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Cross-Linking Agents Induce Rapid Calcium Release from Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: The passive permeability of skeletal muscle sarcoplasmic reticulum vesicles to Ca^{2+} ions is drastically increased upon addition of the oxidizing agent cupric phenanthroline. The permeability change, which occurs very rapidly, is partially reversed by reducing agents and cannot be explained by a direct effect of cupric phenanthroline on the lipid moiety of the membranes. The rapid efflux phenomenon is due to protein cross-linking induced by the cupric phenanthroline catalyzed oxidation of SH groups to disulfide bridges. Similar effects are also induced by cross-linking sarcoplasmic reticulum proteins with dithiodipropionic acid disuccinimido ester. The rapid Ca^{2+} efflux is inhibited by micromolar concentrations of lanthanum and by labeling the Ca^{2+}-ATPase with dicyclohexylcarbodiimide. These observations suggest that Ca^{2+} channels are formed by chemical modification of the ATPase. The Ca^{2+} permeability rate of sarcoplasmic reticulum obtained after cross-linking is compatible with the requirements of Ca^{2+} release in vivo. The possibility that Ca^{2+}-ATPase oligomers might mediate the release process is discussed.

The fast regulation of the cytosolic free Ca^{2+} concentration in fast skeletal muscle cells is assured mainly by the action of a highly specialized membrane system, the sarcoplasmic reticulum (SR).[1] Fast removal of Ca^{2+} ions from the cytosol, which induces relaxation, occurs via an efficient Ca^{2+}-pumping ATPase which is the major protein component of SR membranes. Many details of the mechanism of action of this ATPase have been clarified [for a review, see Tada et al. (1978) and deMeis (1981)]. The enzyme has now became a model of reference for the study of energy conversion by other ion-motive ATPases. However, the phenomenon which precedes contraction of the myofilaments, i.e., the factors which induce the rapid Ca^{2+} outflow from the intracellular Ca^{2+} stores [mainly from the terminal cisternae of the SR (Huxley & Taylor, 1958)], is still poorly understood. It seems plausible that the action potential at the sarcolemma and its invaginations (T system) is transmitted either via electrical or by chemical coupling to the SR cisternae, which respond by opening Ca^{2+} channels. Experiments with both isolated skinned fibers and fragmented SR vesicles have shown that a multitude of manipulations can induce contractures which are correlated to a massive release of Ca^{2+} ions from the SR network [for a review, see Endo (1977)]. The physiological significance of these experiments, however, is still a matter of speculation. An interesting hypothesis was formulated by Vanderkooi et al. (1977), who suggested that a reversible transient association of several ATPase molecules could provide the structural basis for the rapid Ca^{2+} release occurring during excitation.

In this paper, we will present evidence that, indeed, accompanying the formation of ATPase oligomers by means of covalent cross-linking a dramatic increase of the passive permeability of SR vesicles occurs. The possibility of a noncasual link between the Ca^{2+}-release phenomenon and the presence of ATPase oligomers will be discussed.

Materials and Methods

Materials. *N,N'*-[^{14}C]Dicyclohexylcarbodiimide (DCCD) was obtained from the Commisariat à l'Energie Atomique (CEA, France); 3,3'-dithiodipropionic acid disuccinimido ester (DSP) was obtained from Fluka, AG Buchs (Switzerland). All other reagents were of the highest grade commercially available. SR vesicles were isolated from white muscles of the hind legs of rabbits according to Eletr & Inesi (1972). The preparation obtained, which consisted of fragments derived from both the longitudinal and cisternal systems, was used in most of the experiments. When required, SR vesicles were subfractionated on a linear sucrose gradient (0.85-1.6 M) after centrifugation in a swinging bucket rotor (Beckman SW 27) at 25 000 rpm for 16 h (Myiamoto & Racker, 1981) in order to separate the light fraction (0.9-1.05 M) from the heavy vesicles (1.2-1.4 M sucrose). Cross-linking of SR proteins was induced by either cupric phenanthroline (CuPh) or DSP. SR vesicles (usually 1 mg/mL) were incubated at room temperature in 100 mM NaCl (or KCl), 20 mM MOPS, pH 7, 20 μM $CaCl_2$, and various amounts of 1:3 $CuSO_4$:1,10-orthophenanthroline. At various time intervals, oxidation was either stopped in SDS sample buffer supplemented with 10 mM EDTA and 50 mM *N*-ethylmaleimide and samples were analyzed by gel electrophoresis or stopped by dilution in an ice-cold medium (100 mM NaCl, 20 mM MOPS, pH 7, and

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[1] Abbreviations: SR, sarcoplasmic reticulum; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CuPh, cupric phenanthroline; DTT, 1,4-dithiothreitol; DSP, 3,3'-dithiodipropionic acid disuccinimido ester; DCCD, *N,N'*-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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1 mM EDTA). When the ATPase or Ca^{2+}-uptake activity was to be measured, the CuPh-treated vesicles were directly diluted (200-fold) into the appropriate reaction mixtures. Alternatively, the SR vesicles were incubated at room temperature with DSP, basically as previously described (Louis & Holroyd, 1978). The reaction was stopped by neutralizing unreacted DSP with excess glycine (50 mM).

Assays. ATPase was measured at room temperature essentially as described by Neet & Green (1977). SR vesicles (ca. 5 μg/mL) were incubated in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 20 mM MOPS, pH 7, 1 mM EGTA, 2 units of pyruvate kinase/mL, 2 units of lactate dehydrogenase/mL, 5 mM phosphoenolpyruvate, and 200 μM NADH. Basal ATPase activity was measured after the addition of 0.5 mM ATP in a dual-wavelength spectrophotometer (550 and 360 nm). Thereafter, 1 mM $CaCl_2$ was added, and Ca-dependent ATPase activity was measured. ATPase activity of leaky vesicles was determined in the presence of 2 μM A23187.

Ca^{2+} uptake was measured by using radioactivity labeled ^{45}Ca and the Millipore filtration technique (Martonosi & Feretos, 1964). The uptake medium was composed of 100 mM NaCl, 5 mM $MgCl_2$, 20 mM MOPS, pH 7, ^{45}CaEGTA buffers, and, when required, 5 mM oxalate. Ca^{2+} transport was blocked on 0.45-μm filters, and vesicles were washed with ice-cold medium containing 2 mM $LaCl_3$ (Chiesi & Inesi, 1979).

Passive efflux of Ca^{2+} was measured after passive loading of the vesicles (10 mg/mL, 0 °C, overnight) in 100 mM NaCl, 20 mM MOPS, pH 7, and 1 mM $^{45}CaCl_2$. Efflux experiments were carried out at room temperature by diluting the loaded vesicles 50 times into 100 mM NaCl, 20 mM MOPS, pH 7, and 1 mM EGTA. The reaction was stopped on Millipore filters using a La^{3+} solution. The ^{45}Ca–Ca exchange rate was measured in a similar way but with 1 mM $CaCl_2$ instead of EGTA in the dilution medium. A rapid-mixing device with a time resolution of approximately 200 ms was also used to measure Ca–Ca exchange (Kessler et al., 1977). ^{45}Ca-loaded vesicles were diluted 20-fold to initiate exchange. The reaction was stopped by quenching in a cold solution containing 2 mM $LaCl_3$. The quenching mixture was filtered (within 5 s), and the filters were washed again before radioactivity was counted.

