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A SODIUM AND POTASSIUM-STIMULATED ADENOSINE TRIPHOSPHATASE FROM CARDIAC TISSUES—III.

THE PROPERTIES OF AN ENDOGENOUS INHIBITORY FACTOR

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Abstract—An endogenous factor has been solubilized from cardiac muscle preparations, which exerts a preferential inhibitory action on the Mg^{2+}-dependent site of a heart microsomal ATPase system. Similar factors have been isolated from brain and parotid gland. The effects of this material resemble those induced by certain nitrogenous compounds and those observed after 'aging' of the enzyme system. Possible relationships to basic polypeptides are discussed.

SKOU[1] described in 1957, the properties of an ATPase* enzyme system, isolated from crab nerve membranes, which he believed to be involved in active cation transport. He subsequently developed procedures for isolating similar enzymes with high activities from brain and kidney.[2] These studies were extended to many other tissues by other investigators[3–9] and today represent an important area of research in the field of active transport mechanisms.

As indicated by Skou,[10] the separation of this membrane ATPase from heart muscle is difficult because an active basic Mg^{2+}-dependent ATPase appears to obscure the $Na^+ + K^+$ stimulation. The procedure for the preparation of the muscle enzyme system, which involves aging, was developed by one of us (A.S.)[7] and appears to be adequate for use in studying various properties. Skou[10] has reported that small amounts of the supernate derived from heart muscle suspensions apparently preferentially inhibits the Mg^{2+} site of the cardiac ATPase system, thus revealing the stimulation induced by the addition of $Na^+ + K^+$ in the presence of Mg^{2+}. This effect is quite similar to the inhibitory actions of sodium azide and azide-like compounds on the heart muscle membrane ATPase which we have recently reported.[11–13] In addition, the aging phenomenon[7] cited above is also apparently analagous to the 'supernatant effect'.

It appears possible, therefore, that a common mechanism exists for selective inhibition of one site of the transport enzyme system from heart muscle, which might include the effects of azide compounds with similar configuration, aging and, more recently, what appears to be an endogenous inhibitory factor.

In view of the interest in the relationship between the sarcoplasmic reticulum (and the ATPase associated with it) and the relaxing system of muscle,[14, 15] it seemed worthwhile to investigate the properties and characteristics of this inhibitory substance or substances located in the supernate of heart muscle suspensions.

* Abbreviations: ATPase, adenosine triphosphatase; TSF, treated solubilized factor.

METHODS

Preparation of the solubilized factor

An aliquot of the supernate derived from the last centrifugation of heart muscle suspensions (prepared as previously described[7, 13] in the presence of 0·2% sodium deoxycholate (150,929 *g*, average, for 30 min)) is heated in a water bath at 85° for 15 min and then centrifuged at maximum speed (2,500 rev/min) for 15 min in a Clay-Adams table-top centrifuge. In some experiments the supernate was heated for 2 hr or longer at 85° to 95° before centrifugation, and the results were the same as those obtained with the 15-min heating method. The longer period is more suitable since more of the extraneous 'soluble' protein is eliminated. The supernate may be used without heat denaturation and the 'factor' may still be observed.

Particle-free solubilized factor

In a number of experiments, all sedimentable material was removed from the freshly prepared untreated supernate derived from the last centrifugation (150,929 *g* for 30 min). An aliquot was retained and stored at 5°; the remainder was then centrifuged at 150,929 *g* for 2 to 3 hr. The translucent, pale-yellowish pellet was washed by swirling and decantation with suspending medium ('SM')—0·25 M sucrose, 30 mM histidine, 1 mM EDTA, and 30 mM AMPD (2-amino-2-methyl-1,3-propanediol), pH 7·0—and then lightly suspended in the same volume of 'SM' from which the pellet was derived. This postmicrosomal material was labeled 'PMP'; and the clear particle-free supernate was labeled 'PMS'; aliquots of both PMP and PMS were heated as described for the supernatant factor (TSF).

The solubilized factor derived from other tissues (brain, kidney, or parotid glands) was prepared in the same manner.

All the 'factors' were stored frozen, usually at −15° but in some experiments they were stored at various other temperatures.

Dialysis of the 'factors'

Aliquots (usually 1 ml) of the supernates, either before or after heating, were placed in cellophane dialysis bags (Visking tubing) and dialyzed against distilled water for varying periods of time either in a rocking dialyzer or in a simple 1-l. beaker arrangement with magnetic stirring device. Usually three changes of water were made. The dialysates were lyophilized in a VirTis freeze-dry apparatus, and the dried material was suspended in a volume of distilled water equivalent to the original aliquot of supernatant material. The dialyzed fluid was retained and stored at −5° to −15°.

