Biochimica et Biophysica Acta, 1064 (1991) 321–328

ADONIS 0005273691002053

BBAMEM 75221

Covalent and non-covalent inhibitors of the phosphate transporter of sarcoplasmic reticulum

H.I. Stefanova, J.M. East and A.G. Lee

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton (U.K.)

(Received 16 October 1990)

Key words: ATPase, (Ca^{2+} + Mg^{2+})-; Sarcoplasmic reticulum; Phosphate transporter; Phosphonocarboxylic acid

The sarcoplasmic reticulum (SR) of skeletal muscle contains a P_i transporter which transports P_i into the lumen of the SR, increasing the level of accumulation of Ca^{2+} by SR by forming insoluble salts with Ca^{2+}. Phosphonocarboxylic acids inhibit the transport of P_i by the transporter, phosphonoformic acid itself being transported into the SR increasing the level of accumulation of Ca^{2+}. Phenylphosphonic acid also inhibits P_i transport, distinguishing the P_i transporter of SR from the Na^+/P_i transporter of brush-border membranes. Oxalate transport is also inhibited by the phosphonocarboxylic acids, consistent with the suggestion that oxalate and phosphate are carried on the same transporter. The effects of maleate are, however, not inhibited, suggesting a separate carrier for the dicarboxylic acids. Acetic annydride and phenylglyoxal inhibit the transporter, P_i providing protection against the effects of acetic anhydride, suggesting the presence of a lysine residue at the P_i binding site. ATP provides protection against the effects of acetic anhydride and phenylglyoxal, suggesting the presence of an ATP binding site on the transporter.

Introduction

Contraction and relaxation of skeletal muscle follow from the release and reaccumulation of Ca^{2+} by the sarcoplasmic reticulum (SR). The protein responsible for the accumulation of Ca^{2+}, the (Ca^{2+}-Mg^{2+})-ATPase, has been well characterised [1]. Less well characterised are the proteins responsible for the release of Ca^{2+} and for the storage of Ca^{2+} within the lumen of the SR. An acidic Ca^{2+} binding protein, calsequestrin, is found in the lumen of the sarcoplasmic reticulum (SR) and is thought to act as a Ca^{2+}-sequestering agent, reducing the concentration of free Ca^{2+} within the SR [2–4]. Nevertheless, the total amount of Ca^{2+} taken up by isolated SR vesicles is relatively low in the absence of ions such as P_i, oxalate or pyrophosphate; in the presence of these ions, uptake is increased several-fold [5,6]. It was suggested that these ions could diffuse through the membrane and, because of the low solubilities of their calcium salts, form precipitates of calcium salts within the SR, lowering the free Ca^{2+} concentration; such precipitates have been observed in electron micrographs [7,8]. Subsequent studies showed that movement of ions across the membrane was mediated by a specific transporter [9]. In particular, Knowles and Racker [10] showed that although Ca^{2+} uptake by reconstituted vesicles containing the purified (Ca^{2+}-Mg^{2+})-ATPase was increased if oxalate or P_i was trapped inside the vesicles, addition of the ions to the external medium had no effect, indicating that transport of oxalate or P_i was not an intrinsic property of the ATPase. Hasselbach and Weber [9] showed that oxalate, P_i and pyrophosphate competed for a single carrier. It has also been shown that calcium-chelating dicarboxylates such as maleate and succinate also increase the level of Ca^{2+} accumulated by SR vesicles [11–13], and it was suggested that these anions were also transported on the same transporter as oxalate and P_i [13].

Little is yet known about the mechanism of this transporter, referred to for convenience as the P_i transporter. The SR is known to be permeable to anions such as Cl^- [14] and a Cl^--selective ion channel has now been characterised in SR [15,16]. This channel is, however, impermeable to P_i [16]. It has also been shown that the passive permeability of the SR membrane to P_i is very much less than to Cl^- [14]. In a series of studies

Abbreviations: SR, sarcoplasmic reticulum; ATPase, (Ca^{2+}-Mg^{2+})-ATPase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PFA, phosphonoformic acid; PAA, phosphonoacetic acid; PPA, 3-phosphonopropionic acid; PhPA, phenylphosphonic acid.

Correspondence: A.G. Lee, Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

it has been shown that stilbene sulphonates inhibit anion movement across SR, and therefore it was suggested that the anion transporter of SR could be similar to Band 3 of red blood cells which is also inhibited by stilbene sulphonates [17–21]. Kasai and Taguchi [19] reported that stilbene sulphonates label a protein of molecular weight about 100 000 which was suggested to be the transporter. However, subsequent studies by Byers and Meissner (see Ref. 14) suggested that most labelling was of the ATPase rather than of an anion transporter; stilbene sulphonates have also been shown to inhibit the activity of the ATPase (Lee, unpublished observations). MacLennan et al. [22] suggested that the anion transporter could be the 53-kDa glycoprotein which has now been sequenced [23]. This is made less likely by the observation that SR from lobster muscle lacks the glycoprotein [24] and yet still transports oxalate [25].