The passive permeability of liposomes was mesured with a dialysis procedure. Liposomes were formed from a mixture of phosphatidylethanolamine and phosphatidic acid (95:5) by the sonication procedure (30 min at maximal energy output in the pulsed mode) in 100 mM KCl, 10 mM Tris, pH 8, and 1 mM $^{45}CaCl_2$ at a concentration of 20 mg/mL. Monolamellar liposomes were obtained in the supernatant after centrifugation of the sonicated mixture. Extravesicular ^{45}Ca was removed by treating the liposomes with Chelex resin (batch procedure). Samples of liposomes (1 mL) were then dialyzed at room temperature against 20 mL of 100 mM KCl and 10 mM Tris, pH 8, and Ca efflux was measured by counting the radioactivity leaking out of the dialysis bags. The effect of CuPh and Ca ionophore on the efflux rate was determined by adding 1 mM CuPh or 10 μM A23187 to the liposomal suspension at zero time.

Gel electrophoresis was carried out as described by Laemmli (1970) with the minor modifications previously reported (Chiesi & Carafoli, 1982). Usually, 5% or 7% gels were utilized, and the sample buffer contained no reducing agents. Coomassie blue stained gels were analyzed densitometrically.

SR ATPase was modified by DCCD treatment as described by Pick & Racker (1979). SR vesicles (0.3 mg/mL) were incubated at room temperature in 100 mM NaCl, 50 mM MOPS, pH 6, 1 mM EGTA, and 30 μM DCCD. After 1 h, the mixture was cooled and centrifuged, and the vesicles were resuspended (10 mg/mL) in 100 mM NaCl, 50 mM MOPS, pH 7, and 1 mM $^{45}CaCl_2$. After being passively loaded overnight at 0 °C, ^{45}Ca–Ca exchange was measured. Control vesicles were treated in a similar way but in the absence of DCCD. In parallel experiments, the vesicles were treated with [^{14}C]DCCD in a similar way, and then the proteins were separated on 12% Laemmli gels. After being fixed, the gels were saturated with 22.2% diphenyloxazole in dimethyl sulfoxide (w/v). Autofluorography was carried out on Kodak films (XS-5) at −70 °C after the gels were dried.

Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as a standard.

Results

CuPh catalyzes the air oxidation of sulfhydryl groups to disulfides (Kobashi, 1968) and is an efficient reagent to cross-link SR Ca-ATPase molecules (Louis et al., 1977; Chyn & Martonosi, 1977; Hebdon et al., 1979). This observation, as well as evidence coming from electron microscopy (Scales & Inesi, 1976; Dux & Martonosi, 1983a), fluorescence energy transfer (Vanderkooi et al., 1977), excimer formation of pyrene-labeled ATPase (Lüdi & Hasselbach, 1982), rotational mobility (Hoffmann et al., 1979), radiation inactivation analysis (Maurer et al., 1983), and other experimental lines, has led to the concept that the ATPase molecules may (or may have the tendency to) be organized as oligomers in the SR membranes. Since oligomeric and monomeric structures can exist in a dynamic equilibrium which could be rapidly shifted by physiological effectors, it is of interest to investigate whether the various aggregational states of the ATPase could perform different functions. Therefore, we have studied the effect of CuPh-catalyzed oligomerization on the activities of SR vesicles. Figure 1B shows that the amount of monomeric ATPase (as determined after SDS gel electrophoresis in the absence of reducing agents) decreased with time after addition of CuPh. As judged from polyacrylamide gels, the ATPase was the only visible protein of SR to undergo aggregation, as already observed by others (Murphy, 1976; Chyn & Martonosi, 1977; Louis et al., 1977; Hebdon et al., 1979). The Ca^{2+}-dependent ATPase activity of membranes made leaky by the Ca^{2+} ionophore A23187 decreased in parallel with the disappearance of the monomeric ATPase species (Figure 1A), indicating that the cross-linking of ATPase molecules also involved sulfhydryl groups essential for catalysis. On the other hand, the Ca^{2+}-independent (basal) ATPase activity was not significantly affected by CuPh-induced oligomerization (Figure 1A). Steady-state Ca^{2+}-uptake activity of SR vesicles was studied under the same cross-linking conditions and found to be greatly impaired even during the early stages of oligomerization (Figure 1B), indicating that uncoupling between hydrolytic and transport activity was also occurring. In a recent report (Bailing, 1980), a similar observation was made by using 1,5-difluoro-2,4-dinitrobenzene to cross-link the ATPase. It was concluded that the cross-linking agent might have caused disruption of ATPase sites involved in the translocation reaction. However, another potentially interesting explanation could be that cross-linking had rendered the vesicles leaky, thus, impairing their retention of accumulated Ca^{2+} ions. Strong support for this interpretation was obtained from experiments on the effect of CuPh on the Ca^{2+}-dependent ATPase activity of intact vesicles in the absence of precipitating anions (i.e., oxalate) and Ca^{2+} ionophores. Figure 1A shows that the steady-state rate of the Ca^{2+}-stimulated hydrolytic

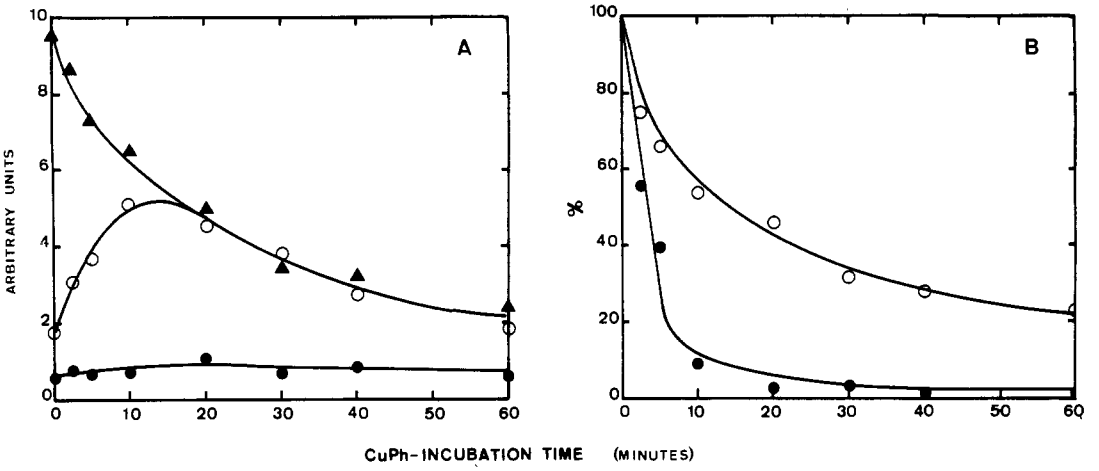

FIGURE 1: (A) Effect of CuPH on ATPase activity. SR vesicles were incubated at room temperature in a medium containing 50 mM MOPS, pH 7, 100 mM KCl, 5 mM $MgCl_2$, and 20 μM $CaCl_2$ at a concentration of 1 mg/mL. At zero time, 500 μM phenanthroline and 160 μM $CuSO_4$ were added. After various time invervals, aliquots were withdrawn, and ATPase activities were tested with a coupled enzyme assay as described under Materials and Methods. (●) Basal ATPase activity; (○) Ca-activated ATPase activity; (▲) Ca-dependent ATPase activity of uncoupled vesicles, obtained by the addition of 5 μM A23187 to the ATPase reaction medium. (B) Effect of CuPh on Ca uptake and oligomerization. The experiment was performed exactly as described for (A). After CuPh addition, aliquots were analyzed for Ca uptake activity (●) in the presence of oxalate with the Millipore filtration technique as described under Materials and Methods. The Ca uptake rate is given as a percent of the activity of untreated vesicles. Simultaneously, the degree of oligomerization of the ATPase was checked after the CuPh-induced reaction was quenched with 10 mM EDTA and 1% SDS. The protein composition of the quenched samples was analyzed by gel electrophoresis (see Materials and Methods), and the disappearance of the monomeric species of the ATPase was determined by densitometric scanning after Coomassie Blue staining (○).

