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THE LABELING WITH 8-AZIDO-CYCLIC ADENOSINE MONOPHOSPHATE OF PROTEINS IN VESICLES OF SARCOPLASMIC RETICULUM FROM RABBIT SKELETAL MUSCLE

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Photoinduced labeling with 8-azido-cyclic AMP of proteins in vesicles from sarcoplasmic reticulum rabbit skeletal muscle has been examined. At concentrations of 0.1 μM or less, specific labeling of three or four bands, representing trace components in the SDS gels, was seen. This labeling was prevented by cyclic AMP. Some of these bands correspond approximately in apparent molecular weight to subunits of cyclic AMP-dependent protein kinases reported in other systems. Attempts to use this reagent as a probe for protein position in the membrane have been unsuccessful since 8-azido-cyclic AMP passes into and through the membrane. The reagent also appears to partition with a linear concentration dependence into the vesicular membrane itself, presumably into the lipid portion.

Introduction

Previous experiments have demonstrated the utility of photoactivatable reagents as probes for membrane proteins (see Ref. 1). We felt that such a reagent could be used to explore the asymmetric disposition of the Ca^{2+}-ATPase in sarcoplasmic reticulum (SR) vesicles isolated from rabbit skeletal muscles. Since biological studies had indicated that cyclic AMP is poorly diffusable through cell membranes, we thought that a photoactive analog of this reagent would be suitable for these studies. Additionally, since previous experiments [18] had indicated that cyclic AMP did not alter Ca^{2+} transport in skeletal muscle SR, we expected a cyclic AMP analog would act as a non-specific label for protein surfaces exposed to the aqueous phase. Thus, we chose to label SR vesicles with 8-azido-cyclic [^{32}P]AMP. Instead of vectorial labeling, however, our data show that SR vesicles are permeable to this reagent and suggest that it partitions into the bilayer. In addition, these experiments indicate the presence of specific cyclic AMP binding proteins in SR membranes. Assuming these proteins represent cyclic AMP-dependent protein kinases, this data adds to the accumulating evidence that the regulation of Ca^{2+} transport in skeletal muscle SR may be mediated by protein phosphorylation [2–5].

Experimental procedures

Materials and Methods

Cyclic AMP was purchased from Aldrich Chemicals, ATP from Sigma Biochemicals, and [γ-^{32}P]-ATP and $^{45}CaCl_2$ from New England Nuclear. 8-Azido-cyclic [^{32}P]AMP was purchased from ICN Radioisotopes and diluted with unlabeled 8-N_3-cyclic AMP, kindly prepared and provided by Dr. Ullrich Walter, to a specific activity of 10 μCi/mmol.

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Abbreviations: SDS, sodium dodecyl sulfate; Tes, *N*-tris-[hydroxymethyl]methyl-2-aminoethanesulfonic acid; Tes-S buffer, 10 mM Tes, pH 7.2/10% sucrose; SR, saroplasmic reticulum; STI, soybean trypsin inhibitor.

Sarcoplasmic reticulum vesicles were prepared from rabbit white skeletal muscles (back and leg muscles) essentially as described by McFarland and Inesi [6], substituting 10 mM Tes buffer, pH 7.2, for histidine. SR vesicles which included [^{14}C]inulin or 8-azido-cyclic [^{32}P]AMP were prepared by adding these reagents during the initial homogenization of rabbit muscle. These homogenizations were performed in a small blender container on 5–10 g of rabbit muscle. The final SR suspensions were characterized by chromatography on Sephadex G-75 (1 × 50 cm), eluting with 10 mM Tes, pH 7.2/10% sucrose (Tes-S buffer). Calcium uptake was measured in the presence of oxalate by the filtration method of Martonosi and Feretos [7], measuring the radioactivity remaining on the filter. SR vesicle concentrations were expressed as protein as determined by the method of Lowry et al. [8] modified to include 0.5% SDS in the carbonate reagent. Bovine serum albumin was used as a standard.

8-Azido-cyclic [^{32}P]AMP incorporation

Samples for photoactivated incorporation of 8-azido-cyclic [^{32}P]AMP were prepared by two procedures, both carried out at 0 or 4°C in dim light.

(1) Samples of 50–100 μl were prepared by gently mixing SR vesicles and various concentrations of 8-azido-cyclic [^{32}P]AMP in Tes-S buffer supplemented with 10 mM $MgCl_2$.

(2) Samples of a final volume of 0.2 ml containing Tes-S buffer, 10 mM $MgCl_2$, various amounts of SR vesicles (up to 20 mg/ml) and various concentrations of 8-azido-cyclic [^{32}P]AMP were homogenized in a 2 ml Ten Broeck Pyrex tissue grinder for 1 min at 10 min intervals for a total of 30 min. The homogenate was chromatographed on Sephadex G-50 fine (1 × 25 cm), eluting with Tes-S buffer. Void volume fractions containing the SR vesicles were used for irradiation.

The suspensions were irradiated at 4°C for 30 min with a Hanovia 450 W medium pressure mercury light source; light was filtered through a water-cooled Pyrex glass jacket and a 1.5 mm uranium filter. Aliquots containing 50 μg of SR protein (concentrated by lyophilization if necessary) were then subjected to SDS-polyacrylamide (10%) slab gel electrophoresis as described by Laemmli [9], with some modifications. Samples were mixed with SDS buffer, to final concentrations of 0.05 M Tris-HCl, pH 6.8, 0.005% pyronine Y, 2% SDS, 2% sucrose, 1% β-mercaptoethanol and 0.4 mM EDTA. These solutions were incubated at 37°C for at least 30 min prior to electrophoresis. Directly before applying the samples to the gel, β-mercaptoethanol was added to a final concentration of 50 mM. The running buffer was 0.025 M Tris-HCl/0.19 M glycine, pH 8.6/0.1% SDS/1 mM β-mercaptoethanol. The 13 × 13 cm gels were usually run at 50 V for 14 h and then stained for protein with 0.02% Coomassie blue in 10% ethanol/10% acetic acid. The destained gels were dried and subjected to autoradiography at −80°C using Kodak X-Omat R film and intensifying screens from DuPont.

