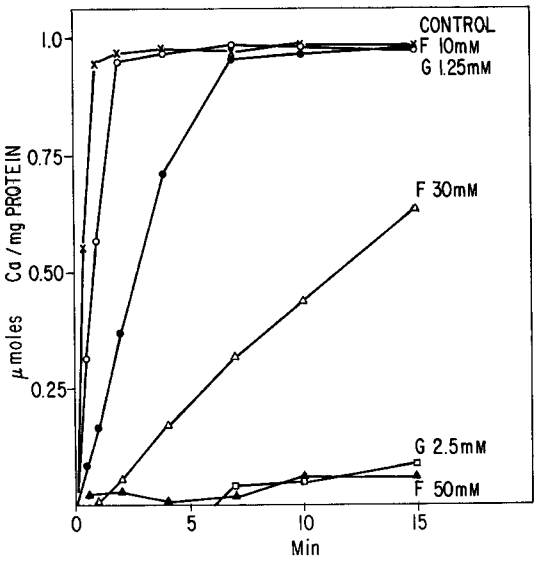

FIGURE 2 Calcium uptake after preincubation (1 hr) of SR vesicles with different concentrations of glutaraldehyde and formaldehyde. Control (-×-); 10 mM formaldehyde (-○-); 1.25 mM glutaraldehyde (-●-); 30 mM formaldehyde (-△-); 2.5 mM glutaraldehyde (-□-); 50 mM formaldehyde (-▲-). See Fig. 1, phosphate release at 2.5 mM glutaraldehyde and at 50 mM formaldehyde without ATP.

The present work suggests that previous cytochemical studies on the ATPases of the SR after formaldehyde or glutaraldehyde fixation probably demonstrated ATPases unrelated to Ca^{++} uptake. However, to conclude that the aldehyde concentrations used in the cytochemical studies are incompatible with preservation of the Ca-pump ATPase is to disregard diffusion gradients in tissue sections, a factor that was practically eliminated in the present in vitro experiments. It is virtually impossible to make any concise statement about accurate concentrations of fixative, substrate, or components of the reaction mixture at that particular level of the tissue at which the final favorable section for electron microscopy is cut. Therefore, as regards cytochemical investigations, the possibility cannot be excluded that concen-

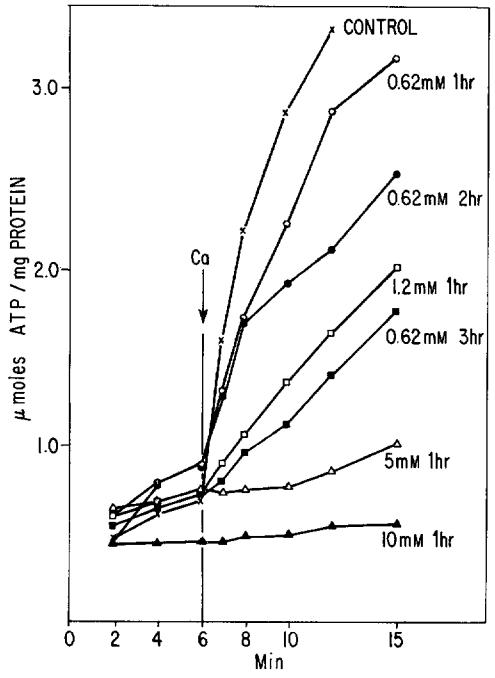

FIGURE 3 Phosphate release after preincubation of SR vesicles with various concentrations of glutaraldehyde over various periods of time. Control (-X-); 0.62 mM for 1 hr (-O-), for 2 hr (-●-), and for 3 hr (-■-); 1.2 mM for 1 hr (-□-). The data for 5 mM glutaraldehyde for 1 hr (-△-), and for 10 mM for 1 hr (-▲-)have been taken from another experiment (Fig. 4) to emphasize, in one plot, concentration-dependent spread.

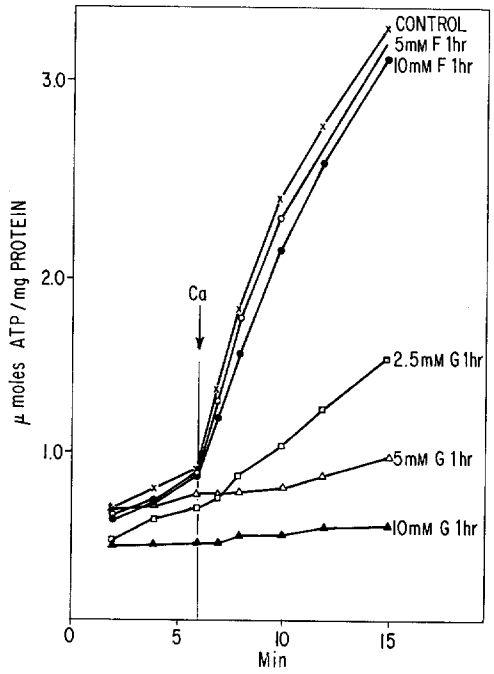

FIGURE 4 Comparison between equimolar concentrations of glutaraldehyde and formaldehyde, respectively. Glutaraldehyde 10 mM for 1 hr (-▲-), 5 mM for 1 hr (-△-), and 2.5 mM for 1 hr (-□-); formaldehyde 10 mM for 1 hr (-●-), 5 mM for 1 hr (-O-). Control (-X-): Phosphate release by SR vesicles that had not been preincubated with either aldehyde.

trations of the fixative favorable to the demonstration of the Ca-pump ATPase did, in fact, exist at a particular level of sectioning. Poor tissue preservation is often found in those areas in which the deposition of lead-phosphate reaction product is the most striking. This is what one would expect after fixation with low concentrations of aldehydes. Nevertheless, because the basic ATPase has greater resistance to aldehyde inactivation, it is probably the basic ATPase that is responsible for the lead-phosphate reaction product observed in cytochemical studies. It would be important to know whether or not the Ca-pump ATPase is further compartmentalized within the SR as regards its localization, for example, in the terminal cisterna to the exclusion of the remainder of the SR, or vice versa. Unfortunately, at the moment, a direct cytochemical demonstration of the calcium-dependent Ca-pump ATPase is frustrated because lead successfully competes with calcium for chelation by EGTA, and because no specific inhibitors of the basic ATPase are known. However, our work also shows that, if the preparation has been preincubated with ATP (see reference 20), appreciable calcium uptake can be observed even after fixation with aldehydes. The resulting improvement of preservation of cellular structure, especially with respect to the *in situ* distribution of the SR, may help to analyze possible differential localization of calcium within the SR. While the aldehyde concentrations necessary to preserve enzyme activity seem too low to provide proper structural preservation, preliminary experiments on the effect of these low concentrations on very small intact "strands" of cardiac muscle have revealed rather good fixation, provided that the tissue is postfixed in 200–500 mM (2–5%) glutaraldehyde after incubation for ATPase activity.

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