activity was low under these conditions (zero time), since a high-Ca^{2+} gradient across the membranes was established. However, the Ca^{2+}-dependent ATPase rate was stimulated severalfold during the early stages of cross-linking (Figure 1A) and reached values which were comparable to those obtained in the presence of the Ca^{2+} ionophore. This indicates the incapability of the vesicles to maintain a Ca^{2+} gradient after CuPh treatment. To reinforce this conclusion, the effect of CuPh on the passive Ca^{2+} permeability of SR vesicles was investigated. To this purpose, SR vesicles were passively loaded with ^{45}Ca and then incubated a short time at room temperature with CuPh, before diluting them into an isoosmotic medium containing EGTA, which also chelates Cu ions and thus terminates the catalyzing action of CuPh. Figure 2A shows that CuPh indeed had drastic effects on the Ca^{2+} efflux kinetics from SR vesicles. Although control efflux data could be fitted by a single exponential, typical biphasic curves were obtained in the presence of CuPh. Thus, after the rapid phase of Ca^{2+} release, which depends on the preincubation conditions in the presence of CuPh, the efflux rate resumed the slow control value. Such biphasic efflux kinetics suggest the presence of a heterogeneous population of SR vesicles: a fraction of the vesicles exhibit normal Ca^{2+} permeability characteristics, while the remainder are strongly affected by CuPh (i.e., the Ca^{2+} permeability was drastically increased). As the first component of Ca^{2+} efflux was too rapid to be followed by normal mixing techniques, the possibility existed that CuPh treatment had somehow damaged some of the vesicles. To rule out this explanation of the biphasic efflux curves, the effect of CuPh on the efflux of a slowly permeating molecule such as [^{14}C]sucrose was investigated. Sucrose efflux experiments from passively loaded vesicles treated with CuPh gave results which were qualitatively similar to those on Ca^{2+} (Figure 2B). However, the sucrose efflux rates were slow enough to enable the resolution also of the first rapid component, indicating that all the vesicles were intact after controlled CuPh treatment. The increased Ca^{2+} and sucrose permeability of some of the vesicles seemed to be due to the formation of a common efflux pathway. Therefore, these results suggested that the size of the molecular structures allowing for the rapid Ca^{2+} efflux was large enough to accommodate sucrose molecules.

The experiments presented so far provide little information on the mechanism underlying the large permeability change observed after CuPh treatment of SR vesicles. Several explanations for the release phenomenon are possible. For instance, CuPh might have a direct effect on membrane permeability or catalyze lipid peroxidation reactions which could then lead to massive Ca^{2+} efflux. Alternatively, membrane permeability could be affected by CuPh-induced modification of protein components other than the formation of oligomeric structures. Oxidation of sulfhydryl groups to disulfides can occur intermolecularly as well as intramolecularly. The covalent modification of specific SH groups within a monomeric chain could also account for the observed ionophoric activity. The following experiments were carried out to test the validity of the various possibilities outlined above.

Effect of CuPh on Liposome Permeability. Monolamellar liposomes were prepared from a mixture of egg lecithin and phosphatidic acid (95:5) in 100 mM KCl, 10 mM Tris, pH 8, and 1 mM $^{45}CaCl_2$ by the sonication procedure. Extravesicular Ca was removed by passing the liposomes through a Chelex column, and Ca^{2+} permeability was measured with the dialysis procedure as described under Materials and Methods. Accordingly, we carried out Ca^{2+} efflux experiments by using control liposomes and liposomes treated either with 1 mM CuPh or with a 10 μM sample of the Ca^{2+} ionophore A23187. Such experiments could unequivocally demonstrate (not shown) that the low Ca^{2+} permeability rate of normal liposomes was drastically stimulated by the inclusion of the Ca^{2+} ionophore, while the treatment with CuPh caused no measurable effect on the passive efflux rate. Therefore, one can state that CuPh has no direct effect on the permeability characteristics of lipid bilayers and that its mode of action on SR membranes must involve the chemical modification of some protein components.

Effect of Reducing Agents on the CuPh-Induced Permeability Change. Protecting agents of SH groups, such as 1,4-dithiothreitol (DTT) or mercaptoethanol, prevented almost completely the CuPh-induced cross-linking and ATPase oli-