Preparation of the adenosine triphosphatase

The procedure of homogenization, centrifugation, and storage of the ATPase from heart muscle, brain, or parotid gland has been previously described in detail.[6, 7, 9, 13]

The cardiac and cerebral tissues were derived from guinea pigs which were stunned by a blow on the neck and exsanguinated before removal of the organs. The preparative manipulations were carried out at, or nearly at, 0°. The parotid gland preparations were obtained from dogs or rats; this procedure has been described in detail.[9]

Of importance is the fact that, unless specified otherwise, all enzyme preparations were derived from homogenates to which 0·2% sodium deoxycholate was added. The homogenizing medium also included 0·25 M sucrose, 5 mM EDTA, 30 mM histidine, and 50 mM AMPD, at pH 6·8.

Storage. The enzyme-containing fractions were kept in suspending medium, usually at −5°.

Assays

1. *ATPase activity.* The methods previously described were employed.[6, 7] Briefly, they consisted of the measurement of inorganic phosphate released after a 30-min incubation period in the presence of enzyme, ATP, various ions, and chemical additions.

2. *Sulfhydryl group content (–SH).* Three different procedures were utilized for the measurement of –SH content of the 'supernatant factor': (a) an amperometric titration procedure[16] using a Sargent polarographic recording device with a rotating platinum cathode and a $Hg\text{-}Hg_2Cl_2$ anode fitted with an agar-saturated KCl bridge; (b) a N-ethylmaleimide procedure;[17] and (c) a colorimetric method involving DPPH[18] (2,2-diphenyl-1-picrylhydrazyl).

All three methods were employed because of the variability in sulfhydryl group measurements.[19]

3. *Deoxycholic acid assay.* The colorimetric method of Szalkowski and Mader[20] was employed. Sodium desoxycholate (Fisher) was used as standard.

4. *Protein.* The methods used in previous communications were employed.[6, 7]

Reagents

Tris–adenosine triphosphate (ATP) was purchased from Sigma Chemical Co. and routinely tested for Na^+ and K^+, both of which were generally less than 0·1 mM. In some experiments, Na-free ATP was prepared as described previously.[6] All chemicals were of reagent grade.

RESULTS

Inhibitory action of a heart muscle factor in the supernate

When the treated supernate derived from heart muscle suspensions was added to an active microsomal ATPase preparation obtained from the same tissue, a marked inhibition of the Mg^{2+} component was observed, which was accompanied by a much less marked depression of the $Mg^{2+} + Na^+ + K^+$ site (Fig. 1). The proposed two sites on the enzyme—namely, Mg^{2+} — and $Mg^{2+} + Na^+ + K^+$ — dependent respectively, are based upon the previous studies of the active transport enzyme system.[7, 13] If enough supernate is added, the Mg^{2+} site can be completely inhibited or eliminated (Fig. 1). The preferential inhibition of the Mg^{2+} site in the presence of TSF was maintained for most of the period of incubation (Fig. 2), resulting in about a 52% depression of Mg^{2+}–ATPase activity and a 15% depression of the $Mg^{2+} + Na^+ + K^+$–ATPase activity at the 30-min period, which is the usual incubation interval for the measurement of inorganic phosphate release by the enzyme system.

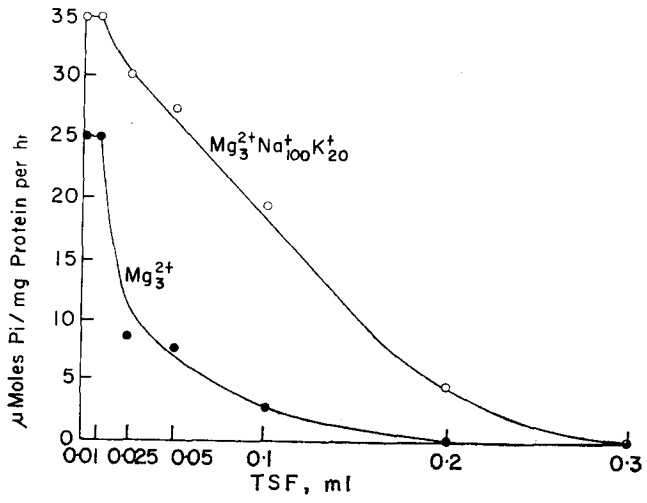

FIG. 1. The effect of TSF on a cardiac microsomal ATPase from guinea pig. TSF was prepared as described in the text and 0·1 ml was added to the incubation mixture (total volume, 1·0 ml) prior to the addition of the enzyme. The concentrations of ions used are indicated as subscripts. The conditions of assay are described in the text. The values are the mean of at least five separate experiments. These conditions prevail for the subsequent figures unless otherwise indicated. The ATPase varied in age from 1 to 3 days.