Endogenous phosphorylation

Endogenous phosphorylation was measured as described by Walter et al. [10]. Reaction mixtures (final volume 0.1 ml) contained buffer, 10 mM $MgCl_2$, 4 μM [γ-^{32}P]ATP (10 Ci/mmol) and up to 400 μg SR protein in the absence or presence of cyclic AMP. The reaction was started by addition of [γ-^{32}P]ATP, incubated at room temperature for 10 min and terminated by the addition of 25 μl of 5-fold-concentrated sample buffer for SDS-gel electrophoresis. The samples were heated at 100°C for 2 min, incubated at 37°C and subjected to electrophoresis and autoradiography as described above.

Two-dimensional SDS-gel electrophoresis

Two-dimensional gels were run as described by Michalak et al. [11]; the first dimension was a 7.5% gel run according to Weber and Osborn [12] and the second dimension was a 7.5% gel run according to Laemmli [9].

Determination of space accessible to azido-cyclic AMP in SR suspensions

Samples were prepared on ice in the dark in 1.5 ml Eppendorf tubes as follows: 1.5 mg of SR vesicles were suspended in 50 μl of Tes-S buffer supplemented with 10 mM $MgCl_2$ and 80 μM cyclic AMP. To four tubes were added 2, 3, 5 and 10 μl of STI (50 mg/ml) in Tes-S in ^3H_2O; to a second series of four tubes were added 2, 3, 5 and 10 μl of 10 μM 8-azido-cyclic [^{32}P]AMP in Tes-S. After 30 min at 4°C, the tubes held in rubber adaptors were centrifuged at 30 000 × *g* for 60 min at 4°C. Duplicate

samples of 5 μl were removed from the supernatants and assayed for ^{3}H or ^{32}P by scintillation counting in Aquasol or for protein by the method of Lowry et al. [8], using STI as a standard. The data were normalized to a 50 μl volume for each sample, and were plotted as the quantity added vs. concentration as the measured quantity/ml.

Results

Photoincorporation of 8-azido-cyclic [^{32}P]AMP into SR proteins was studied by two procedures: (1) label was added to vesicle preparations and photolyzed; (2) label was added to vesicle preparations, followed by homogenization and chromatography on Sephadex G-50; fractions from the void volume were photolyzed. The first procedure was intended to label proteins on the external surface of the vesicle while the second was intended to label proteins on the internal surface of the vesicles. Control experiments indicate that these expectations were incorrect.

Fig. 1 shows the autoradiogram obtained when SR is labeled by procedure 1 as a function of concentration of labeling reagent. Bands 3, 4 and 5 are clearly labeled at low concentrations. In contrast, when the concentration of 8-azido-cyclic [^{32}P]AMP is 1 μM (lane 6) bands 1, 2, 6, 7 and 8 are heavily labeled. In lane 7 one can see that the addition of cold cyclic AMP results in the loss of bands 3 and 4 and apparently the top portion of band 5. If one assumes that band 5 is actually two unresolved bands, as seen in the Coomassie blue stain pattern (Fig. 1), then one can concluded that bands 3, 4 and the top of 5 are proteins whose labeling with 8-azido-cyclic [^{32}P]AMP is specifically displaced by cyclic AMP. The different intensities of these three bands as a function of label concentration may reflect either the relative concentrations of these proteins or their relative affinities for 8-azido-cyclic AMP. The labeling of other bands is apparently not related to the structural similarity of 8-azido-cyclic AMP to cyclic AMP. In lane 8 one can see that the addition of ATP does not prevent labeling of any band, but may reduce labeling somewhat in bands 2 and 5. Thus, 8-azido-cyclic AMP is not simply labeling ATP binding sites by acting as an ATP analog. If one compares lanes 7 and 8 it is clear that the effects of cyclic AMP are different from those of ATP. This indicates that at least some of the observed labeling is specific for the cyclic nucleotide.

In the autoradiogram obtained when SR vesicles are labeled by procedure 2 (Fig. 2) only four bands are labeled after photolysis; these correspond to the bands labeled 3, 4, 5 and 6 in Fig. 1. As seen in lane 7, the addition of cyclic AMP virtually eliminates labeling of all these bands. These results indicate that all the labeling of samples prepared by procedure 2 is dependent on the structural similarity of azido-cyclic AMP to cyclic AMP. This is consistent with the hypothesis that 8-azido-cyclic AMP binds at specific sites in the SR membrane. Then after chromatography on Sephadex G-50, most of the label found in the vesicle preparation will be associated with these sites which are then covalently labeled by photolysis. When the samples are prepared by procedure 1 without Sephadex G-50 chromatography, then the labeling is not only to these specific sites but also to sites available to random collision with the photoreactive reagent.

Two additional observations can be made from Fig. 2. First, band 6 is more heavily labeled at lower concentrations when compared with Fig. 1. Secondly, in lane 8, the addition of ATP appears to: (1) reduce label in bands 3, 4, and 5; (2) increase label in band 6; and (3) result in the appearance of a new band labeled 6a.

The hypothesis that 8-azido-cyclic AMP binds to the SR vesicles is also evident when the elution profiles of the Sephadex G-50 columns are quantitated. Fig. 3 presents data from several experiments at a constant concentration of SR vesicles showing the percent of radioactivity which is found in the void volume fractions. If the only factor of importance here were the volume of solution entrapped within the vesicle, then this percent should not vary with either the total concentration or the specific activity of label. As seen in Fig. 3, this is not the case, indicating that at least some of the label found in the void volume is bound to the membrane of the vesicles. In this data the percent of label bound is greater at lower label concentrations. This is consistent with the curve expected for a constant number of saturatable binding sites when the concentration range tested includes the region of significant saturation.