As a starting point for the study of the P_i transporter in SR we looked for specific inhibitors of the transporter, to be used both as structural probes and as the basis for the development of affinity probes to be used in label studies and for affinity purification. It has been reported that phosphonocarboxylic acids specifically inhibit the Na^+/P_i co-transport system in brush-border membranes [26,27]. We show here that these same compounds inhibit the P_i transporter in SR, but with a different structural specificity. We also show that transport is inhibited by acetic anhydride and phenylglyoxal, reagents reacting preferentially with lysine and arginine residues respectively; such residues are commonly found at anion binding sites [28,29].

Materials and Methods

AnalaR reagents and murexide were obtained from BDH Chemicals and Hepes (Ultrol) was from Calbiochem. Phosphonates were obtained from Sigma and Aldrich. SR was prepared largely as described in McWhirter et al. [30]. White muscle was dissected from the hind legs of a female (New Zealand White) rabbit, and fat and connective tissue was removed, this and all subsequent procedures being performed at 4°C. The muscle was homogenized in buffer (0.3 M sucrose, 20 mM histidine-HCl (pH 8.0)) containing dithiothreitol (1 mM) and phenylmethanesulphonyl fluoride (5 μM) in a Waring Blendor for 15 s. The homogenate was spun at $8000 \times g$ for 20 min and the supernatant was filtered through muslin and then spun at $37000 \times g$ for 90 min. The resulting pellet was resuspended in ice-cold buffer (0.3 M sucrose, 10 mM histidine-HCl, 0.6 M KCl (pH 8.0)) containing 1 mM dithiothreitol and 5 μM phenylmethanesulphonyl fluoride. The suspension was left to stand for 30 min to precipitate actomyosin before centrifugation at $5000 \times g$ for 20 min. The supernatant was decanted off the soft actomyosin pellet and centrifuged at $37000 \times g$ for 90 min. The resulting pellet was rehomogenized in buffer (40 mM Hepes-KOH, 100 mM KCl (pH 6.3)) containing 1 mM dithiothreitol to give 10-20 mg protein/ml and stored frozen. In our previous preparative procedure [30] the final SR preparation was washed twice in buffer before storage, but we have found that this results in decreased uptake of Ca^{2+} measured in the presence of P_i. Purified ATPase was prepared as described in East and Lee [31].

Ca^{2+} fluxes were monitored photometrically by using the dye murexide. Spectra were run on a Shimadzu UV3000 dual-wavelength spectrophotometer with a wavelength pair of 507 nm-542 nm, at 25°C. Sample were stirred with a Cell-Spinbar magnet (Bel-Art Products) in the sample cuvette, and reagents were injected directly into the cuvette from Hamilton syringes through a modified lid. A saturated solution of murexide was prepared by addition of 2.4 mg of murexide to 1 ml of water, leaving the resulting suspension to stand on ice for 30 min, followed by filtration to give a clear solution. For assays of Ca^{2+}, 160 μl of this stock solution was added to 3 ml of buffer containing SR membrane (usually at 0.08 mg protein/ml) in the assay cuvette, to give a final murexide concentration of 0.45 mM. To calibrate the optical signal, samples of a concentrated stock solution of $CaCl_2$ were added, to a final concentration of 50 μM: over the concentration range used here, the murexide response was linear with Ca^{2+} concentration. Ca^{2+} uptake was initiated by addition of ATP; at the Mg^{2+} concentration usually employed (5 mM) the change in free Ca^{2+} concentration due to binding of Ca^{2+} to ATP was shown to be negligible (data not shown).

Uptake of [^{32}P]P_i by SR vesicles was determined by Millipore filtration. SR vesicles (0.06 mg/ml) were incubated at room temperature in 40 mM Hepes-KOH, 100 mM KCl, 5.0 mM $MgSO_4$, 50 μM $CaCl_2$ (pH 6.3) containing 5.0 mM P_i. Active Ca^{2+} uptake was initiated by addition of 0.5 mM ATP. At given times, 200-μl samples were removed and rapidly filtered on Milipore HA filters (0.45 μm) and washed with 15 ml ice-cold buffer (20 mM P_i, 40 mM Hepes-KOH, 100 mM KCl (pH 6.3)). Filters were counted in OptiPhase HiSafe 3.

For covalent modification, SR vesicles (0.9 mg protein/ml) in buffer (40 mM Hepes-KOH, 100 mM KCl (pH 7.0)) were incubated with the required concentrations of acetic anhydride or phenylglyoxal. For modification with acetic anhydride, SR was incubated with acetic anhydride for 5 min at 25°C, and the reaction was stopped by addition of lysine to a final concentration of 20 mM. For modification with phenylglyoxal, SR was incubated with phenylglyoxal for 30 min at 25°C, and the reaction was stopped by addition of arginine to a final concentration of 20 mM. Samples were then diluted into the appropriate buffers for assay of Ca^{2+} accumulation. In experiments where ATP was

included in the modification medium, this procedure resulted in significant concentrations of ATP in the assay buffer, so that reactions could not be started in the usual way by addition of Ca^{2+}. For these experiments, therefore, modified SR was added to buffer containing the required concentration of ATP in the absence of Ca^{2+}. The reaction was then started by the addition of Ca^{2+}. Using this protocol it was not possible to record the initial fast phase of accumulation of Ca^{2+}, but the second, slower phase of accumulation observed in the presence of P_i could be recorded.