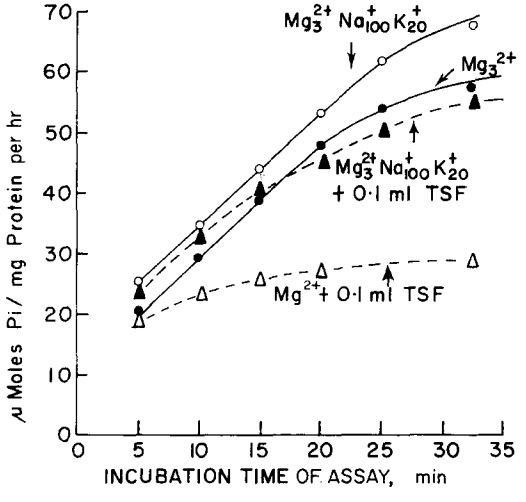

FIG. 2. The relationship between incubation time and TSF activity. Aliquots were withdrawn from the incubating mixture at various time periods.

The inhibitory factor was found in heart muscle obtained from a number of different species in numerous experiments (Table 1). The preferential depression of the Mg^{2+}–ATPase activities as compared to control values seemed to be quite similar among the different species. There appeared to be a tendency toward a reduction of inhibition (expressed as per cent of control) as the enzyme preparation increased in age. Thus in the case of the guinea pig, e.g., the fresh enzyme preparation showed a 69% reduction

TABLE 1. THE EFFECTS OF SOLUBILIZED FACTOR FROM HEART ON CARDIAC MICROSOMAL ATPASE ACTIVITY*

Species	Age of preparation	After addition of TSF Depression of control ATPase activity Mg (%)	Mg + Na + K (%)
Guinea pig	Fresh	69 ± 4·1 (6)	33 ± 3·7 (6)
	1 Week	71 ± 5·9 (7)	36 ± 5·3 (7)
	1 Month	47 ± 4·4 (6)	9 ± 2·3 (6)
	2 Months	76 ± 3·2 (6)	26 ± 4·0 (6)
	3 Months	36 ± 5·5 (11)	12 ± 1·7 (11)
Rabbit	1 Week	69 (2)	43 (2)
	1 Month	73 ± 2·2 (7)	47 ± 1·5 (7)
Mouse	1 Week	41 ± 10 (7)	18 ± 7·5 (7)
Rat	Fresh	60 ± 8 (4)	27 ± 5 (4)
	1 Week	31 ± 2 (5)	11 ± 1 (5)
	1 Month	30 ± 4·7 (8)	10 ± 3·5 (8)

* The ATPase preparations were derived from microsomal fractions of heart muscle from the various species as indicated, and stored at −5° for periods cited in the table. Conditions of assay, preparation, and incubation are described in the text. In all experiments, 0·1 ml of the solubilized factor (TSF) from the particular preparation was added to the mixture of ions, enzyme, and substrate just prior to incubation. In this and subsequent tables, the concentrations of ions included are as follows: Mg = 3 mM; Na = 100 mM; K = 20 mM. The incubations were carried out in Tris buffer, 30 mM, pH 7·0, in the presence of Tris–ATP, 3 mM, for 30 min. The reaction was stopped by the addition of ice-cold trichloroacetic acid (0·1 ml of 50%) and the tubes immersed in ice for 15 min before assay. The values are ± the standard errors of the means; number of experiments in parentheses.

of ATPase activity in the presence of TSF and Mg^{2+}, whereas after 3 months the same preparation exhibited a 36% inhibition.

Similarity between the solubilized factor, aging, and azide treatment

The fact that storage of the heart muscle ATPase preparation for extended periods in the cold appeared to reduce the sensitivity of the Mg^{2+}–ATPase to TSF as cited above, suggested a relationship between aging and TSF action. Figure 3 is representa-

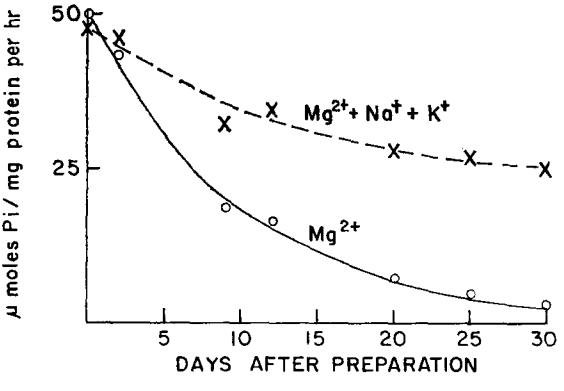

FIG. 3. The effect of cold storage on ATPase activity of cardiac muscle microsomes from rat heart. The enzyme preparation was stored at 5°, divided into separate tubes. Assays were conducted at the indicated periods.

tive of the storage or aging phenomenon. After 20 days of aging at 5°, the Mg^{2+} activity was reduced about 75% of the initial value, whereas the $Mg^{2+} + Na^+ + K^+$ site retained 72% of its full initial activity, an effect that appears to be at least qualitatively analagous to the inhibitory effects observed after the addition of the solubilized factor to fresh muscle preparations.