This conclusion is further supported by the data from the Sephadex G-50 column profiles observed in the presence of the competitor cyclic AMP. At a

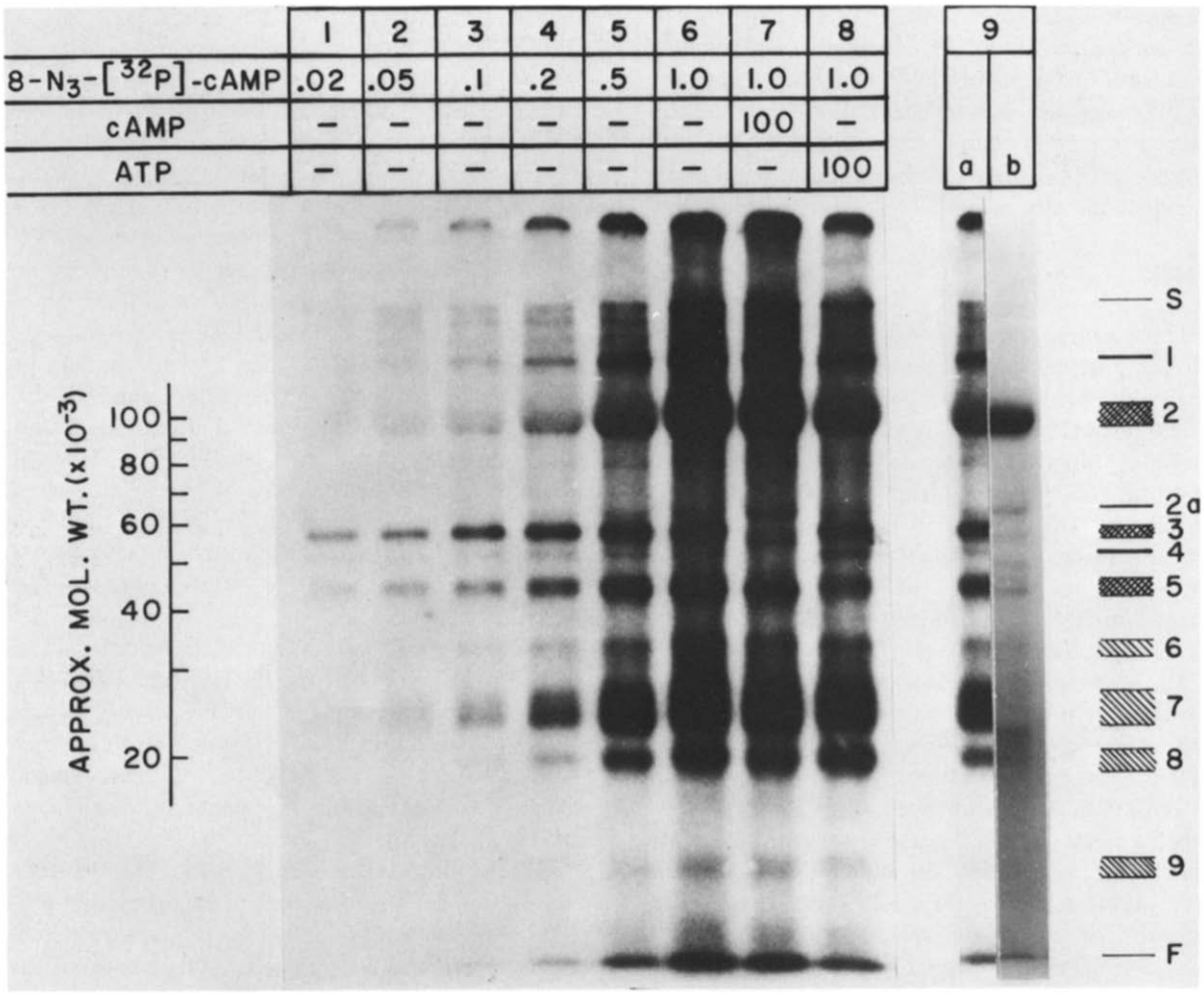

Fig. 1. SR vesicles labeled by procedure 1. Samples containing Tes-S buffer, 10 mM $MgCl_2$, and 1.3 mg/ml SR vesicles were gently mixed with various concentrations of 8-azido-cyclic [^{32}P]AMP. When cyclic AMP or ATP was present, it was added directly before the photolabel. After 30 min on ice in the dark, the samples were photolyzed and subjected to SDS-polyacrylamide (10%) electrophoresis [9]. Each lane contained 50 μg of protein. Lanes 1–8 and 9a are the autoradiograms obtained from these samples while lane 9b is the Coomassie blue staining pattern. All reagent concentrations are μM. The symbol S indicates the division between the stacking and running gels, and F the buffer front. cAMP, cyclic AMP; 8-N_3-[^{32}P]-cAMP, 8-azido-cyclic [^{32}P]AMP.

concentration of 8-azido-cyclic AMP of 0.05 μM with no added cyclic AMP, 10.5% of the counts are found in the void volume. When 5 μM cyclic AMP is added to this sample before homogenization, only 2.6% of the counts are found in the void volume. Similar results are seen at other concentrations of label and cyclic AMP. Thus, the addition of cyclic AMP leads to a significant reduction in the amount of 8-azido-cyclic AMP found in the Sephadex G-50 void volume fractions as would be expected if there are specific sites on the SR vesicles.