ATPase activity was measured using the coupled enzyme system described in East and Lee [31]. Samples (12 μl, equivalent to 6 μg of ATPase) were added to a medium containing 40 mM Hepes-KOH, (pH 7.2), 5 mM $MgSO_4$, 1.01 mM EGTA, 0.42 mM phospho*enol*-pyruvate, 0.15 mM NADH, 2.1 mM ATP, 7.5 IU pyruvate kinase and 18 IU lactate dehydrogenase in a total volume of 2.5 ml, with $CaCl_2$ added to give maximal activity.

Concentrations of protein were estimated by using the absorption coefficient given by Hardwicke and Green [32].

Results

Transport of phosphate

Addition of ATP to a suspension of SR vesicles leads to uptake of Ca^{2+}, which can be monitored spectrophotometrically by using murexide to determine the concentration of Ca^{2+} in the external medium (Fig. 1). As shown in Fig. 1, in the absence of P_i a rapid accumulation of Ca^{2+} to a level corresponding to 198 nmol Ca^{2+}/mg protein is observed, the rate of uptake being

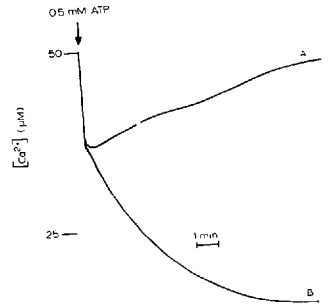

Fig. 1. The uptake and release of Ca^{2+} are shown following addition of ATP (0.5 mM) to SR vesicles (0.08 mg protein/ml) in buffer (40 mM Hepes-KOH, 100 mM KCl, 5 mM $MgSO_4$ (pH 6.3)) at an initial Ca^{2+} concentration of 50 μM, in the absence (A) or presence (B) of 5 mM P_i.

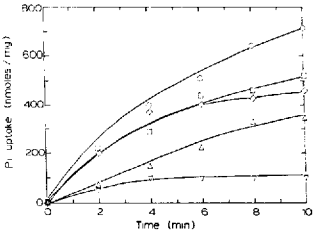

Fig. 2. The uptake of $[^{32}P]P_i$ following addition of ATP (0.5 mM) to SR vesicles (0.06 mg protein/ml) in buffer (40 mM Hepes-KOH, 100 mM KCl, 5 mM $MgSO_4$ (pH 6.3)) at an initial Ca^{2+} concentration of 50 μM, in the absence (○) or presence of 1 mM: PhPA (□), PPA (◇), PAA (△), PFA (▽).

too fast to follow in these experiments. Following uptake of Ca^{2+}, Ca^{2+} is released in two phases, an initial slow phase and a second faster phase, the faster phase starting when the ATP concentration has been reduced by hydrolysis to a value comparable to the K_d value of the ATPase for ATP (approx. 5 μM) [30]. As reported previously [30], we have found that the maximal extent of Ca^{2+} accumulation varies between preparations of SR. As shown in Fig. 1, the pattern of uptake of Ca^{2+} is distinctly different in the presence of P_i. The fast, initial phase of Ca^{2+} uptake is unaltered by the addition of P_i, but, in the presence of P_i, the initial uptake is followed by a second, slower phase of accumulation of Ca^{2+} which continues until either the Ca^{2+} in the external medium or the ATP is depleted. This slow second phase has been attributed to the transport of P_i across the membrane, leading to the precipitation of $CaHPO_4$ within the SR vesicles [33,34]. Accumulation of P_i measured directly is shown in Fig. 2. The ratio of extra Ca^{2+} accumulated in the presence of P_i to P_i accumulated is close to 1 : 1. Thus, after 2 min, the extra Ca^{2+} accumulated is 293 nmol/mg protein (Fig. 1), and the P_i accumulated at this time is 300 nmol/mg protein (Fig. 2).

Inhibition by phosphonates

Fig. 2 shows the effects of a series of phosphonyl derivatives on the active accumulation of P_i by SR vesicles. As shown, at 1 mM all inhibit P_i transport, the greatest effect being observed with PFA. The concentration of PPA giving 50% inhibition of P_i transport at 5 mM P_i is 1.4 mM (data not shown).

Fig. 3 shows the effect of 1 mM PFA on the uptake of Ca^{2+} in the absence of P_i. Surprisingly, a slow second phase of uptake of Ca^{2+} is observed in the presence of PFA after the initial fast phase, comparable to that seen in the presence of P_i (Fig. 1). Since it is known that PFA will complex Ca^{2+} [35,36], these re-

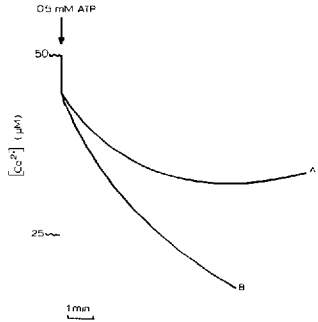

Fig. 3. The uptake of Ca^{2+} is shown following addition of ATP (0.5 mM) to SR vesicles (0.033 mg protein/ml), in (A) the presence of 5 mM P_i and (B) the presence of 1 mM PFA, the other conditions being as in Fig. 1.

