Inhibitors of Calmodulin-Dependent Phosphorylation Simultaneously Inhibit Calcium Uptake and Calcium-Dependent ATPase Activity in Skeletal Muscle Sarcoplasmic Reticulum and Transiently Induce Calcium Release

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Z. Naturforsch. **39 c,** 1189–1191 (1984);
received August 31, 1984

Skeletal Muscle, Sarcoplasmic Reticulum, Calcium Transport, Calcium dependent ATPase, Calmodulin Antagonists

Under adequate experimental conditions calmodulin antagonists like compound 48/80 do not dissociate calcium uptake from the calcium-dependent ATP hydrolysis of skeletal muscle sarcoplasmic reticulum membranes but simultaneously inhibit both processes. Apart from the agent's pump inhibiting effect, they interact with the caffeine sensitive calcium channel in the sarcoplasmic reticulum causing a rapid transient calcium release.

Recently, Tuana and McLennan [1] have reported that reagents like compound 48/80 and calmidazolium which inhibit calmodulin-dependent phosphorylation, are able to uncouple, in native skeletal muscle sarcoplasmic reticulum vesicles, calcium uptake and calcium-dependent ATPase activity. These substances were reported to affect the light as well as the heavy fraction of the vesicular preparation. On the other hand, however, these agents do not interfere with the calcium uptake by liposomes reconstituted from purified preparations nor do they accelerate calcium efflux from calcium loaded vesicles. These observations led the authors to suggest that calmodulin-dependent phosphorylation plays a functional role in the coupling of ATP hydrolysis and calcium transport, perhaps through the regulation of calcium release channels in the reticular membranes. These channels should be different from those present in the terminal cisterna of the sarcoplasmic reticulum heavy fraction because, as reported, the light fraction derived from the free sarcoplasmic reticulum which is largely devoided of these channels, is also affected by the drugs [2]. If such a mechanism would play a major role in the function of the sarcoplasmic reticulum, the invariance of the coupling ratio calcium taken up – ATP hydrolyzed must be considered as a fortuitous property of the preparations [3]. The most important results which are the basis of the authors' hypothesis are measurements of calcium uptake in the presence of oxalate and of the calcium-dependent ATPase activity under identical conditions. Such measurements are made difficult by the fact that the enzyme's activity is rapidly suppressed when during calcium uptake the internal calcium concentration rises and the external concentration declines [4]. The inhibition develops rapidly under experimental conditions where oxalate is absent. In this case, after a few seconds calcium uptake and the calcium activated ATPase slow down [5]. In order to establish reliable correlations between the two events, one has to measure calcium uptake and ATP hydrolysis simultaneously and continuously [5]. In the presence of oxalate the calcium concentration inside the vesicles also initially rises before calcium-oxalate precipitation occurs, because oxalate penetration lags behind calcium accumulation [6]. After this initial period, calcium-oxalate is precipitated and a relatively low calcium concentration inside the vesicles is maintained. Under these conditions it is possible to measure for 1–2 min the appropriate enzyme activity, provided that the amount of calcium offered can be stored by the preparation. If this is the case, the calcium concentration in the solution drops to low values and ATP hydrolysis approaches the activity of the so-called basic ATPase [3]. If, however, calcium is offered in large quantities exceeding the storing capacity of the respective preparation, enzymatic activity prevails, the size of which is determined by the residual calcium concentration in the medium as well as by the calcium concentration inside the vesicles. In this situation, direct inhibition of the enzyme by an inhibitor can result in an apparent activation of ATP hydrolysis because inhibition prevents the decline of the activity resulting from the reduction of the calcium concentration in the medium and the rise of the calcium concentration in the vesicular volume. There are two possibilities to avoid these complications. First, one has to use relatively small calcium quantities so that calcium uptake is not limited by the storing capacity of the

Abbreviations: Compound 48/80 is a condensation product of N-methyl-*p*-methoxy-phenethylamine with formaldehyd. SR, sarcoplasmic reticulum.

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0341-0382/84/1100-1189 $ 01.30/0

preparation, and one has to follow the time course of the reaction continuously or in short time intervals since activity measurements during one fixed time interval give erroneous results [7]. Secondly, the relatively time-consuming procedure can be avoided if changes in the internal and the external calcium concentration are prevented by making the vesicular membranes calcium permeable with an effective calcium ionophore A 23187. This procedure has been applied in order to correlate calcium-dependent ATPase activity and the calcium transport in the presence of the calmodulin antagonist compound 48/80. As reported by Tuana and McLennan [1], the compound 48/80 effectively suppresses the oxalate supported calcium uptake. Half maximal inhibition is achieved at a concentration of ~ 50 µg/ml (Table I). The same concentration of the drug suppresses initial calcium uptake in the absence of oxalate. When the reaction in the presence of oxalate further proceeds, all calcium offered to the vesicles is taken up, while in the absence of oxalate the residual calcium in the medium increases with increasing concentrations of the drug. The severe inhibition of initial calcium uptake in presence and absence of oxalate is difficult to reconcile with the authors' sole explanation of a drug-induced calcium channel opening. In fact, as shown in Fig. 1, the calcium-dependent ATPase is suppressed by the