Since it is clear from Fig. 3 that at least some of the ^{32}P found in the void volume is bound to the vesicles, it was necessary to demonstrate that homogenization of SR vesicles in the presence of 8-azido-cyclic AMP does actually entrap the label within the interior space in the vesicles. Comparison of samples prepared with and without homogenization shows no

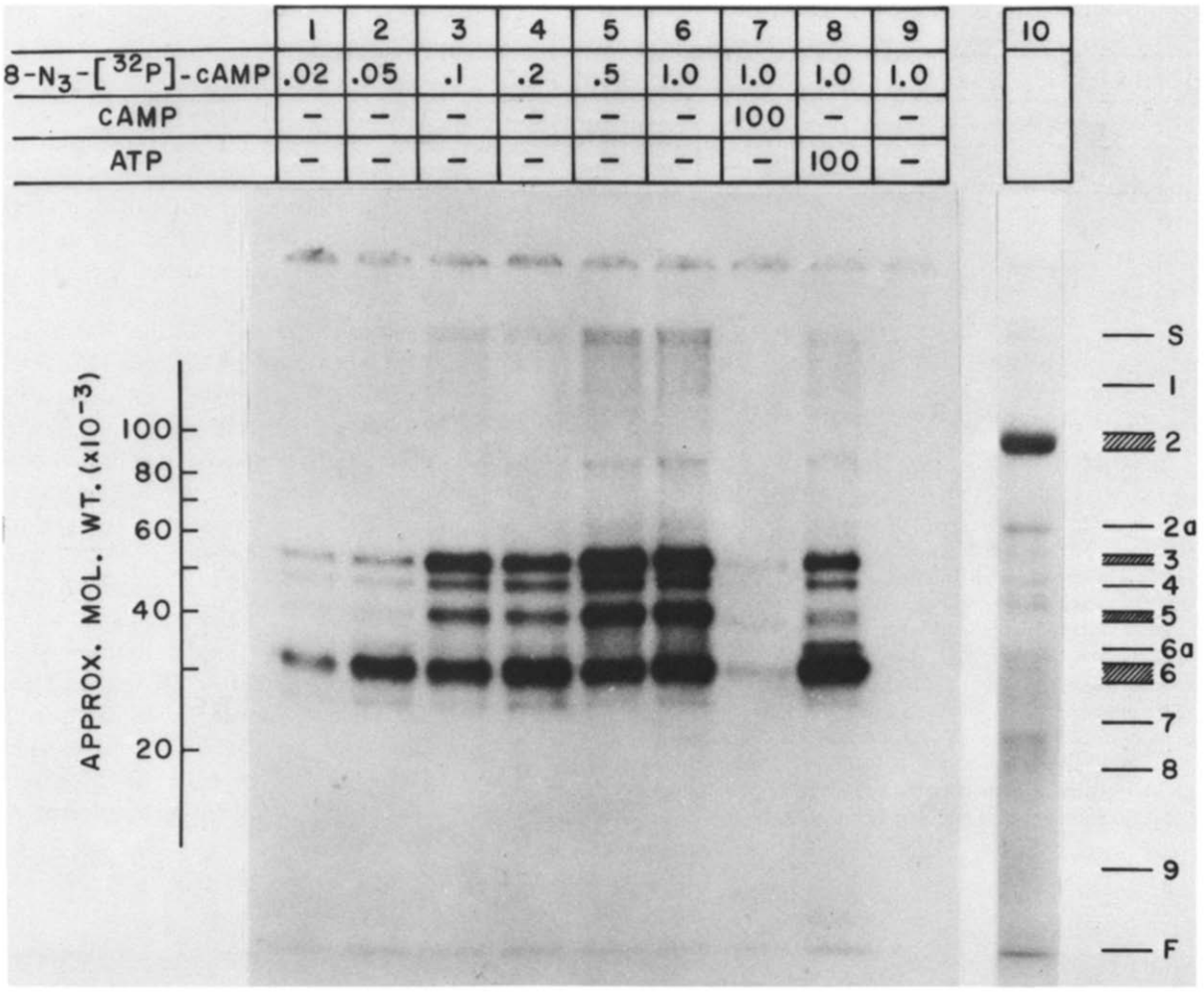

Fig. 2. SR vesicles labeled by procedure 2. Samples, final volume 0.2 ml, in Tes-S buffer, 10 mM $MgCl_2$, 5.7 mg/ml SR vesicles and various concentrations of 8-azido-cyclic [^{32}P]AMP were homogenized in a 2 ml Ten Broeck Pyrex tissue grinder for 1 min at 10 min intervals for a total of 30 min. This homogenate was chromatographed over Sephadex G-50 fine, eluting with Tes-S buffer. Void volume fractions containing the SR vesicles were photolyzed and subjected to SDS-polyacrylamide electrophoresis as in Fig. 1. Lanes 1–9 represent the autoradiogram obtained from these samples. Lanes 1–8 are as in Fig. 1 while lane 9 is a sample which was not photolyzed and lane 10 is the Coomassie blue staining pattern.

change in the labeling pattern. After chromatography on G-50, as reported in Table I, the void volume fractions of both samples (cf. 1 and 2, also 4 and 5) contain a significant number of counts. Additionally, the percent recovery varies with label concentration in the same manner as seen in Fig. 3. This result implies that label is tightly bound to the vesicle and has a slow off-rate. This conclusion receives further support when the void volume fractions are run over Sephadex G-50 a second time. The percent of the counts which were retained in the void volume lies between 65 and 80%, irrespective of the method of initial sample preparation, Table I. Thus, the binding of label to vesicles forms a stable complex, with or without homogenization. The labeling patterns for various preparative routes are shown in Fig. 4. Homogenization did not affect the pattern (lanes 1 vs. 3 or 4 vs. 6; compare also 2 and 5 with Fig. 2).