sults suggest that PFA can be accumulated by SR vesicles, complexation between PFA and Ca^{2+} within the SR lumen decreasing the free concentration of Ca^{2+}, as for P_i. Effects of PFA are comparable at Mg^{2+} concentrations between 3 and 10 mM (data not shown). In the presence of PPA or PhPA (Fig. 4) and the absence of P_i, although no slow second phase of uptake of Ca^{2+} is observed, the rate of release of Ca^{2+} is slower than for the control, so that higher levels of accumulation of Ca^{2+} are measured at long times after the addition of ATP in the presence of PPA or PhPA than in their absence. Effects of PAA at 1 mM are comparable to those of PPA (data not shown). However, at higher (2.5 mM) concentrations, PAA reduces the magnitude of the initial, fast phase of accumulation of Ca^{2+}, an effect which can probably be attributed to

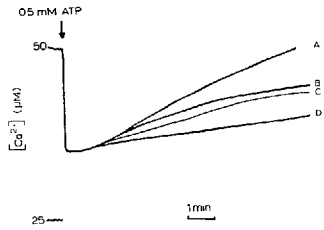

Fig. 4. The uptake of Ca^{2+} is shown following addition of ATP (0.5 mM) to SR vesicles (0.08 mg protein/ml), in the absence (A) or presence of: (B) 1 mM PPA, (C) 2.5 mM PhPA, (D) 2.5 mM PPA. Other conditions were as in Fig. 1.

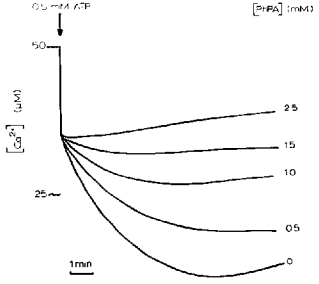

Fig. 5. The uptake of Ca^{2+} is shown following addition of ATP (0.5 mM) to SR vesicles (0.08 mg protein/ml) in the presence of 5 mM P_i and the given concentrations (mM) of PhPA. Other conditions were as in Fig. 1.

its inhibitory effect on ATPase activity (see below). The smaller effects of PPA and PhPA then PFA would suggest that these derivatives are transported less well.

As shown in Fig. 5, PhPA markedly reduces the magnitude of the slow, second phase of Ca^{2+} accumulation seen in the presence of 5 mM P_i, so that at 2.5 mM PhPA, the pattern of uptake is comparable to that seen in the absence of P_i (Fig. 1). Effects of PAA and PPA are comparable to those seen with PhPA except that, as described above, 2.5 mM PAA reduces the magnitude of the initial, fast phase of accumulation of Ca^{2+} (data not shown).

The amino derivative 4-aminophenylphosphonic acid had no effect on uptake of Ca^{2+}, either in the absence or presence of P_i (data not shown).

Addition of oxalate has also been observed to result in increased accumulation of Ca^{2+} [7] (Fig. 6). As shown in Fig. 6, the slow second phase of accumulation

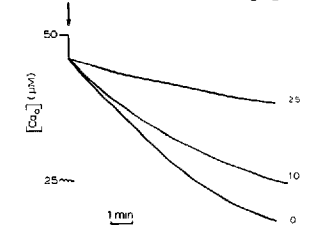

Fig. 6. The uptake of Ca^{2+} is shown following addition of ATP (0.5 mM) to SR vesicles (0.02 mg protein/ml) in the presence of 1 mM oxalate and the given concentrations of PPA. Other conditions were as in Fig. 1.

TABLE I

Accumulation of Ca^{2+} in the presence of maleate and succinate

Maximal uptake was measured following addition of ATP (0.5 mM) to SR vesicles (0.08 mg protein/ml) in buffer (40 mM Hepes-KOH, 100 mM KCl, 5 mM $MgSO_4$ (pH 6.3)) at an initial Ca^{2+} concentration of 50 μM.

Additions	Maximal Ca^{2+} accumulation (nmol/mg protein)
None	146
40 mM maleate	236
+ 2.5 mM PPA	274
+ 2.5 mM PhPA	300
40 mM succinate	195
+ 2.5 mM PPA	256
+ 2.5 mM PhPA	255

of Ca^{2+} seen in the presence of oxalate is also inhibited by PPA. Uptake of Ca^{2+} has also been reported to be increased by addition of dicarboxylic acids such as maleate [11,30]. Effects of maleate are very different to those of P_i or oxalate, with no marked second phase of uptake, but rather an increase in the level of accumulation of Ca^{2+} in the initial, fast phase, giving rise to a more marked 'overshoot' in the profile of Ca^{2+} uptake and release [11,30]. Addition of PPA or PhPA leads to a slight increase in the level of maximal accumulation of Ca^{2+} seen in the presence of maleate, although the effect is probably not significant (Table I). Effects of succinate are comparable to those seen with maleate (Table I).