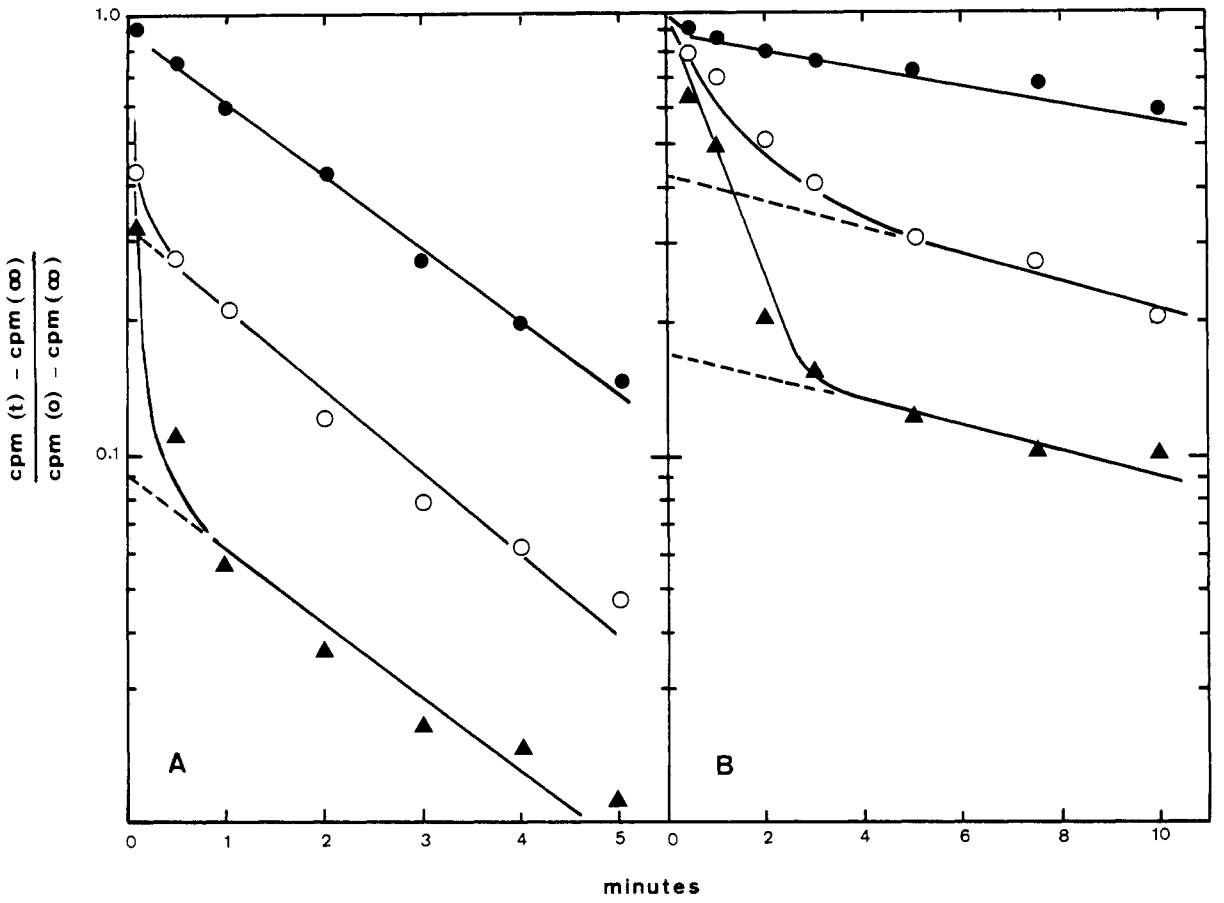

FIGURE 2: Effect of CuPh on passive Ca and sucrose efflux. SR vesicles suspended in 100 mM NaCl, 20 mM MOPS, pH 7, 10 mM sucrose, and 2 mM $CaCl_2$ were incubated overnight at 0 °C in the prescence of either ^{45}Ca (A) or [^{14}C]sucrose (B) to study passive sucrose or Ca efflux. Loaded SR vesicles were then incubated for 3 min at room temperature. When required, CuPh was added during this incubation time. (●) Control efflux (no CuPh); (○) 500 μM CuPh; (▲) 1000 μM CuPh. Efflux studies were initiated immediately after CuPh incubation, by dilution of the vesicles at room temperature into an isoosmotic medium in the presence of EGTA, with the Millipore filtration technique as described (see Materials and Methods). cpm(∞) were obtained by measuring the radioactivity content of the vesicles (▲) after 60 min of efflux. cpm(0) were obtained by extrapolation of efflux curves when 200 μM $LaCl_3$ was present in the dilution medium (see Figure 4).

gomerization reactions and the concomitant increase in Ca^{2+} permeability of SR vesicles. Table I shows the effect of DTT on the Ca^{2+} uptake activity of SR vesicles. It can be seen that the inhibitory effect induced by a short treatment of the vesicles with CuPh, which reduced the Ca^{2+} uptake level to about 60% of the control value, could be completely prevented in the presence of 2 mM DTT. Under these mild conditions of CuPh treatment, no significant effect on the Ca-dependent ATPase activity in the presence of a Ca ionophore could be observed (not shown). Therefore, the maximal Ca^{2+} levels which SR vesicles could accumulate in the presence of ATP were directly related to the capability of the vesicles to retain the Ca^{2+} ions translocated across the membranes and could be considered as an indirect measure of the passive Ca^{2+} permeability. Table I also shows that the reducing agent could partially reverse the inhibitory action of the previous CuPh treatment. Thus, it is clear that the major chemical modification induced by CuPh, which was responsible for the drastic increase in SR permeability to Ca^{2+}, was the oxidation of specific SH groups to disulfides. It still remains to be established whether the oxidation of such groups is merely a means to induce the formation of oligomeric structures, necessary for rapid Ca^{2+} release, or whether some SH groups are somehow involved directly in the efflux phenomenon.

Effect of DSP on SR Activities. DSP is a homobifunctional reagent for primary amino groups and has been used to cross-link proteins in various systems, including SR membranes, where it induces the formation of homopolymers of

Table I: Effect of DTT on Ca Uptake Activity[a]

	max Ca^{2+} uptake (%)
control - DTT	100
control + DTT	100–105
CuPh	57–64
DTT, CuPh	95–100
CuPh, DTT	79–85

[a] SR vesicles were preincubated at room temperature at a concentration of 1 mg/mL in 100 mM KCl, 20 mM MOPS, pH 7, 5 mM $MgCl_2$, 20 μM $CaCl_2$, and various agents as indicated below. Ca uptake activity was measured by diluting the vesicles into 10 volumes of a medium containing 100 mM KCl, 5 mM $MgCl_2$, 20 μM $^{45}CaCl_2$, 20 mM MOPS, pH 7, and 2 mM ATP. Maximal levels of uptake were determined with the Millipore filtration technique. Preincubations were as follows: control - DTT, 2-min preincubation in the medium described above; control + DTT, 2-min preincubation in the presence of 5 mM DTT; CuPh, 1-min preincubation with 150 μM CuPh; DTT, CuPh, 2-min preincubation with both 5 mM DTT and 150 μM CuPh; CuPh, DTT, 1-min preincubation with 150 μM CuPh followed by 1-min preincubation with 5 mM DTT.

ATPase molecules. We have measured the permeability and the ATPase activity of SR vesicles treated with DSP for various times and have found that (i) the basal ATPase activity was only minimally affected, (ii) the Ca^{2+}-dependent ATPase activity (measured in the presence of a Ca^{2+} ionophore) was gradually inhibited and paralleled the level of remaining ATPase monomers (Figure 3A), and (iii) the Ca^{2+}-dependent hydrolytic activity measured in the absence of Ca^{2+} ionophores was markedly stimulated in the early stages of DSP treatment