In previous communications,[11, 13] the selective inhibitory effect of sodium azide and azide-like compounds on the heart ATPase system was described. Again, it was the Mg^{2+} site that was particularly sensitive, thus paralleling the TSF and aging effects.

The influence of solubilized factor and azide on cold storage of the heart ATPase system

Another property of the solubilized factor which resembles the effects of sodium azide and azide-like compounds is its retardation of the loss of activity of the $Mg^{2+} + Na^+ + K^+$ site on the muscle ATPase system. This is exemplified in Fig. 4. During the process of aging at 5°, the $Mg^{2+} + Na^+ + K^+$–ATPase activity dissipated, but not so rapidly as the Mg^{2+}-dependent activity (Fig. 3).

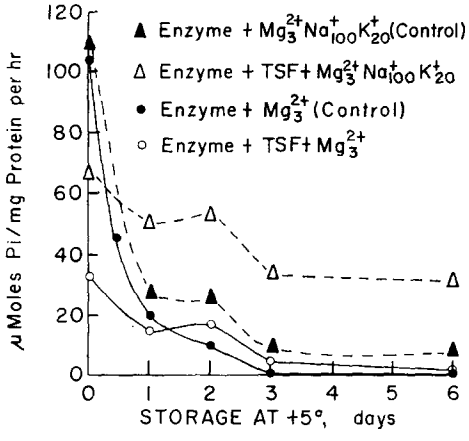

FIG. 4. The reaction between TSF and the cardiac microsomal ATPase during cold storage. The enzyme preparation (isolated from guinea pig) was stored with an equivalent of 0·1 ml TSF per ml for various periods at +5°.

However, TSF is able to slow the loss of activity of the $Mg^{2+} + Na^+ + K^+$ site without affecting the sensitivity of Mg^{2+} site to aging. In other words, TSF appears to have protected the $Mg^{2+} + Na^+ + K^+$ site against the effects of aging at the same time it induced the usual inhibitory action on the Mg^{2+} site. Sodium azide likewise showed a tendency toward protection of the $Mg^{2+} + Na^+ + K^+$ site (Fig. 5). Similar results were observed when the enzyme was stored in the presence of TSF or in the presence of azide at the following temperatures: room, 0°, 5°, −5°, and −15° (data not shown).

Influence of deoxycholate on TSF activity

Since the solubilized factor was obtained when deoxycholate (DOC) was included in the initial muscle homogenate (see Fig. 6 TSF, no DOC), studies were carried out to determine the role of deoxycholate on the inhibitory action of TSF. Deoxycholate

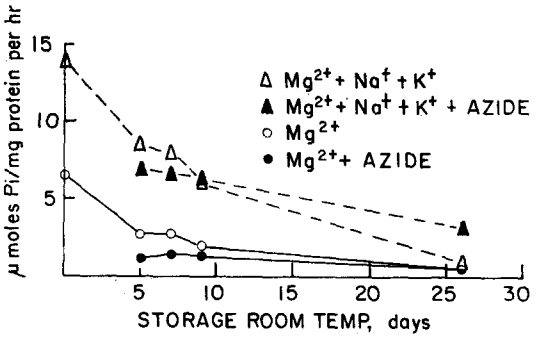

FIG. 5. The reaction between sodium azide and the cardiac microsomal ATPase during cold storage. The enzyme preparation was stored with an equivalent of 5mM sodium azide for various periods at room temperature.

alone inhibited both the Mg^{2+} and $Mg^{2+} + Na^+ + K^+$–ATPase activity, the latter, at least in the presence of low concentrations of DOC, to a greater extent than the former. The treated supernate from cardiac muscle suspensions prepared without deoxycholate showed no activity, nor did the same supernate to which the deoxycholate was added elicit any preferential inhibitory effect (Fig. 6).