Effects of the phosphonates on steady state ATPase activity are shown in Table II. To demonstrate that none of the phosphonates was hydrolyzed under the conditions of these experiments, SR vesicles (0.6 mg/ml) were incubated with the phosphonates (1.0 and 5.0 mM) in a medium containing 40 mM Hepes-KOH, 100 mM KCl, 5.0 mM $MgSO_4$ and 10 μM Ca^{2+} (pH 7.2) for 90 min at room temperature. An equal volume of 10% trichloroacetic acid was then added, and precipitated protein spun down in an Eppendorff centrifuge. The supernatants were assayed for liberated phosphate using

TABLE II

Effects of phosphonates on steady-state ATPase activity

ATPase activities for the purified ATPase were assayed in 40 mM Hepes-KOH, 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 50 μM Ca^{2+} (pH 7.2) at 25°C.

Concentration (mM)	Fractional activity [a]			
	PFA	PAA	PPA	PhPA
0.5	1.0	1.0	1.0	0.98
1.0	0.98	0.99	0.98	0.94
2.5	0.64	0.62	0.93	0.84
5.0	0.33	0.14	0.74	0.57

[a] Activity in the absence of phosphonate equal to 6.8 IU/mg protein.

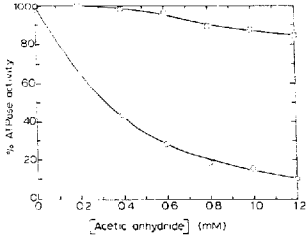

Fig. 7. ATPase activity of SR vesicles modified with the given concentration of acetic anhydride for 5 min in the absence (○) or presence (□) of 5 mM ATP. ATPase assays were performed at 2.1 mM ATP, maximally stimulating concentrations of Ca^{2+}, and pH 7.2. ATPase activities are expressed as percentage of control activity, measured for unmodified SR.

the method of Chen et al. [37]. In no case was any hydrolysis detected.

Effects of covalent modification

As shown in Fig. 7, incubation of SR vesicles with acetic anhydride leads to inhibition of ATPase activity. The presence of a high (5 mM) concentration of ATP during the incubation with acetic anhydride provides a high level of protection against the inhibitory effect (Fig. 7). Effects on accumulation of Ca^{2+} were less marked, so that after modification for 5 min with 0.4

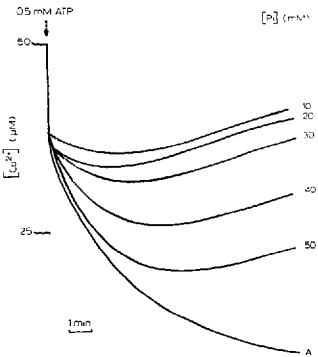

Fig. 8. The uptake of Ca^{2+} is shown following addition of ATP (0.5 mM) to SR vesicles (0.08 mg protein/ml) in the presence of 5 mM P_i, (A) for unmodified SR, and for SR modified with 0.4 mM acetic anhydride for 5 min in the presence of the given concentration (mM) of P_i.

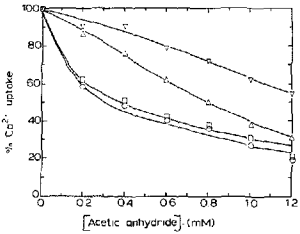

Fig. 9. The level of Ca^{2+} accumulation 5 min after the addition of ATP (0.5 mM) to SR vesicles modified with the given concentrations of acetic anhydride for 5 min, assayed in buffer (40 mM Hepes-KOH, 100 mM KCl, 5.0 mM $MgSO_4$, 50 μM $CaCl_2$ (pH 6.8)) containing 5.0 mM P_i. For this experiment, concentrations of Ca^{2+} in the medium were assayed photometrically using 50 μM arsenazo III. ○, SR modified in the absence of P_i or ATP; □, SR modified in the presence of 5 mM ATP; △, SR modified in the presence of 50 mM P_i; ▽, SR modified in the presence of 50 mM P_i and 50 mM ATP. The control level of Ca^{2+} accumulation was 400 nmol/mg protein.

and 1 mM acetic anhydride, maximal levels of uptake were approx. 80% and 40% of control, respectively (data not shown). However, as shown in Fig. 8, modification of SR with 0.4 mM acetic anhydride resulted in almost complete loss of the secondary, slow phase of Ca^{2+} accumulation observed in the presence of P_i. The presence of P_i during the reaction with acetic anhydride results in protection of the phosphate transporter (Figs. 8 and 9). The presence of 5 mM ATP together with 50 mM P_i during reaction with 0.4 mM acetic anhydride results in almost complete protection of the transporter, although some inhibition is still observed at higher concentrations of acetic anhydride (Fig. 9). The concentration of ATP resulting in 50% extra protection of the transporter modified with 0.4 mM acetic anhydride in the presence of 50 mM P_i is 3.0 mM (data not shown).

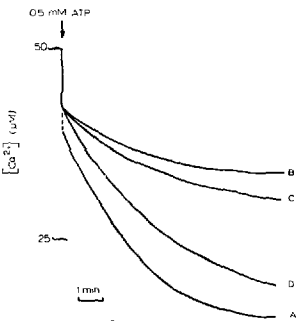

Fig. 10. The uptake of Ca^{2+} is shown following addition of ATP (0.5 mM) to SR vesicles (0.08 mg protein/ml) in the presence of 5 mM P_i, (A) for unmodified SR, and for SR modified with 3 mM phenylglyoxal in the absence of P_i and ATP (B), in the presence of 50 mM P_i (C) and in the presence of 50 mM P_i and 5 mM ATP (D). All measurements of Ca^{2+} accumulation were started by addition of ATP, except for (D) which was started by addition of Ca^{2+} (see Materials and Methods). All other conditions as in Fig. 1.