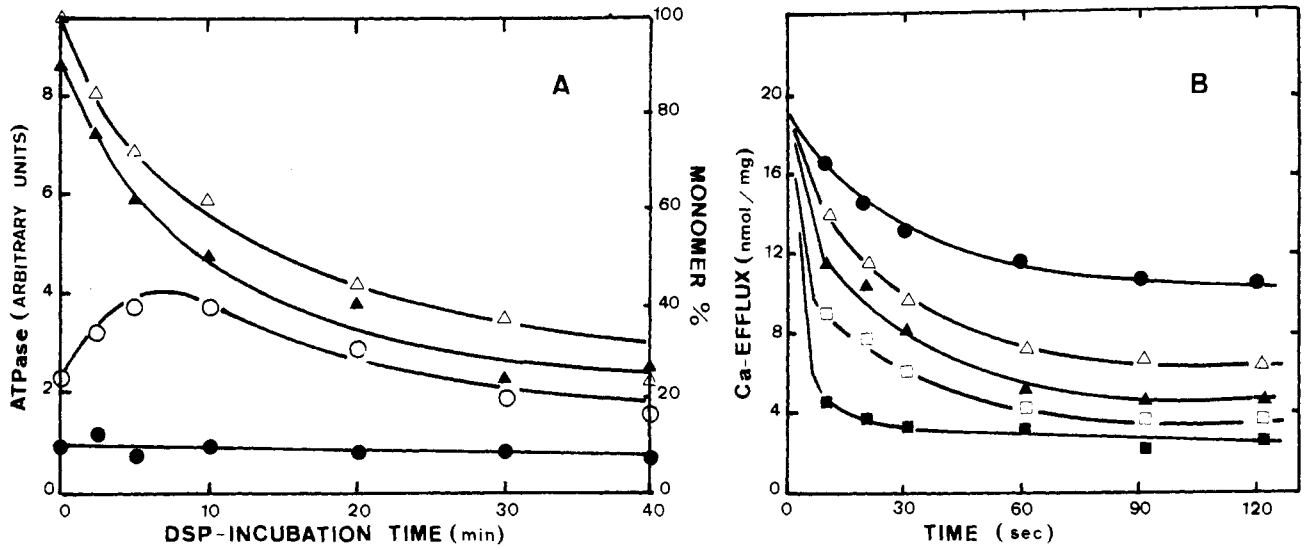

FIGURE 3: (A) Effect of DSP on oligomerization and ATPase activities. Oligomerization was induced at room temperature by the addition of 350 μM DSP to SR vesicles (3 mg/mL) in 50 mM MOPS, pH 7, 100 mM KCl, and 5 mM $MgCl_2$. At sequential time intervals, aliquots were quenched with 50 mM glycine, and ATPase activity was measured as described for Figure 1A. (●) Basal activity; (○) Ca-dependent activity; (▲) Ca-dependent activity of leaky vesicles in the presence of 5 μM A23187. Simultaneously, the disappearance of ATPase monomers (△) was determined after electrophoretic separation of SR proteins, as described under Materials and Methods. (B) Effect of DSP on passive Ca efflux. SR vesicles were loaded overnight at 0 °C with radioactively labeled $^{45}CaCl_2$. The composition of the medium was 3 mg/mL SR, 100 mM KCl, 50 mM MOPS, pH 7, and 1 mM $^{45}CaCl_2$. Passive efflux was measured by diluting loaded vesicles into an isoosmotic medium with EGTA at room temperature, before and after exposing the loaded vesicles to 350 μM DSP. Time after DSP addition: (●) 0 min; (△) 1 min; (▲) 7 min; (□) 20 min; (■) 40 min.

(Figure 3A). The stimulation was due to the drastic increase of the passive Ca^{2+} permeability of the vesicles after DSP treatment (Figure 3B). It is apparent that DSP and CuPh caused strikingly similar functional changes of SR vesicles. These observations indicate that the modification of either SH or NH_2 groups can induce rapid Ca^{2+} efflux.

Identification of the Protein Component of SR Membranes Responsible for the CuPh-Induced Ca^{2+} Release. It has been reported by many laboratories that the ATPase is the only protein of SR vesicles which undergoes oligomerization after CuPh treatment. However, we have observed that large changes in passive membrane permeability occur long before extensive oligomerization of the ATPase molecules was induced. This is emphasized in Figure 4, where the effect of CuPh on ^{45}Ca–Ca exchange is shown. In these experiments, SR vesicles were passively loaded with 1 mM radioactivity labeled Ca^{2+} and then diluted into an identical medium containing 1 mM cold Ca^{2+}. As EGTA was absent from the dilution medium, CuPh-induced oligomerization was initiated at zero time after vesicle dilution as soon as Ca–Ca exchange began. It can be seen from Figure 4 that CuPh induced an immediate (within 5 s) increase in the Ca^{2+} permeability of the vesicles. Parallel experiments showed that at this stage only a minor percentage of the ATPase molecules were cross-linked (not shown). This observation could indicate that the cross-linking (or aggregation) of a minimal amount of ATPase molecules is sufficient to induce massive Ca^{2+} release. Since only a minor amount of cross-linked structures is required, it is in principle also possible that a minor protein component other than the ATPase, barely visible on polyacrylamide gels, could account for the rapid efflux phenomenon. Thus, the effect of ligands, which are expected to interact specifically with the ATPase molecules, on the rapid Ca exchange was investigated. It was found that small concentrations of La^{3+} could inhibit completely the rapid Ca–Ca exchange induced by CuPh (Figure 4). On the other hand, the CuPh-catalyzed formation of oligomers was not affected by millimolar La^{3+} concentrations (not shown). Therefore, the inhibitory effect of La^{3+} was not directed at the formation

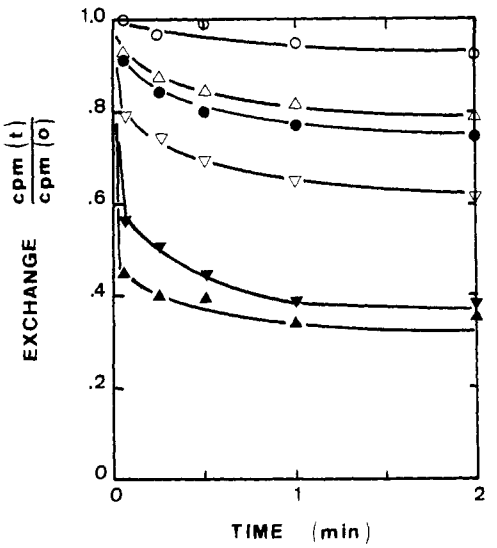

FIGURE 4: Effect of La^{3+} on CuPh-induced Ca release. SR vesicles were passively loaded as described for Figure 3B. ^{45}Ca–Ca exchange was initiated at room temperature by diluting the loaded vesicles 50 times in 100 mM KCl, 50 mM MOPS, pH 7, and 1 mM cold $CaCl_2$ (●). When required, the dilution medium was supplemented with 300 μM CuPh and various amounts of $LaCl_3$: (▲) 0, (▼) 5, (▽) 10, (△) 20, and (○) 200 μM $LaCl_3$. Exchange was measured with the Millipore filtration technique as described under Materials and Methods. The radioactivity content of the vesicles corresponding to zero time was obtained by extrapolation of the exchange curve measured in the presence of 200 μM $LaCl_3$.

of cross-links but rather was manifested by plugging the aqueous pores formed after CuPh treatment. La^{3+} is a nonspecific ligand which interacts with several membrane components and has been successfully used as a quenching agent of passive fluxes across various membrane systems. However, it is interesting to note that half-maximal inhibition of the rapid Ca–Ca exchange induced by CuPh was observed at La^{3+} concentrations below 10 μM in a medium containig 1 mM $CaCl_2$. This strongly indicates that the inhibitory effect of La^{3+} was due to a specific interaction with a site of high

affinity. Interestingly, the La^{3+} affinity of the SR ATPase has been found to be in the micromolar range (Chevallier & Butow, 1971). The experiments of Figure 4 also document the usefulness of La^{3+} in the accurate determination of the level of Ca^{2+} loading of the vesicles at zero time, especially in the efflux or exchange experiments on CuPh-treated vesicles, which were highly permeable.