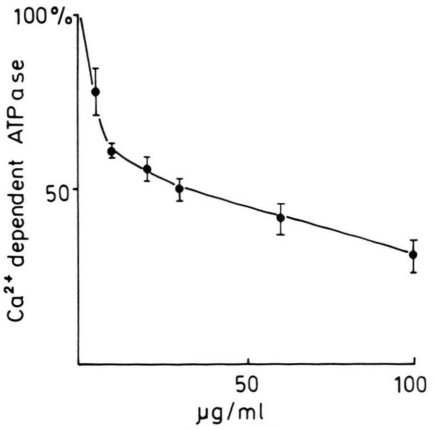

Fig. 1. Inhibition of the calcium dependent ATP hydrolysis of calcium permeable sarcoplasmic reticulum vesicle by compound 48/80. The measurements were performed under the conditions given in Table I. The points in the graph are mean values ± SE of five measurements.

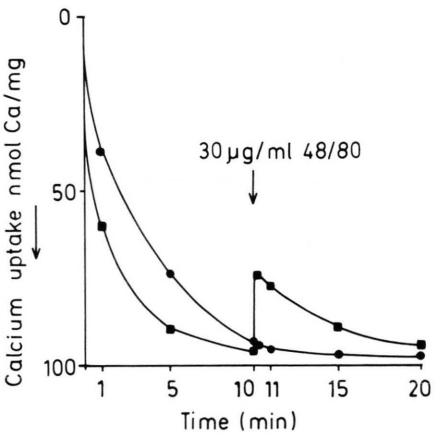

Fig. 2. Transient calcium release induced by compound 48/80 and its inhibition by caffeine. The sarcoplasmic reticulum vesicles were actively loaded with calcium as previously described [11]. At the end of the loading period calcium release was initiated by the addition of 30 µg/ml compound 48/80 (■). In the presence of 10 mM caffeine in the loading assay calcium uptake is significantly retarded. When after 10 min 30 µg/ml compound 48/80 are added, no calcium is released. The drug was added at the arrow. The presence of caffeine in the loading medium also suppresses calcium release by a second addition of caffeine. 0.1 mM trifluoperazine and 0.1 mM quercetin also become ineffective.

Table I. Simultaneous inhibition of calcium uptake and calcium dependent ATPase activity by compound 48/80. The assay for measuring uptake in the presence of oxalate containd 5 mM ATP, 5 mM $MgCl_2$, 5 mM oxalate, 0.1 mM $^{45}CaCl_2$, 40 mM KCl, 50 mM histidine, pH 7.0 and 0.1 mg protein/ml. In the absence of oxalate the assay contained 5 mM ATP, 5 mM $MgCl_2$, 40 mM KCl, 20 µM $^{45}CaCl_2$, 50 mM histidine, pH 7.0 and 0.2 mg protein/ml. The ATPase assay for measuring total activity contained 5 mM ATP, 5 mM $MgCl_2$, 5 mM oxalate, 0.1 mM $CaCl_2$, 40 mM KCl, 50 mM histidine, pH 7.0 and 10 µM calcium ionophore A 23187. Calcium uptake was assayed by the Millipore filtration method [8]. The basal activity was measured in the presence of 2 mM EGTA. The respective concentrations of the inhibition are given in the table. Aliquots were taken at 2 min and 7 min. Inorganic phosphate was measured as previously described [3]. The turbidity arising at concentrations > 100 mg/ml of the inhibitor was removed by centrifugation. The calcium dependence activity amounts to 1.2–1.5 µmol P/mg · min at room temperature of 20–22 °C. The percentage uptake activity observed in the absence of oxalate is given in brackets.

Compound [µg/ml]	Calcium uptake with oxalate [µmol/mg]		Calcium uptake without oxalate [µmol/mg]		Calcium dependent ATPase [%]
	1 min	5 min	1 min	5 min	
0	0.99	1.0	68	72	100
5	0.70	1.0	48 (71)	61	78
20	0.62	1.0	40 (60)	45	62
50	0.56	1.0	35 (51)	32	51
100	0.48	1.0	30 (44)	33	44

agent indicating its interference with the pump's catalytic cycle. This finding fully agrees with previous reports describing the inhibition of the sarcoplasmic calcium transport system by polycyclic amines [9, 10]. We therefore must conclude that the experimental conditions chosen by Tuana and McLennan for measuring ATPase activity were inadequate. The erroneous results, however, do not exclude that the authors' suggestion concerning the interaction of the agents with the calcium channels in the sarcoplasmic reticulum were right. In fact, compound 48/80 and other calmodulin antagonists interact with caffeine sensitive calcium channels in the sarcoplasmic reticulum. Compound 48/80 and trifluoperazine (not shown) produce, like caffeine, a rapid release of a fraction of 30–50% of the stored calcium (Fig. 2). This release is prevented if the preceding active calcium storage has been performed in the presence of caffeine. Thus, calmodulin antagonists and caffeine interact on the same calcium releasing structure in the sarcoplasmic reticulum membranes. Channel opening in a fraction of the vesicular preparation results, in the absence of oxalate, in a dose-dependent reduction of the preparation's calcium uptake capacity. In the presence of oxalate, such an effect cannot be seen because, due to the oxalate enhanced storing capacity, the vesicular fraction devoid of channels can completely store the calcium added to the assay (Table I).

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