Modification of SR with 1.0 to 5.0 mM phenylglyoxal for 30 min results in a reduction in the initial fast phase of Ca^{2+} accumulation to 60% to 40%, respectively, of the unmodified levels (data not shown). As shown in Fig. 10, the slow phase of Ca^{2+} accumulation observed in the presence of 5 mM P_i is also reduced. The presence of 50 mM P_i during modification with phenylglyoxal has little effect, but the presence of 5 mM ATP and 50 mM P_i results in a high level of protection of the transporter (Fig. 10).

Discussion

The role of the SR in muscle is to sequester Ca^{2+} and release it when required to initiate muscle contraction. Calsequestrin and other Ca^{2+}-binding proteins are located in the lumen of the SR and serve to reduce the free internal concentration of Ca^{2+} [2–4]. Further, it has long been known that higher levels of Ca^{2+} are accumulated by isolated SR vesicles in the presence of Ca^{2+}-precipitating anions such as oxalate, P_i or pyrophosphate, attributable to transport of these ions across the membrane with the formation of Ca^{2+} precipitates in the lumen of the SR [5,6,8]. These results have been interpreted in terms of a distinct anion transporter, usually referred to as the P_i transporter. This transporter is distinct from the major protein of the SR, the (Ca^{2+}-Mg^{2+})-ATPase, since no P_i is taken up from the external medium by reconstituted vesicles containing only the purified (Ca^{2+}-Mg^{2+})-ATPase [10]. Hasselbach and Weber [9] showed that oxalate, P_i and pyrophosphate competed for the same carrier, and Chu et al. [12,13] suggested that maleate and succinate, which are also accumulated by SR vesicles, could also be transported by the same carrier.

Active accumulation of P_i by SR vesicles in the presence of Ca^{2+} and ATP can be studied directly, measuring the uptake of [^{32}P]P_i, or indirectly, through the second, slower phase of accumulation of Ca^{2+} observed in the presence of P_i, and attributable to the precipitation of insoluble calcium salt in the lumen of the SR (see Fig. 1). Martonosi and Feretos [6] and Tate et al. [38] have reported that the stoichiometry of uptake of oxalate to Ca^{2+} is 1 : 1, and the data reported in Figs. 1 and 2 show that the stoichiometry of uptake of P_i and Ca^{2+} in the second, slower phase, is also 1 : 1.

Here we report on effects of covalent and non-cova-

lent inhibitors of the P_i transporter. It has been reported that phosphonocarboxylic acids specifically inhibit the Na^+/P_i co-transport system in brush border membranes [26,27]. Since these compounds inhibit viral DNA and RNA polymerases by binding at the pyrophosphate binding site [40] it is likely that they bind at the P_i binding site on the Na^+/P_i transporter. As shown in Fig. 2, the phosphonocarboxylic acids also inhibit P_i transport by SR. For the Na^+/P_i transporter, Szczepanska-Konkel et al. [26] reported that PhPA had no effect at concentrations up to 5 mM, and suggested that the proximity of a carboxyl group to the phosphonyl moiety was an important determinant of inhibitory potency. The data reported in Fig. 2 show that this is not so for the P_i transporter of SR, where PhPA is an inhibitor at 1 mM. The strongest inhibitor of P_i transport is, however, PFA (Fig. 2).

As shown in Fig. 5, PhPA inhibits the second, slow phase of Ca^{2+} uptake observed in the presence of P_i, consistent with the observed inhibition of uptake of P_i (Fig. 2). However, for PFA in the absence of P_i, a slow phase of Ca^{2+} uptake is observed (Fig. 3), comparable to that seen in the presence of P_i (Fig. 1). This we attribute to transport of PFA by the P_i transporter, followed by complexation of Ca^{2+} by PFA in the lumen of the SR; the effective binding constant at pH 6.3 of PFA and Ca^{2+} can be calculated to be 248 from the binding constants given by Heubel and Popov [35] and Farmer et al. [36]. PAA at 1 mM had no effect on Ca^{2+} uptake in the absence of P_i (data not shown) despite an effective binding constant at pH 6.3 for Ca^{2+} of 802 calculated from the data of Heubel and Popov [35] and Farmer et al. [36]. Effects of PPA and PhPA on Ca^{2+} uptake in the absence of P_i are also much smaller than those of PFA (Fig. 4) and, although this can be attributed in part to a weaker binding to Ca^{2+} [35,36], given the results obtained with PAA, the smaller effects of these bulkier derivatives can probably be attributed to a slow rate of transport by the P_i transporter. The positively charged analogue 4-aminophenylphosphonic acid had no effect on uptake of Ca^{2+}, either in the absence or presence of P_i (data not shown).