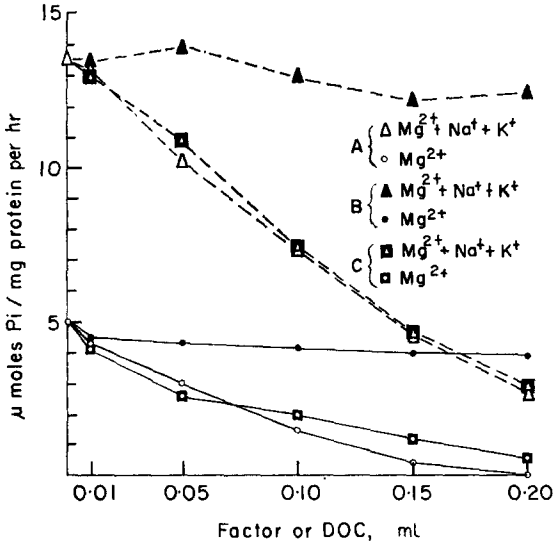

FIG. 6. The effects of deoxycholic acid on the cardiac microsomal ATPase in the presence of TSF. The abscissa represents the volume in milliliters of the following: A = 0·2% DOC; B = TSF prepared from a DOC-free homogenate; C = TSF prepared from a DOC-free homogenate to which 0·2% DOC has been added.

Solubilized factors from other tissues

An examination of other tissues for the presence of the endogenous inhibitory factor revealed its presence particularly in cerebral tissues and in the dog parotid gland. The activity of the brain factor on the heart muscle membrane ATPase appeared to be lower than the factor obtained from heart muscle (Table 2) and was much more variable in activity. This solubilized factor from brain had no significant effect on the microsomal ATPase from the same tissue nor did it have any effect on membrane

ATPases from kidney or liver (unpublished observations). The dog parotid gland exhibited a very active TSF on a microsomal ATPase derived from both the same tissue and an ATPase obtained from heart muscle (Table 2). The heart muscle TSF also displayed a similar inhibitory effect on a parotid ATPase system. The inhibitory effects referred to in this section were all preferentially on the Mg^{2+}-sensitive site of the enzyme system.

TABLE 2. THE EFFECTS OF SOLUBILIZED FACTOR DERIVED FROM DIFFERENT TISSUES ON MICROSOMAL ATPASES FROM HEART, BRAIN, AND PAROTID GLAND*

ATPase preparation	TSF preparation	Depression of control	
		Mg (%)	Mg + Na + K (%)
Heart	Brain (0·1 ml)	29 ± 6·2 (17)	15 ± 4·0 (17)
	Parotid (0·05 ml)	30 (2)	19 (2)
	Parotid (0·1 ml)	43 (2)	23 (2)
Brain	Brain (0·1 ml)	17 (2)	15 (2)
	Heart (0·1 ml)	16 (2)	15 (2)
Parotid	Parotid (0·1 ml)	75 (3)	0 (3)
	Heart (0·1 ml)	83 (3)	5 (3)

* The preparations of the enzymes and the solubilized factors are described in the text. The parotid glands were obtained from mongrel dogs. The brain and heart preparations were from guinea pigs. The 'ages' of the various preparations varied from 'fresh' to one week. The amounts of TSF added are indicated in parentheses after the tissue of origin.

The relationship between sulfhydryl groups and TSF activity

In preliminary studies it was found that sulfhydryl compounds such as cysteine or β-mercaptoethanol could partially reverse some of the effects of TSF. Investigations in greater detail, however, revealed that the basic inhibitory effect of TSF was independent of sulfhydryl groups. It may readily be seen from the representative curves in Fig. 7 that the sulfhydryl inhibitor, *p*-hydroxymericuribenzoate (POMB) selectively

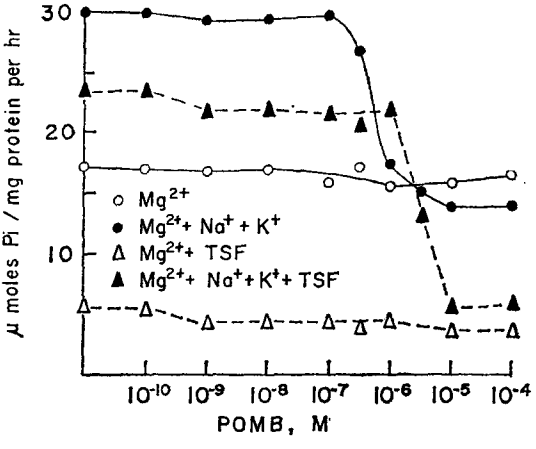

FIG. 7. The effects of *p*-hydroxymercuribenzoate (POMB) on the cardiac microsomal ATPase in the presence of TSF. TSF was added to the preparations indicated, in a concentration of 0·1 ml per ml of incubation mixture. Both POMB and TSF were added prior to the addition of enzyme.