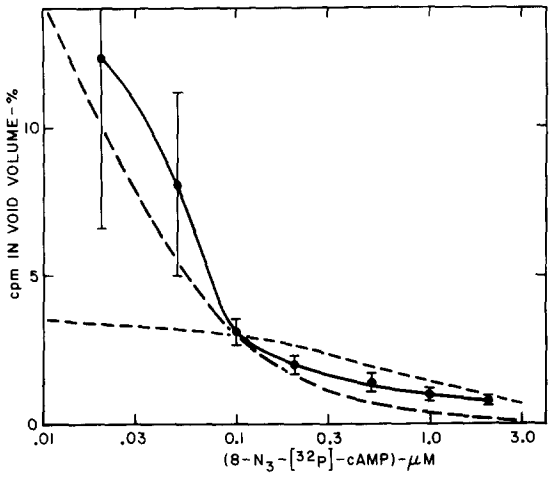

Fig. 3. Association of 8-azido-cyclic [^{32}P]AMP with SR vesicles after Sephadex G-50 chromatography (•). SR samples were prepared and chromatographed as described for Fig. 2. The cpm of each fraction was determined by Cherenkov counting. The % cpm in the void volume is the total number of cpm in the void volume fractions divided by the total counts eluted from the column × 100. The total number of counts eluted from the column varied from 85 to 95% of those applied. The bars represent the standard deviations estimated from counting statistics. The dashed lines are calculated curves for a single class of non-interacting binding sites with two different sets of binding constants and binding site densities, chosen to intersect at 0.1 μM.

At least two explanations could account for these results: (1) homogenization does not disturb the SR vesicle membrane so that label is not internalized or (2) label readily diffuses through the membrane so that only bound label remains associated with the vesicles after chromatography. Experiments were therefore designed to insure the incorporation of the label within the internal space of the vesicles. In these experiments label was added to the buffer during the initial blender homogenization of the rabbit muscle. Since, presumably, it is at this step that the vesicles are formed, then the label added here should be internalized. As a control for these experiments the muscles were also homogenized in the presence of [^{14}C]inulin. The [^{14}C]inulin served as a marker both to follow incorporation and to assess the amount of internalized material which is retained through the SR preparation steps. The final SR suspensions were characterized by Sephadex G-75 chromatography as shown in Fig. 5. These data indicate that greater than 90% of the [^{14}C]inulin internalized during homogenization remains associated with the SR vesicles. When the vesicles are similarly prepared with azido-cyclic AMP (and a 500-fold excess of cyclic AMP to minimize specific binding), the Sephadex G-75 chromatography profile of the SR suspension is different. As

TABLE I

RADIOACTIVITY FOUND IN VOID VOLUME FRACTIONS AFTER SEPHADEX G-50 CHROMATOGRAPHY

Samples for chromatography on Sephadex G-50 were prepared as described for Fig. 1 (samples 2 and 5) or Fig. 2 (samples 1, 3, 4 and 6).

No.	SR (mg/ml)	8-Azido-cyclic [^{32}P]AMP (μM)	Homogenized	% cpm in void volume	
				1st run [a]	Rerun of peak from 1st run [b]
1	4.5	0.1	yes	3.2	n.d.
2	4.5	0.1	no	2.7	79
3	2.3	1.0	yes	0.6	71
4	4.5	1.0	yes	1.0	71
5	4.5	1.0	no	1.3	67
6	6.8	1.0	yes	1.4	68

[a] Number of counts per minute found in the void volume fractions divided by the total counts eluted from the column × 100 (see legend to Fig. 4). The total counts eluted from the column varied from 85 to 95% of the counts applied.

[b] Aliquots from the void volumes of the first Sephadex G-50 run of samples 2–6 were rechromatographed and the percent counts found in the second void volume were determined as for the first.

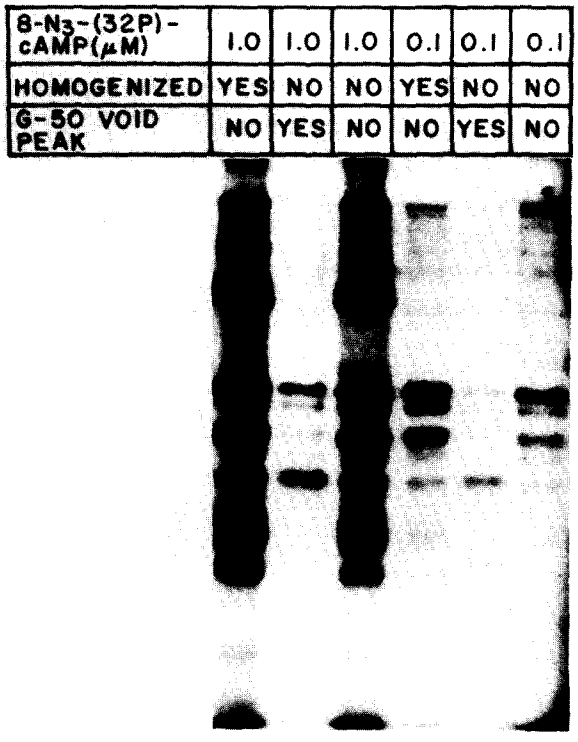

Fig. 4. Comparison of SDS-gel labeling patterns of SR proteins found after various preparative procedures were used prior to photolysis. Samples for lanes 1 and 4 were homogenized as described for Fig. 2 in the presence of 1 μM (lane 1) or 0.1 μM (lane 4) 8-azido-cyclic [^{32}P]AMP. Samples for lanes 2, 3, 5 and 6 were prepared as described for Fig. 1 in the presence of 1 μM (lanes 2 and 3) or 0.1 μM (lanes 5 and 6) 8-azido-cyclic [^{32}P]AMP. For lanes 2 and 5 the samples consisted of the void volume fractions from chromatography on Sephadex G-50 as described in Fig. 2. Each of these six wells contained 50 μg of SR vesicles.