Additional evidence for the implication of the ATPase oligomers in the rapid Ca^{2+} efflux was obtained by using DCCD. Under certain experimental conditions, DCCD interacts specifically with a hydrophobic carboxylic group of the ATPase of SR membranes, which is involved in Ca^{2+} binding and is essential for all Ca^{2+}-dependent activities of the pump (Pick & Racker, 1979). Accordingly, SR vesicles were incubated with 30 μM DCCD for 1 h at room temperature at pH 6 in the presence of 1 mM EGTA and then washed several times to remove unreacted DCCD and EGTA. Measurements of Ca^{2+}-dependent ATPase activity showed that DCCD-treated vesicles displayed only 5–12% of the activity of control vesicles, which were treated similarly but in the absence of DCCD. In parallel experiments using [^{14}C]DCCD, we could confirm that the reagent had interacted specifically with the ATPase (Figure 5A). DCCD-treated and control vesicles were then passively loaded with $^{45}CaCl_2$, and the effect of CuPh on the passive permeability was investigated. DCCD had no significant effect on the normal passive permeability of SR vesicles in the absence of CuPh (Figure 5B1). When 50 μM or higher concentrations of CuPh were included in the dilution medium (Figure 5B2), only a minor effect of DCCD on the rapid Ca–Ca exchange rate could be observed. However, the exchange rate obtained with these CuPh concentrations was too rapid to be resolved by the Millipore filtration technique, and a possible inhibitory effect of DCCD could have escaped detection. Indeed, when the CuPh concentration was reduced (3–6 μM) to allow measurement of initial exchange rates, a strong inhibition (50–60%) of the rapid exchange rate induced by CuPh was apparent in vesicles in which the ATPase was modified by selective DCCD labeling (Figure 5B3,4).

Heterogeneous Response of SR Vesicles to CuPh-Induced Ca^{2+} Release. It is apparent from the results presented in Figure 2 that SR vesicles did not respond homogeneously to the CuPh-induced increase in the passive permeability. The same observation can be made also for all Ca–Ca exchange experiments presented, where only a fraction (50–70%) of the SR vesicles displayed a high exchange rate in the presence of CuPh in the dilution medium within the time of analysis. To improve the analysis of this particular behavior of the SR membranes, the preparation was subfractionated into heavy and light vesicles, which are usually considered to derived from the terminal cisternae and the longitudinal system of the SR network, respectively (Meissner, 1975). Ca–Ca exchange measurements on the various SR fractions have established that, indeed, the heavy vesicles were much more responsive to a CuPh-induced increase in permeability than the light fraction (not shown). Only 20–30% of the total intravesicular Ca^{2+} was rapidly released by the light vesicles, while values as high as 65–75% could be obtained with the heavy fraction. Interestingly, we also could observe that the rate of ATPase oligomerization induced by CuPh is much faster in the heavy fraction as compared to the light SR vesicles (not shown).

Rate of CuPh-Induced Ca^{2+} Release. A question of interest concerns the maximal rates of Ca^{2+} release which can be achieved after CuPh-induced cross-linking. As shown above, complete uncoupling between ATPase activity and Ca^{2+}-loading capacity of SR vesicles was observed even during the early stages of both CuPh- and DSP-induced cross-linking (see Figures 1 and 3, respectively). This could be correlated to the massive changes in the passive Ca^{2+} permeability characteristics produced when even a minor degree of oligomerization was induced by the cross-linking agents. We investigated cross-linking conditions which induced a minimal but measurable fraction of ATPase oligomers, thus permitting the determination of the Ca–Ca exchange rate. Figure 6 shows an experiment in which Ca–Ca exchange induced by the presence of 300 μM CuPh in the dilution medium was measured with the aid of a rapid-mixing device (see Materials and Methods). Exchange rate constants obtained ranged between 1.5 and 2 s^{-1} (giving initial efflux rates of 20–30 nmol of Ca^{2+} mg^{-1} s^{-1}), whereas the total fraction of oligomeric structures (i.e., from dimers up to polymers of the ATPase) formed within 2 s was approximately 3–5% of the total ATPase population. It is evident that the rates measured from such experiments only represent a lower limit. Indeed, the CuPh-induced formation of the putative "channels" or "pores" might have been rate limiting.

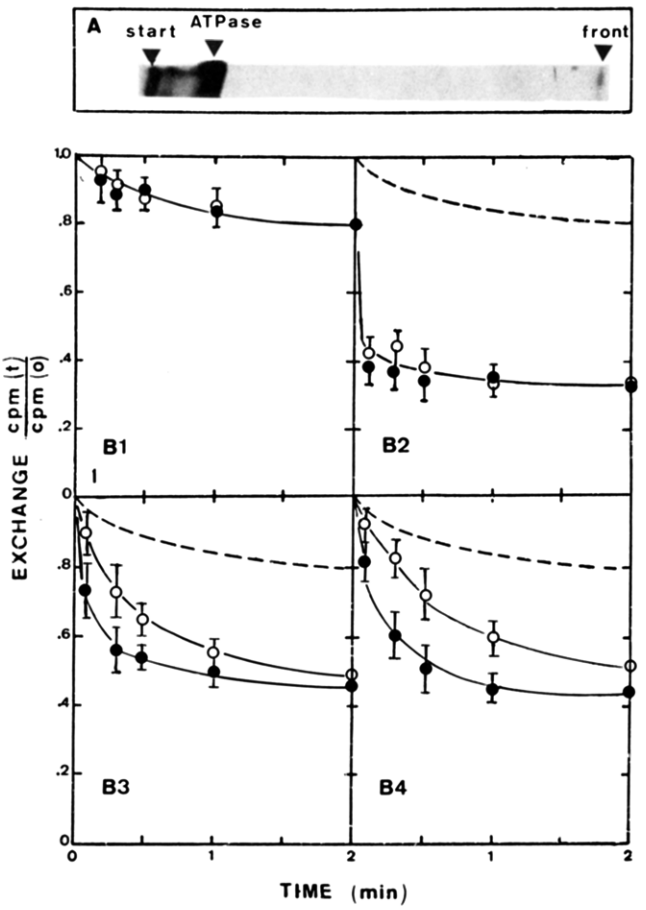

FIGURE 5: (A) [^{14}C]DCCD labeling of SR proteins. SR vesicles (0.3 mg/mL) were incubated for 1 h at room temperature with 30 μM [^{14}C]DCCD in 100 mM KCl, 50 mM MOPS, pH 6, and 1 mM EGTA. The vesicles were then washed 2 times, and labeling of SR proteins was investigated after electrophoresis on 12% acrylamide gels and fluorography as described (see Materials and Methods). (B) Effect of DCCD on the CuPh-induced Ca release. SR vesicles were labeled with cold DCCD as described in (A) and resuspended after being washed in 100 mM KCl, 50 mM MOPS, pH 7, and 1 mM $^{45}CaCl_2$. Control vesicles were treated in the same way but in the absence of DCCD. ^{45}Ca–Ca exchange was measured as described in Figure 4. Closed symbols, control vesicles (–DCCD); open symbols, DCCD-treated vesicles; B1, normal exchange rate; B2, exchange measured in the presence of 50 μM CuPh in the dilution medium; B3, 6 μM CuPh; B4, 3 μM CuPh. Values shown are given as relative $\bar{x} \pm$ SEM (n = 4).