Effects of 1 mM PFA on uptake of Ca^{2+} in the absence of P_i were little changed on changing the Mg^{2+} concentration between 3 and 10 mM (data not shown). From the effective binding constant for Mg^{2+} at pH 6.3 (251, calculated from the data of Heubel and Popov [35] and Farmer et al. [36]), it can be calculated that, at a total PFA concentration of 1 mM, the concentration of uncomplexed PFA changes from 0.6 to 0.3 mM on changing Mg^{2+} from 3 to 10 mM. Since uptake of Ca^{2+} in the absence of PFA also changes little over this range of Mg^{2+} concentrations [41], this implies that MgPFA and PFA are transported equally by the P_i transporter.

As shown in Fig. 6, the slow phase of accumulation of Ca^{2+} observed in the presence of oxalate is also inhibited by PPA, consistent with the suggestion of Hasselbach and Weber [9] that P_i and oxalate are carried on the same carrier. However, PPA had no effect on the extra accumulation of Ca^{2+} observed in the presence of maleate or succinate (Table 1), suggesting that the carrier for the dicarboxylates is distinct from that for P_i and oxalate.

Studies of the effects of covalent modification of SR membranes can provide information about amino acid residues likely to be important in P_i transport. Lysine and arginine residues have been located at the anion binding sites of anion transporters [28,29]. Acetic anhydride modifies lysine residues but also histidine and cysteine, whereas phenylglyoxal is fairly specific for arginine residues [42,43]. A complication in studies of the P_i transporter is that both the transporter and the $(Ca^{2+}$-$Mg^{2+})$-ATPase are likely to be affected. Thus Murphy [44] has demonstrated the presence of arginyl residues on the ATPase whose modification affects activity. Fig. 7 shows that the ATPase activity of SR vesicles is reduced by modification with acetic anhydride. Since the effect of acetic anhydride is largely prevented by the presence of ATP (Fig. 7) it is likely that the sites whose modification affects activity are at the ATP binding site. Similar effects were seen for acetic anhydride modification of the purified ATPase (data not shown). Phenylglyoxal was also observed to inhibit ATPase activity, with some protection being provided by ATP (data not shown).

Effects of modification of SR vesicles with acetic anhydride on maximal levels of accumulation of Ca^{2+} are less marked than effects on ATPase activity. Thus modification with 0.4 mM acetic anhydride leads to a reduction in ATPase activity to 40% of control, but the maximal level of accumulation of Ca^{2+} is only reduced to 80% of control (data not shown, but see Fig. 8); levels of accumulation of Ca^{2+} measured at greater times after initiation of uptake are more affected by modification, suggesting an effect of modification on leak of Ca^{2+} from the vesicles (data not shown). Effects on the slow phase of accumulation seen in the presence of P_i are, however, very marked, and the slow phase of uptake is almost abolished after 5 min modification with 0.4 mM acetic anhydride (Fig. 8). The presence of P_i during reaction with acetic anhydride protects against the inhibitory effect of acetic anhydride (Figs. 8 and 9). Although ATP (5 mM) alone does not reduce the effect of acetic anhydride on Ca^{2+} accumulation, the presence of both 5 mM ATP and 50 mM P_i provides almost complete protection against modification with 0.4 mM acetic anhydride, although inhibition is still observed at higher concentrations of acetic anhydride (Fig. 9). These results suggest the presence of lysine residues at the P_i binding site on the P_i transporter, and the presence of an ATP binding site on the transporter. As described in [45] there is evidence for ATP stimulation of the trans-

porter. Modification with acetic anhydride also decreases the magnitude of the slow phase of Ca^{2+} uptake seen in the presence of oxalate, and again the presence of ATP protects against this effect (data not shown).

Shoshan-Barmatz [46] also studied the effect of modification with acetic anhydride on Ca^{2+} accumulation by SR vesicles in the presence of P_i. Shoshan-Barmatz [46] observed an inhibition of accumulation of Ca^{2+}, as observed here, but attributed it to an effect on a Ca^{2+} channel in the membrane, increasing the permeability of the membrane to Ca^{2+}. Although an effect on the rate of Ca^{2+} efflux from SR vesicles is likely (see above), the major effect of modification with acetic anhydride on accumulation of Ca^{2+} in the presence of P_i is likely to follow from the effects on the P_i transporter described above.

Modification of SR with phenylglyoxal also inhibits the slow phase of uptake seen in the presence of P_i (Fig. 10). In this case, however, the presence of 50 mM P_i during modification provides little protection, although 5 mM ATP does reduce inhibition very markedly. This argues again for an ATP binding site on the P_i transporter, the binding site including at least one arginine residue.

Acknowledgement

We thank the SERC for financial support.