inhibited only the Mg^{2+} + Na^+ + K^+-dependent site of the cardiac microsomal ATPase system.[13] In the presence of TSF this curve is shifted slightly but significantly to the right, indicating the possible presence of sulfhydryl groups in the solubilized supernatant factor. However, the absence of effect of POMB on the Mg^{2+} activity either in the presence or absence of TSF is noteworthy. The inhibitory factor produced its characteristic effect, then, in the presence of high concentrations of POMB. In order to confirm the actual presence of –SH groups in TSF, an analysis for sulfhydryl content in the muscle solubilized factor was carried out which revealed the presence of approximately 30 × 10^{-4} moles of –SH/l TSF. It is significant that a factor derived from brain tissues possessed the same sulfhydryl content. The TSF of brain had, as shown in Fig. 8 and cited above, very little effect on the ATPase enzyme. However, the free sulfhydryl groups present in the brain TSF shifted the curves to the right, indicating a competition for the POMB with –SH groups of the brain ATPase.

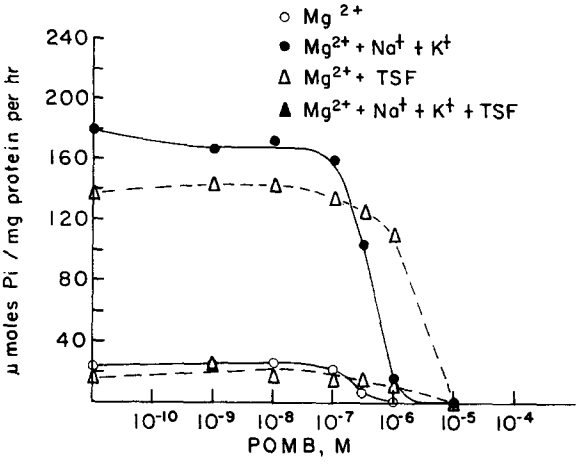

FIG. 8. The effects of POMB on the cerebral microsomal ATPase in the presence of TSF. The conditions were the same as in Fig. 7.

The effects of particle-free inhibitory factor on heart muscle ATPase activity

Electron micrographs of TSF indicated the presence of fragments of sarcoplasmic reticulum (unpublished observations). In order to eliminate the possibility that this particulate material affected the observations presented herein, the supernate derived from heart and brain homogenates (after the 30-min centrifugation at 150,929 *g*) was subjected to further and more intense centrifugal forces, resulting in a supernate which was particle free. Almost all the inhibitory activity was found in the clear super nate (Table 3). In fact, the activity had increased as compared to the particle-containing supernate.

The influence of the solubilized factor on the effect of ouabain on the cardiac ATPase

It was previously noted that azide and azide-like compounds selectively inhibited the Mg^{2+}–ATPase activity and increased the sensitivity of the Mg^{2+} + Na^+ + K^+ -stimulated activity to ouabain.[11, 13] Since these effects appeared to be similar to those

TABLE 3. THE EFFECTS OF PARTICLE-FREE SOLUBILIZED FACTORS ON MICROSOMAL ATPASE ACTIVITY OF CARDIAC MUSCLE*

Expt.	Factor tissue	Addition	Mg (μmoles P_i/mg protein/hr)	Mg + Na + K (μmoles P_i/mg protein/hr)
1		None	74	81
(a)	Heart	PMS, 0·1 ml	11	42
	Heart	PMP, 0·1 ml	55	63
(b)	Brain	PMS, 0·1 ml	22	41
	Brain	PMP, 0·1 ml	58	63
2		None	69	67
(a)	Heart	PMS, 0·1 ml	7	45
	Heart	PMS heated	7	45
(b)	Brain	PMS, 0·1 ml	20	40
	Brain	PMS heated	12	42
3		None	94	100
(a)	Brain	PMS, 0·1 ml	65	79
	Brain	PMP, 0·1 ml	73	89

* The heart ATPase preparation was derived from guinea pig cardiac muscle microsomes as indicated in the text and was freshly isolated. The particle-free supernatant factors were prepared as cited in the text, from guinea pig tissues. PMS = post-microsomal supernate (not heated) obtained after 150,929 *g*/2 hr. PMP = post-microsomal pellet (not heated) obtained after 150,929 *g*/2 hr. In experiment 2 (a) and (b), 'PMS heated' refers to a supernatant factor which was heated at 90° for 20 min, and centrifuged for 15 min at 2,500 rev/min. The sediment was discarded, and the remaining supernate was employed in the experiments cited above.