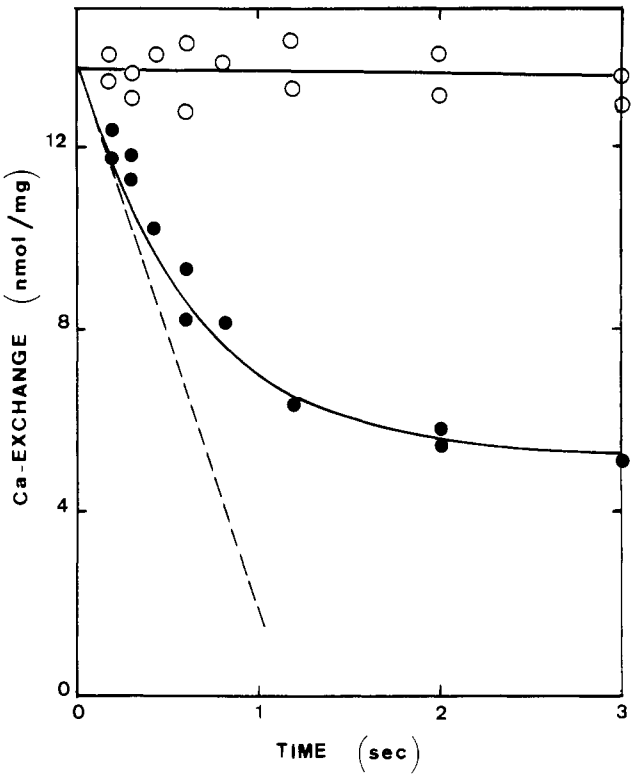

FIGURE 6: Rate of ^{45}Ca–Ca exchange induced by CuPh. ^{45}Ca–Ca exchange was carried out as described in Figure 4 after passive loading of SR vesicles with 1 mM $^{45}CaCl_2$. (○) Control exchange rate; (●) exchange rate obtained with 300 μM CuPh in the dilution medium. The experiments were carried out with the Millipore filtration technique and rapid-mixing equipment as described under Materials and Methods.

Prolonged incubation of SR vesicles in the presence of CuPh produced higher levels of oligomerization and Ca^{2+} efflux rates which were beyond the detection limits of the rapid-quenching apparatus. Therefore, it was not possible to establish whether the Ca^{2+} efflux process was related somehow to the cross-linking level of the ATPase. We could circumvent these methodological limitations by measuring the characteristics of the passive exchange rate of sucrose at 0 °C. As shown in Figure 2, the channels formed by CuPh treatment are also permeable to sucrose although the passive efflux rate is considerably slower than that of Ca^{2+} ions. Figure 7 shows that sucrose permeability increased as a function of the time of CuPh treatment of the vesicles, whereas the fraction of ATPase monomers on polyacrylamide gels progressively decreased. The exact relationship between oligomers formed and efflux rates cannot be obtained by this simple experimental approach. However, the results clearly demonstrate that a certain correlation between increasing passive permeability and increasing oligomerization levels exists. Therefore, these observations indicate that maximal Ca^{2+} release rate constants of SR vesicles extensively cross-linked might be in fact 1 or 2 orders of magnitude higher than those calculated from Figure 6.

Discussion

CuPh is a mild oxidant which catalyzes the formation of disulfide bridges (Kobashi, 1968). The formation of oligomeric structures induced by CuPh requires that SH groups on different monomeric species are in the proper orientation and distance (less than 2 Å). Thus, CuPh is considered to be a reliable and specific cross-linking agent. Indeed, when applied to SR vesicles, it induces the exclusive formation of homopolymers of the ATPase (Murphy, 1976; Louis et al., 1977; Chyn & Martonosi, 1977; Hebdon et al., 1979). The results

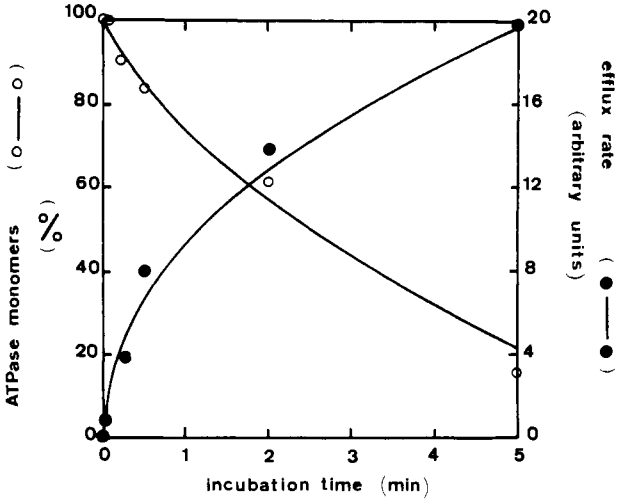

FIGURE 7: Sucrose efflux rate after extensive oligomerization. The heavy subfraction of SR vesicles (see Materials and Methods) was passively loaded at 0 °C with 10 mM [^{14}C]sucrose in the presence of 100 mM NaCl–20 mM MOPS, pH 7, at a concentration of about 10 mg/mL. Aliquots of loaded vesicles were then warmed up to 30 °C, and 1.5 mM CuPh was added. At sequential time intervals, the action of the catalyzer was quenched by addition of 2 mM EDTA and cooling of the suspension down to 0 °C. Aliquots of treated vesicles were analyzed by gel electrophoresis in the absence of reducing agents, and the amount of ATPase monomers was determined densitometrically. In parallel, the sucrose efflux rate was determined at 0 °C after 50-fold dilution into a medium of identical composition but in the absence of radioactivity (see Figure 2).

obtained in this study show definitely that CuPh treatment of SR vesicles induces a sudden and drastic change in the passive permeability characteristics of the vesicles. The increase in permeability is not due to disruption of membrane integrity or to a direct effect of CuPh on the lipid bilayer. Instead, our observations suggest that the chemical modification of specific membrane components, which can partially be reversed by reducing agents, is responsible for the formation of channels in the membrane. CuPh-induced Ca^{2+} release is inhibited by micromolar concentrations of La^{3+} and by DCCD. The ATPase has high-affinity La-binding sites facing the cytosol (Chevallier & Butow, 1971) and is the only protein in the SR membranes specifically labeled by DCCD under the conditions used here to inhibit rapid efflux [see Figure 5A and Pick & Racker (1979)]. These combined observations indicate strongly that the ATPase could be directly involved in the release phenomenon.

A very important aspect of the cross-linking-induced Ca^{2+} release process concerns the high permeability coefficients obtained. In experiments similar to that shown in Figure 6, where only a minimal degree of cross-linking was permitted, rate constants of Ca^{2+} release on the order of 1.5–2 s^{-1} were obtained. In such experiments, SR vesicles were passively loaded with 1 mM $CaCl_2$, so that initial efflux rates of 20–30 nmol of Ca^{2+} mg^{-1} s^{-1} can be calculated. Much higher release rates can be obtained for higher (and possibly closer to physiological) levels of Ca^{2+} loading of the vesicles (i.e., 10–20 mM $CaCl_2$ instead of 1 mM) and at a temperature of 37 °C. In addition, as shown in Figure 7, the passive permeability of SR vesicles increases in parallel with CuPh-induced cross-linking (as judged from the disappearance of ATPase monomers on polyacrylamide gels) so that one should expect that the maximal rates of Ca^{2+} release might be 1 or 2 orders of magnitude higher than those described above. Such permeability rates (i.e., initial velocities higher than 1 μmol of Ca^{2+} mg^{-1} s^{-1}) are compatible with those suggested to be acting during excitation of skeletal muscle cells (Endo, 1977).