References

1 De Meis, L. (1981) The Sarcoplasmic Reticulum, John Wiley, New York.
2 Ostwald, T.J. and MacLennan, D.H. (1974) J. Biol. Chem. 249, 5867–5871.
3 Meissner, G. (1975) Biochim. Biophys. Acta 389, 51–68.
4 Fliegel, L., Ohnishi, M., Carpenter, M.R., Khanna, V.K., Reithmeier, R.A.F. and MacLennan, D.H. (1987) Proc. Natl. Acad. Sci. USA 84, 1167–1171.
5 Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518–528.
6 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648–658.
7 Hasselbach, W. (1964) Prog. Biophys. Mol. Biol. 14, 169–222.
8 De Meis, L., Hasselbach, W. and Machado, R.D. (1974) J. Cell. Biol. 62, 505–509.
9 Hasselbach, W. and Weber, H.H. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G.F., Klingenberg, M.E. and Siliprandi, N., eds.), pp. 103–111, North-Holland Publishing Company, Amsterdam.
10 Knowles, A. and Racker, E. (1975) J. Biol Chem. 250, 3538–3544.
11 Sorenson, M.M. and De Meis, L. (1977) Biochim. Biophys. Acta 465, 210–223.
12 Chu, A., Tate, C.A., Bick, R.J., Van Winkle, W.B. and Entman, M.L. (1983) J. Biol. Chem. 258, 1656–1664.
13 Chu, A., Bick, R.J., Tate, C.A., Van Winkle, W.B. and Entman, M.L. (1983) J. Biol. Chem. 258, 10543–10550.
14 Meissner, G. (1986) in Sarcoplasmic Reticulum in Muscle Physiology, Vol. 2 (Entman, M.L. and Van Winkle, W.B., eds.), pp. 21–29, CRC Press, Boca Raton.
15 Tanifjui, M., Sokabe, M. and Kasai, M. (1987) J. Membr. Biol. 99, 103–111.
16 Rousseau, E., Roberson, M. and Meissner, G. (1988) Eur. Biophys. J. 16, 143–151.
17 Kasai, M. and Kometani, T. (1979) Biochim. Biophys. Acta 557, 243–247.
18 Kasai, M. (1981) J. Biochem. (Tokyo) 89, 943–953.
19 Kasai, M. and Taguchi, T. (1981) Biochim. Biophys. Acta 644, 213–219.
20 Yamamoto, N. and Kasai, M. (1981) J. Biochem. (Tokyo) 89, 1521–1531.
21 Campbell, K.P. and MacLennan, D.H. (1980) Ann. N.Y. Acad. Sci. 358, 328–331.
22 MacLennan, D.H., Klip, A., Reithmeier, R., Michalak, M. and Campbell, K.P. (1979) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.), pp. 255–266, Addison-Wesley, New York.
23 Leberer, E., Charuk, J.H.M., Clarke, D.M., Green, N.M., Zubrzycka-Gaarn, E. and MacLennan, D.H. (1989) J. Biol. Chem. 264, 3484–3493.
24 Carafoli, E., Niggli, V., Malmstrom, K. and Caroni, P. (1980) Ann. N.Y. Acad. Sci. 356, 258–266.
25 Van der Kloot, W. (1969) Science 164, 1294–1295.
26 Szczepanska-Konkel, M., Yusufi, A.N.K., VanScoy, M., Webster, S.K. and Dousa, T.P. (1986) J. Biol. Chem. 261, 6375–6383.
27 Loghman-Adham, M., Szczepanska-Konkel, M., Yusufi, A.N.K., VanScoy, M. and Dousa, T.P. (1987) Am. J. Physiol. 252, G244–G249.
28 Zaki, L. (1983) Biochem. Biophys. Res. Commun. 110, 616–624.
29 Falke, J.J. and Chan, S.I. (1984) Biophys. J. 45, 91–92.
30 McWhirter, J.M., Gould, G.W., East, J.M. and Lee, A.G. (1987) Biochem. J. 245, 731–738.
31 East, J.M. and Lee, A.G. (1982) Biochemistry 21, 4144–4151.
32 Hardwicke, P.M.D. and Green, N.M. (1974) Eur. J. Biochem. 42, 183–193.
33 Weber, A., Herz, R. and Reiss, I. (1964) Fed. Proc. Fed. Am. Soc. Exp. Biol. 23, 896–900.
34 Mermier, P. and Hasselbach, W. (1976) Eur. J. Biochem. 64, 613–620.
35 Heubel, P.H.C. and Popov, A.I. (1979) J. Solution Chem. 8, 615–625.
36 Farmer, R.M., Heubel, P.H.C. and Popov, A.I. (1981) J. Solut. Chem. 10, 523–532.
37 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758.
38 Tate, C.A., Chu, A., McMillin-Wood, J., Van Winkle, W.B. and Entman, M.L. (1981) J. Biol. Chem. 256, 2934–2939.
39 Carley, W.W. and Racker, E. (1982) Biochim. Biophys. Acta 680, 187–193.
40 Oberg, B. (1983) Pharmacol. Ther. 19, 387–415.
41 Gould, G.W., McWhirter, J.M., East, J.M. and Lee, A.G. (1987) Biochem. J. 245, 739–749.
42 Takahashi, K. (1968) J. Biol. Chem. 243, 6171–6179.
43 Glazer, A.N. (1976) in The Proteins (Neurath, H. and Hill, R.L., eds.), 3rd Edn., Vol. 2, pp. 1–103, Academic Press, New York.
44 Murphy, A.J. (1976) Biochem. Biophys. Res. Commun. 70, 1048–1054.
45 Stefanova, H.I., Jane, S.D., East, J.M. and Lee, A.G. (1991) Biochim. Biophys. Acta 1064, 329–334.
46 Shoshan-Barmatz, V. (1986) Biochem. J. 240, 509–517.