TABLE 4. THE EFFECT OF SOLUBILIZED FACTOR FROM HEART MUSCLE ON THE SENSITIVITY OF CARDIAC MICROSOMAL ATPASE TO OUABAIN*

Preparation	Age	Addition	Mg (μmoles P_i/mg protein/hr)	Mg + Na + K (μmoles P_i/mg protein/hr)
Guinea pig	Fresh	None	44	62
		Ouabain	44	50
		TSF, 0·05 ml	20	44
		Ouabain + TSF, 0·05 ml	20	25
Guinea pig	2 Day	None	23	27
		Ouabain	23	22
		TSF, 0·1 ml	5	19
		Ouabain + TSF, 0·1 ml	5	10
Rat	2 Day	None	65	105
		Ouabain	65	90
		TSF, 0·1 ml	59	100
		Ouabain + TSF, 0·1 ml	59	83

* All enzyme preparations were derived from microsomal fractions as described. The conditions of incubation and assay were the same as indicated in previous tables. Ouabain was used in a final concentration of 10^{-4} M. Analyses are the mean values from three experiments in each case.

of TSF as well as to the storage phenomenon reported in this paper the possible influence of TSF on ouabain's action was studied. The data of Table 4 further substantiate the similarity between the endogenous factor and azide. An apparent increase in sensitivity to ouabain, accompanied by a preferential inhibition of the Mg^{2+} activity, was observed. The combination of TSF plus ouabain yielded a greater depression of the Mg^{2+} + Na^+ + K^+–ATPase activity than did either agent alone.

Dialysis of the solubilized factor

Most of the protein material in the supernate is removed by prolonged heating, leaving the inhibitory factor in the soluble portion. A series of dialysis studies was carried out to gain additional information concerning the nature of the substance or substances. Figure 9 indicates that the factor is slowly dialyzable. After about 24 hr

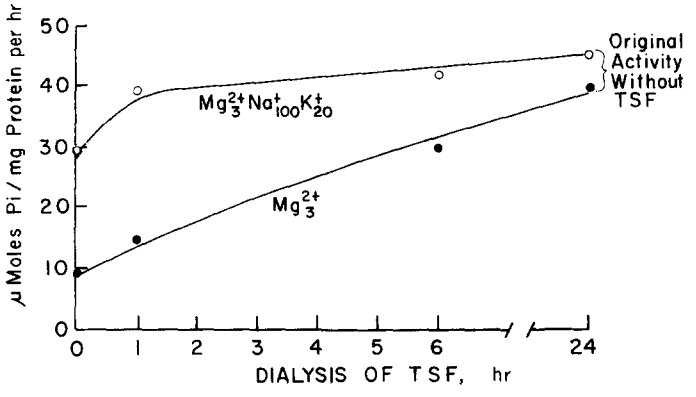

FIG. 9. Dialysis of TSF was carried out as described in the text. At the various periods, 0·1 ml of the dialyzed material was added to the cardiac microsomal ATPase preparation and assays conducted. The zero point indicates the activity in the presence of nondialyzed TSF. After 24 hr, it is seen that the TSF retained no inhibitory activity.

of dialysis, the material remaining within the dialysis bag had lost all the inhibitory activity and, when added back to a cardiac ATPase preparation, induced no effect. Before dialysis, the material produced a marked inhibition of the Mg^{2+} activity, resulting in an activity ratio of 3·0 (Mg^{2+} + Na^+ + K^+/Mg^{2+}). Shorter periods of dialysis have been employed wherein the DOC, EDTA, and histidine were removed (as determined by chemical analysis), but the inhibitory activity was retained.

DISCUSSION

The present communication confirms the presence of an endogenous inhibitory substance, originally observed by Skou,[10] which is present in the supernate of heart muscle suspensions. This factor (TSF) preferentially inhibits the Mg^{2+}-dependent site of the ATPase transport system, thus presenting a similarity in activity to certain nitrogenous compounds, [13] and to the aforementioned 'aging' process.[7] These observations further substantiate the presence of at least two sites of activity associated with the heart microsomal ATPase enzyme complex. From the present and previous reports,[7, 9, 11–13] it is suggested that one site of activity is Mg^{2+}

dependent and does not involve sulfhydryl groups, but possibly does require specific acidic groupings. The other site depends upon the presence of Mg^{2+}, Na^+, and K^+ for optimal activity and is sensitive to sulfhydryl inhibitors and to inhibitors of active transport (cardiac glycosides, detergents, calcium ions, and sodium amytal). The evidence suggests the requirement for a sulfhydryl linkage in the $Mg^{2+} + Na^+ + K^+$-dependent activation. It should be emphasized that none of the inhibitors of the Mg^{2+}-dependent site exhibited absolute specificity, whereas the blocking agents of sulfhydryl groups and active transport did react specifically with the $Mg^{2+} + Na^+ + K^+$ site of the transport ATPase associated with heart muscle. This phenomenon may not be true of membrane ATPases from other tissues.