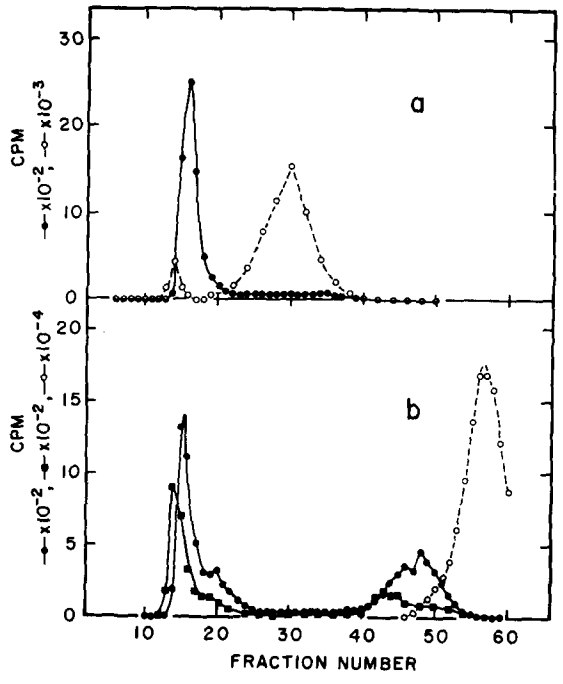

Fig. 5. G-75 Sephadex chromatography profiles. (a) A sample of SR vesicles prepared from rabbit muscle in the presence of [^{14}C]inulin (•) and free [^{14}C]inulin (○) were chromatographed on Sephadex G-75 eluting with Tes-S buffer. (b) Samples of SR vesicles prepared from rabbit skeletal muscle in the presence of 8-azido-cyclic [^{32}P]AMP before photolysis (•), after photolysis (▪), and 8-azido-cyclic [^{32}P]AMP alone (○) were chromatographed as in 5a, except that these were run in dim light and the column covered in foil.

shown in Fig. 5, only 60–70% of the radioactivity is associated with the SR vesicles. These data support the conclusion that the SR membrane is permeable to azido-cyclic AMP.

A fraction of the SR vesicles prepared in this manner was photolyzed before Sephadex G-75 chromatography. The G-75 profile of this sample was the same as that seen prior to photolysis, indicating that photolysis itself does not alter the permeability of the SR membranes significantly. When this sample is run on a 10% SDS-gel, the autoradiogram pattern is essentially the same as that seen in lane 4 of Fig. 4, except that band 6 is relatively less intense.

To demonstrate further the possibility that azido-cyclic AMP can diffuse through the SR membranes, experiments were run comparing the volume accessible to ^3H_2O, 8-azido-cyclic [^{32}P]AMP and STI when they are added to a suspension of SR vesicles. It is assumed that ^3H_2O is fully permeable measuring the total aqueous phase and that STI is fully excluded measuring only the external aqueous phase.

Let: V_0 = volume of liquid outside of vesicles (μl); V_i = volume of liquid inside vesicles (μl); V_m = volume of vesicle membrane phase (μl); A = total amount of solute added (quantity as mass (mg) or mass equivalents (cpm)); c_0, c_i, c_m = concentration of solute in V_0, V_i, V_m, respectively (quantity/μl); f_i = c_i/c_0 and $f_m = c_m/c_0$, partition coefficients.

Then: $A = c_0V_0 + c_iV_i + c_mV_m$
$= c_0V_0 + c_0f_iV_i + c_0f_mV_m$
$= c_0(V_0 + f_iV_i + f_mV_m)$ (1)

For ^3H_2O: Assume $f_m = 0$ and $f_i = 1$

$$A/c_0 = V_0 + V_i \quad (2)$$

For STI: Assume $f_m = 0$ and $f_i = 0$

$$A/c_0 = V_0 \quad (3)$$

For 8-azido-cyclic AMP: Assume $f_i = 1$

$$A/c_0 = (V_0 + V_i) + f_m V_m \quad (4)$$

The results, presented in Fig. 6, indicate that azido-cyclic AMP resembles ^3H_2O more than STI when comparing accessible volume. The observation that azido-cyclic AMP appears to be accessible to a volume greater than that of ^3H_2O implies that azido-cyclic AMP may partition into the lipid phase of the vesicle suspension. The amount of material bound to specific receptors could not account for the magnitude of the

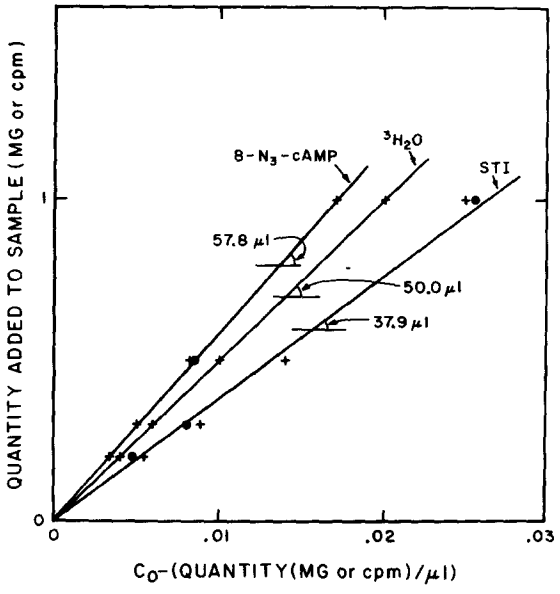

Fig. 6. Accessible volumes in an SR suspension. Samples (50 µl) contained 1.5 mg SR vesicles, Tes-S buffer, 10 mM $MgCl_2$ and 80 mM cyclic AMP. After 5 min on ice, aliquots of either STI (50 mg/ml) in ^3H_2O or 8-azido-cyclic [^{32}P]-AMP (10 µM) in Tes-S were added. After incubating at 4°C for 30 min, the samples were centrifuged for 60 min in an SS-34 rotor with adaptors for Eppendorf tubes at 19 500 rev./min. Aliquots of the clear supernatants were assayed for protein, ^3H or ^{32}P. The quantities added and the measured concentrations were normalized for presentation on the same graph, and direct estimates of accessible volumes were made from the observed slopes. The crosses and dots refer to duplicate experiments.