For a correct interpretation of the CuPh-induced Ca^{2+} release, it is important to establish whether the permeability increase is due to mere chemical modification or to intermolecular cross-linking of the ATPase molecules. The simplest interpretation of our observations is that CuPh induces the oxidation of SH groups (with no casual relation to the formation of oligomers), thereby affecting changes in Ca^{2+} permeability. Indeed, this would seem quite reasonable as SH reagents are also known to induce an increase in the Ca^{2+} permeability of SR vesicles (Martonosi & Feretos, 1964; Fairhurst & Hasselbach, 1970; Abramson et al., 1983; Bindoli & Fleischer, 1983). One should note, however, that hydrophilic SH reagents have been reported to be the most effective in inducing Ca^{2+} release. Therefore, it is not excluded that a modification of the hydrophilic portion of a specific membrane component induced by these reagents might stimulate the occurrence of self-association phenomena between the modified proteins.[2]

On the other hand, it is possible that the formation of rapid efflux pathways is associated with the assembly of macromolecular complexes composed by ATPase units which can be observed after cross-linking of the vesicles. Indeed, it was recently shown by Denticke et al. (1983) that oxidative cross-linking of erythrocyte membrane induces the formation of big aqueous pores. In that report, evidence was provided which supported intermolecular cross-linking as the basic event underlying the permeability change. Several observations made during the course of this study also support the idea that the formation of oligomeric structures rather than the modification of the ATPase chains is responsible for inducing rapid Ca^{2+} release from SR vesicles. (i) Strikingly similar permeability changes could be obtained by using either CuPh or DSP as a cross-linking agent. Therefore, it is likely that the appearance of Ca^{2+} efflux pathways is not related to specific chemical modification of amino acid residues by CuPh or DSP, since different reactive groups are usually involved in the two cases (SH and NH_2 groups, respectively). Rather, it is dependent upon the formation of covalently linked oligomeric structures, which are a common product of both treatments. (ii) Amino acid modification due to intrachain cross-linking should occur with equal probability in both heavy and light fractions of the SR, while the results have shown that the heavy subfraction is much more sensitive to CuPh-induced Ca^{2+} release (and, in parallel, is more susceptible to form ATPase oligomers). On the other hand, a different lipid composition and/or ATPase concentration in the two SR regions might affect the probability and the degree of self-association between ATPase molecules. (iii) The increase in passive permeability is nicely correlated to the increase of ATPase oligomeric structures (Figure 7). (iv) The channels formed have the characteristics of large aqueous pores since they allow the efflux of large molecules such as sucrose.

The interpretation based upon the formation of ATPase oligomers is particularly attractive, especially with regard to its possible physiological significance in the mechanism of excitation–contraction coupling in muscle. One should note that the concentration of ATPase in the SR membrane is extremely high (about 50 mM) and that collision between functional units may occur within 1 μs at room temperature (Poo & Cone, 1974). Therefore, in such a particular membrane, it is plausible that a self-associating protein might exist in different, rapidly interconvertible oligomeric states. A rapid shift from one protein conformation to the other could be induced by a sudden change in the concentration or the distribution of particular ligands (i.e., Ca^{2+} or H^+)[3] or in the membrane or surface potential (Dux & Martonosi, 1983b,c). This could represent a major mechanism of physiological effectors to rapidly regulate membrane characteristics like passive permeability to electrolytes. Vanderkooi et al. (1977) have proposed that such a regulatory system might be responsible for the rapid Ca^{2+} release from Ca^{2+}-loaded SR vesicles during excitation–contraction coupling in muscle tissue. Accordingly, ATPase monomers would be responsible for the active Ca^{2+} translocation from the cytosol to the lumen of the SR membranes, whereas the transient oligomerization of ATPase molecules would allow massive Ca^{2+} efflux through newly formed Ca^{2+} channels. This hypothesis does not necessarily exclude the possibility that Ca^{2+}-pumping activity might also require direct interaction and cooperativity between ATPase monomers. Indeed, this latter possibility is supported by several kinds of evidence, such as kinetic considerations (Rassold et al., 1981; Verjowsky-Almeida & Silva, 1981) and inhibition studies (Pick & Racker, 1979). Accordingly, the transporting conformer of the ATPase might be a dimeric structure, in which case a higher degree of oligomerization (aggregation) might be required for the transient formation of releasing channels. In this context, it is interesting to note that ATPase molecules readily form two-dimensional crystalline arrays (whose unit cell dimensions correspond to those of ATPase dimers) if the enzyme is held in a specific conformational state (E_2) by vanadate (Dux & Martonosi, 1983a). This important observation actually demonstrates that the ATPase possesses specific interaction sites and that a dynamic equilibrium between ATPase dimers and ATPase polymers is possible.

Acknowledgments

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Registry No. DSP, 7558-79-4; DCCD, 538-75-0; ATPase, 9000-83-3; CuPh, 15823-71-9; Ca, 7440-70-2; La, 7439-91-0; sucrose, 57-50-1.

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[2] We have noticed that incubation of SR vesicles with the water-soluble SH reagent *N*-ethylmaleimide in the presence of ATP induces the formation of covalently linked ATPase oligomers. Under such experimental conditions, all but a minor fraction of the SH groups of the ATPase were alkylated by the reagent. Apparently, in spite of the small number of SH groups available, the endogenous formation of intermolecular disulfide bridges was drastically increased. Moreover, a subsequent addition of CuPh to *N*-ethylmaleimide-treated vesicles caused an almost immediate oligomerization of all remaining monomeric ATPase chains. These observations show that chemical modification of some SH groups of the ATPase can indeed increase its ability to form aggregates.

[3] Very recently, Goldkorn et al. (1984) have found by radiation inactivation analysis that a proton electrochemical gradient causes an alteration in the subunit interaction of the *lac* carrier in *Escherichia coli*. The target mass of the carrier doubled in the energized membranes.

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Interaction of Estrogen Receptor of Calf Uterus with a Monoclonal Antibody: Probing of Various Molecular Forms†

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ABSTRACT: A monoclonal antibody to estrogen receptor (JS34/32) is able to recognize, in the calf uterine cytosol, a protein (~65 000 daltons) giving a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Two molecules of this antibody are able to simultaneously interact with the native 8S form of the receptor present in the calf uterine cytosol ("twin antibody" assay). This indicates the presence of two antigenic determinants on the "low-salt" 8S form of the receptor. This form of the receptor shows an increase in M_r from 345 000 to 665 000 after interaction with the soluble antibody. Dissociating agents that induce the dissociation of the 8S form to smaller forms also induce the dissociation of the two antigenic determinants. The 4S "high-salt" form of the estrogen receptor has one determinant per molecule, appearing to be the smallest form of the receptor not containing repetitive structures associated with the steroid binding site. The nuclear receptor also shows the presence of more than one antigenic determinant on its molecule.

The estrogen receptor is present in the cytosolic fraction prepared in low ionic strength buffer from unstimulated target tissue as a large macromolecular complex (Toft & Gorski, 1966; Jensen et al., 1969; Stancel et al., 1973). This complex, defined by its sedimentation coefficient (8 S), is able to reversibly dissociate to a "high-salt" form that sediments in the 4–5S region of a sucrose density gradient (Korenmann & Rao, 1968). Furthermore, in a target tissue exposed to the hormone, the estrogen receptor is present mainly in the nuclei as a smaller 4.5–5S form (Puca & Bresciani, 1969; Puca et al., 1970). All the antibodies to the estrogen receptor so far described recognize both the cytoplasmic and the nuclear receptor (Greene et al., 1977, 1979, 1980a,b; Radanyi et al., 1979; Moncharmont et al., 1982); this further supports the postulated "two-step" mechanism of action (Jensen et al., 1972) for the steroid receptor. The physicochemical properties

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