Some of the characteristics of the endogenous inhibitory factor are of interest. Although the evidence suggests a nonprotein nature, the fact that it is slowly dialyzable implies the presence of a polypeptide or some medium molecular weight compound. Preliminary amino acid analyses (data in preparation) suggest a polypeptide structure.

The factor has been dissociated from particulate material. The fact that a detergent compound, deoxycholic acid is necessary for its preparation implies that the localization of TSF may not be cytoplasmic. Other membrane-solubilizing substances have been used (Triton X-100, digitonin) without much success. The combination of DOC, a steroid-like substance, with a membrane constituent to produce a substance with inhibitory properties was therefore considered. However, the current observations would discourage this idea, since neither DOC alone nor in combination with the supernate obtained from non-DOC homogenates induced any effect. These results, however, do not rule out completely the possibility that the DOC may be interacting with some membrane component when the tissue is in a homogenate form, resulting in what we call TSF. An interaction of DOC with membrane material has been recently reported.[21] As a working hypothesis it is suggested that TSF is located in a particulate region of the cell and is leached off by DOC treatment. The presence of other possible inhibitory compounds such as EDTA, histidine, calcium, or sulfhydryl groups has been eliminated.

The endogenous factor apparently reacts with the $Mg^{2+} + Na^+ + K^+$ site as well as with the Mg^{2+} site, but the effect on the former is noted only after storage of the enzyme with TSF and, in addition, the type of reactivity is different from the effect of TSF on with Mg^{2+} site. Apparently the factor can prevent the loss of activity of the $Mg^{2+} + Na^+ + K^+$-stimulated site of the ATPase system which occurs during storage in the cold. The nature of this observation is unknown. Analagous to the effects of nitrogenous compounds previously cited,[11, 13] TSF increases the apparent sensitivity of the $Mg^{2+} + Na^+ + K^+$-dependent site to ouabain. It is possible that the factor may increase the availability of certain ouabain-sensitive groups on the enzyme or perhaps may alter the charged environment in or around the site of activity.[13] Recently an important new concept of inhibition has been developed, involving two loci of enzymatic activity, both being affected differentially by selected inhibitors but both structurally oriented in such a way that alterations of one site may affect the other. This has been referred to as *allosteric* inhibition[22, 23] and may be useful in explaining the selective inhibitory actions described herein. The compounds, e.g. which affect the Mg^{2+} site, may induce an alteration of the ouabain-sensitive site. It is hoped that studies of this type may yield information concerning the molecular requirements for the action of ouabain on the transport enzyme system.

It is noteworthy that TSF has been found in tissues other than heart muscle. In the brain, however, the factor affects only the heart muscle ATPase and is less active than the heart TSF. It is possible that the factor from the brain is not solubilized by the DOC as completely as is the heart TSF. This would not explain, however, the minimal effect of the heart TSF on the brain microsomal ATPase system as observed in the present report. The only tissue examined thus far that possesses a very active TSF both on its parent ATPase and on the heart ATPase is the parotid gland. A more detailed report of this observation has been previously published.[9]

The present observations offer a possible explanation for the relatively low activation of freshly prepared microsomal ATPases from heart muscle, by $Mg^{2+} + Na^{+} + K^{+}$. The muscle microsomal suspension as prepared may possibly be devoid of a basic polypeptide or protein factor which has been solubilized and appears in the supernate. Addition of the solubilized factor back to the ATPase system in the microsomes would 'reveal' the $Mg^{2+} + Na^{+} + K^{+}$-dependent site. It may be that membrane preparations from other tissues retain, or bind more tightly this endogenous factor.

During the aging process of the heart muscle preparation the endogenous factor may possibly recombine with a membrane constituent. If this is so, a reversal of the TSF effect might be expected after DOC treatment of the microsomal pellet. This has been unsuccessful thus far. On the other hand, different phenomena may be involved in the aging and TSF effects, involving however, a common site.

The basic proteins represent an interesting class of substances and have been implicated recently in active transport mechanisms[6, 24] and in oxidative phosphorylation.[25] It has also been shown that basic proteins 'migrate' from the nuclear fraction of cerebral cortical slices to the microsomal fraction, during cold storage or other treatments, resulting in a loss of metabolic response to electrical stimulation.[24] The present results would suggest that some charged protein or polypeptide fraction of a protein is leached from an organelle within the heart muscle cell by DOC treatment, during the isolation of a microsomal ATPase system. This material appears to place a 'restraint' on one locus of enzymatic activity associated with the ATPase system. Current investigations have revealed a number of interesting aspects concerning possible localization of this endogenous material and its similiarity to specific purified basic proteins.[26] Since this factor also exerts inhibitory action on ATPases derived from cardiac myofibrils,[12] a possible involvement in the muscle-relaxing system is being considered.

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