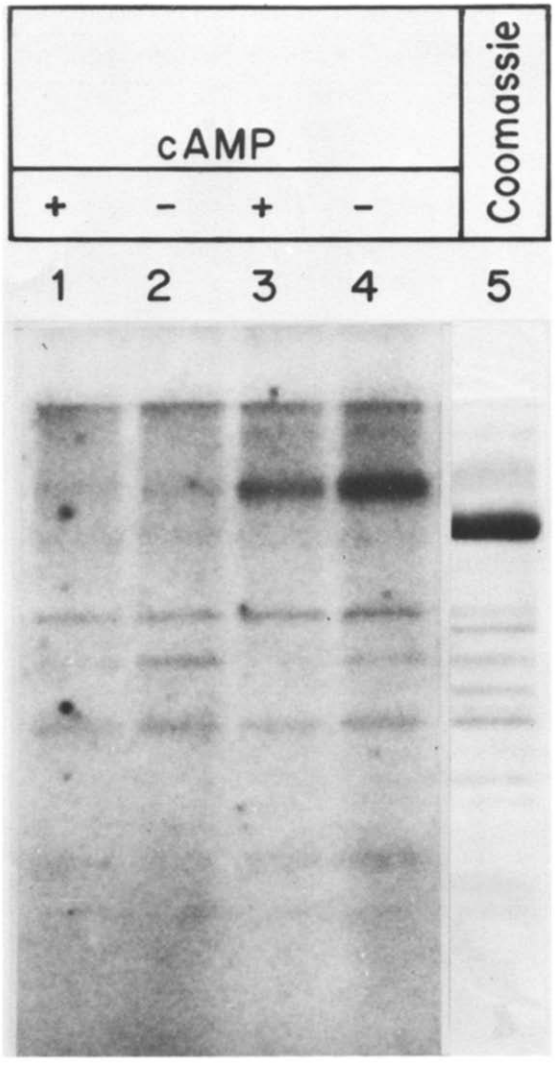

Fig. 7. Endogenous phosphorylation with [γ-^{32}P]ATP. The reaction mixtures (0.1 ml) contained either 50 mM Tris-HCl, pH 6.5, 10 mM $MgCl_2$, 100 mM KCl, 4 µM [γ-^{32}P]-ATP (10 Ci/mmol) and 0.5 mg/ml SR vesicles in the presence (lane 1) or absence (lane 2) of 10 µM cyclic AMP (cAMP); or 10 mM Tes, pH 7.2, 5 mM $MgCl_2$, 120 mM KCl, 0.025 mM $CaCl_2$, 4 µM [γ-^{32}P]ATP (10 Ci/mmol) and 1 mg/ml SR vesicles in the presence (lane 3) or absence (lane 4) of 10 µM cyclic AMP. The reaction was started by the addition of ATP, incubated at ambient temperature for 10 min and terminated by the addition of 25 µl of 5-fold-concentrated sample buffer for SDS electrophoresis. The samples were electrophoresed as described in Fig. 1. Lane 5 is the Coomassie blue staining pattern.

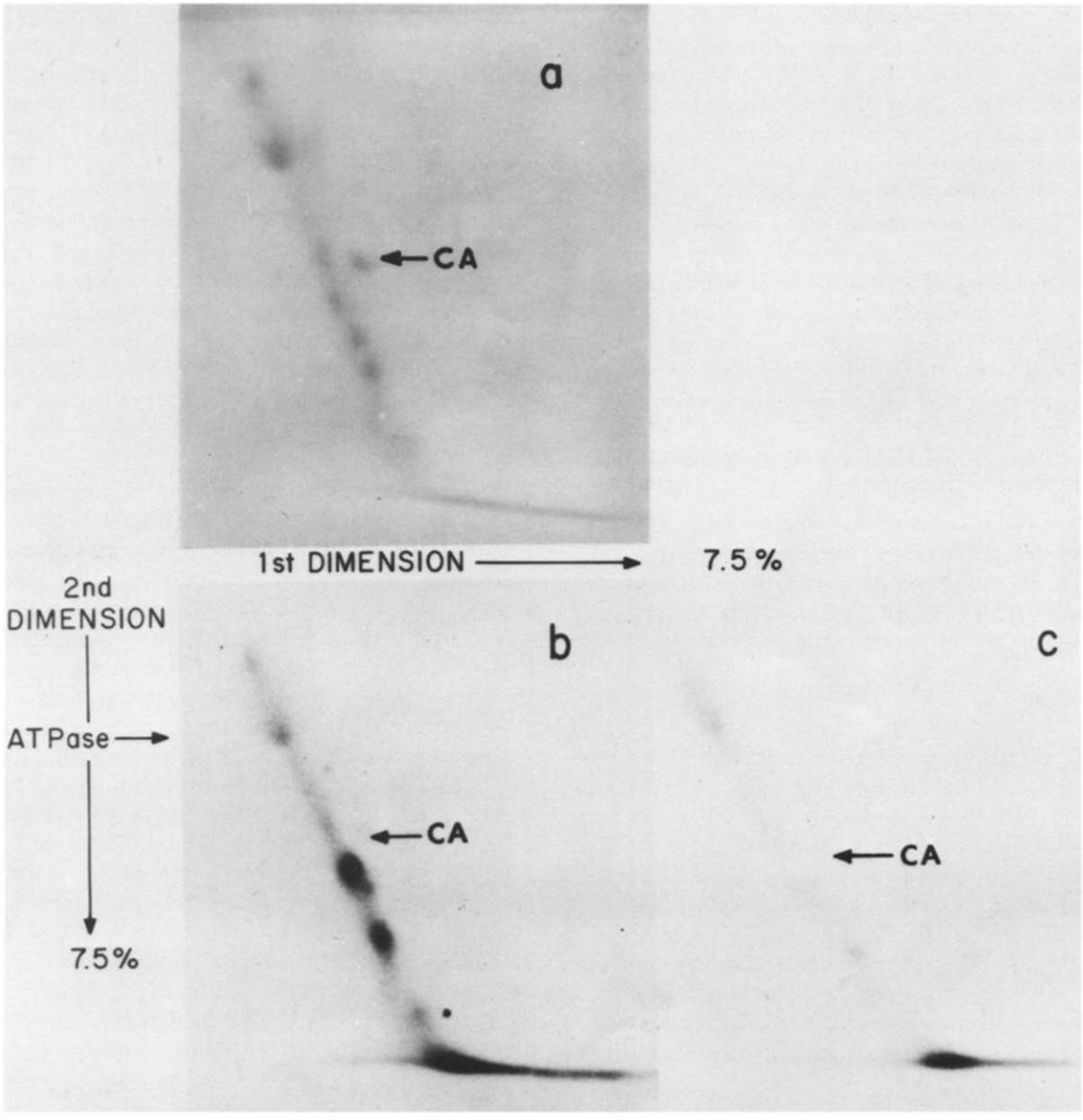

Fig. 8. Identification of labeled bands by two-dimensional SDS-gel electrophoresis. Two-dimensional gels were run as described by Michalak et al. [11]; the first dimension was a 7.5% gel run according to Weber and Osborn [12] while the second dimension was a 7.5% gel run according to Laemmli [9]. Picture a shows the Coomassie blue staining pattern, pictures b and c are the autoradiograms from similar two-dimensional gels where the SR samples were prepared as described in Fig. 1 with 1 μM 8-azido-cyclic [^{32}P]AMP in the absence (b) or presence (c) of 100 μM cyclic AMP. In this system calsequestrin (CA) runs as a cleanly separated spot and is shown not to be specifically labeled by 8-azido-cyclic [^{32}P]AMP.

difference seen. The value of $f_m V_m$ may be derived from the data in Fig. 6 and Eqns. 1–4, and is equal to 7.8 μl. The value of V_m may be considered to represent the lipid phase of the vesicles and would be about 1.5 μl in these experiments. The partition coefficient, f_m, for 8-azido-cyclic AMP between the lipid

and aqueous phases would thus be of the order of 5.

In other studies following photoincorporation of 8-azido-cyclic AMP [10] it has been observed that some proteins which photoincorporate this label are also labeled by [γ-^{32}P]ATP in a manner which may be influenced by the presence of cyclic AMP. As seen in Fig. 7, several SR bands are labeled by [γ-^{32}P]-ATP. Three of these correspond to bands (labeled 3, 5 and 7 in Fig. 1) labeled with 8-azido-cyclic AMP. The presence of cyclic AMP during endogenous phosphorylation gives inconsistent patterns; the addition of cyclic AMP sometimes reduces labeling of band 3, as shown in Fig. 7, but this effect was not always observed. The source of this variation was not determined.

Since it has been reported that calsequestrin has protein kinase activity [4], we ran experiments to see if this is one of the proteins being labeled by 8-azido-cyclic AMP. Samples were prepared as for Fig. 1 and run on the two-dimensional gel system described by Michalak et al. [11]. The results, presented in Fig. 8, clearly indicate that calsequestrin is not labeled.

Discussion

These experiments began as an attempt to demonstrate that the Ca^{2+}-ATPase in SR presents a part of its structure to the internal milieu of the vesicle. However, since it was found that the reagent chosen to label this structure diffuses through the SR membrane, no conclusions can be drawn regarding the localization of the labeled proteins. This reagent did demonstrate the presence of several minor protein bands as well as the major proteins. Several of these bands were apparently specifically labeled since their labeling intensities were significantly reduced by the addition of cyclic AMP.

A possible identification of (at least) two of the specifically labeled bands as being the regulatory subunits of cyclic AMP-dependent protein kinases can be based on several characteristics. The apparent molecular weight of bands 5 and 3, respectively, are approximately those reported in the literature for R_I (the regulatory subunit of type I cyclic AMP-dependent protein kinase) = 47 000 [13] and R_{II} (the regulatory subunit of type II cyclic AMP-dependent protein kinase) = 52 000–58 000 [13,14]. The labeling patterns for these two bands as a function of label concentration indicate K_d values of less than 0.5 μM, consistent with the reported affinities for these kinases [13]. Additionally, the apparent cyclic AMP-dependent autophosphorylation of band 3 is consistent with its identity as R_{II} [10]. The more recent data [14] indicates the presence of two R_{II} type subunits whose 8-azido-cyclic [^{32}P]AMP labeling patterns can resemble those of bands 3 and 4. It is also concluded in that report that membrane bound type II and type I cyclic AMP-dependent protein kinase regulatory subunits are very similar to the corresponding regulatory subunits of soluble protein kinases. (The identity of band 6 in Fig. 2 does not follow from this discussion. Perhaps this band arises from proteolysis of the higher molecular weight species.)

The experiments indicating that SR membranes are permeable to azido-cyclic AMP (and therefore presumably to cyclic AMP) are consistent with the observations of Duggan and Martonosi [15] who showed that these vesicles are permeable to several other small organic anions. The data presented in Fig. 6 indicate not only that SR vesicles are permeable to azido-cyclic AMP but also that azido-cyclic AMP partitions favorably into the SR membrane. The concentrations used are far above those rquired to saturate the specific binding proteins. Thus, it may be assumed that the linear concentration dependence reflects solubility in the lipid phase. The profile presented in Fig. 5 is consistent with this hypothesis since that sample contained a 500-fold excess of cyclic AMP over azido-cyclic AMP so that specific binding of the label to receptor proteins should also be greatly reduced. This partitioning is consistent with data reported by Gerlt et al. [16,17] who concluded that solvation effects appear to be important in the observed thermodynamic instability of cyclic AMP in water. Thus, this molecule may show a preference for a hydrophobic environment, especially one of the amphipathic nature of a membrane.

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