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THE MECHANISM BY WHICH QUININE INHIBITS THE Ca^{2+} TRANSPORT OF SARCOPLASMIC RETICULUM

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Abstract—The effect of quinine on Ca^{2+} transport was investigated in sarcoplasmic reticulum vesicles isolated from skeletal muscle. Both the initial velocity of Ca^{2+} transport and the steady state level of Ca^{2+} accumulation were decreased by quinine. This inhibition varied with the free Ca^{2+} concentration in the assay medium. The rates of ATP $\rightleftharpoons$ P_i exchange, Ca^{2+} efflux coupled with ATP synthesis and the degree of enzyme phosphorylation by P_i were decreased by quinine. In presence of 3 mM quinine, the Ca^{2+} concentration required to attain 50 per cent maximal membrane phosphorylation by ATP, ATP hydrolysis and ATP $\rightleftharpoons$ P_i exchange in each case increased by approximately 2-fold. ATPase inhibition by excess Ca^{2+} and the Ca^{2+} inhibitory effect on membrane phosphorylation by P_i were both attenuated by quinine.

Sarcoplasmic reticulum vesicles isolated from skeletal muscle homogenates retain a membrane-bound ATPase which is highly sensitive to the free Ca^{2+} concentration on each side of the membrane [1–7]. This ATPase can either promote the accumulation of Ca^{2+} by the vesicles at the expense of ATP hydrolysis or, in vesicles previously loaded with Ca^{2+}, promote a fast release of Ca^{2+} coupled with ATP synthesis from ADP and P_i [8–12]. Depending on the experimental conditions used, the transport ATPase (E) is phosphorylated either by the γ-phosphate of ATP or by orthophosphate [1, 5, 6, 12–19]. This phosphoprotein (E-P) represents an intermediate product in the sequence of reactions leading to either ATP hydrolysis and Ca^{2+} accumulation or, alternatively, to ATP synthesis and Ca^{2+} release. Under certain experimental conditions, the Ca^{2+} transport enzyme is also able to catalyze an ATP $\rightleftharpoons$ P_i exchange [4, 8, 20]. This exchange has been shown to be the result of the Ca^{2+} transport enzyme operating simultaneously forward (ATP hydrolysis) and backward (ATP synthesis).

The active Ca^{2+} transport of the sarcoplasmic reticulum plays a key role in the process of skeletal muscle contraction and relaxation [1, 2]. Quinine and its optical isomer quinidine potentiate twitch tension and at higher concentrations cause contracture of skeletal muscle [21–23]. These alkaloids have been shown to inhibit the active Ca^{2+} transport of isolated sarcoplasmic reticulum vesicles [24–29].

In this work we investigated further the effect of quinine on Ca^{2+} accumulation and release by sarcoplasmic reticulum vesicles as well as its effect on ATP hydrolysis, ATP synthesis, ATP $\rightleftharpoons$ P_i exchange and membrane phosphorylation by both ATP and orthophosphate.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles were prepared as described elsewhere [30]. Leaky vesicles were prepared by incubating intact vesicles at room temperature in 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid) at pH 9.0 for 20 min followed by readjustment of the pH to 7.0 with Tris–maleate buffer [31]. After this treatment, although the ATPase activity is maintained, the Ca^{2+} permeability of the membrane is increased and the vesicles are no longer able to accumulate Ca^{2+}.

Ca^{2+} uptake. Ca^{2+} uptake by intact vesicles was measured with ^{45}Ca using Millipore filters [30].

Membrane phosphorylation. Membrane phosphorylation from either [γ-^{32}P]ATP or $^{32}P_i$ was measured as previously described and corrected for non-specific binding [12].

ATPase activity. ATPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP. After precipitation of the protein with 1.5 vol. TCA (10% w/v), the $^{32}P_i$ was extracted as phosphomolybdate complex using a mixture of isobutyl alcohol and benzene [4, 32].

Two different ATPase activities can be distinguished in sarcoplasmic reticulum vesicles. The Mg^{2+}-dependent ATPase requires only Mg^{2+} for its activation and is measured in the presence of EGTA to remove contaminating Ca^{2+}. The ATPase which is correlated with Ca^{2+} transport [1] is Ca^{2+}-activated and requires Ca^{2+} and Mg^{2+} for full activity. It is calculated by subtracting the Mg^{2+}-dependent activity from the total activity measured in presence of Mg^{2+} and Ca^{2+}.

ATP $\rightleftharpoons$ P_i exchange. ATP $\rightleftharpoons$ P_i exchange was determined by measuring [γ-^{32}P]ATP formed from $^{32}P_i$. After precipitation of the protein with TCA (10%, w/v), the $^{32}P_i$ present in the aqueous phase was extracted as a phosphomolybdate complex as described above [4, 32].

Ca^{2+} release. Ca^{2+} release was assayed by following the appearance of ^{45}Ca in the medium. Vesicles were preloaded with Ca by incubation in a medium containing 20 mM Tris–maleate buffer (pH 7.0), 1 mM $^{45}CaCl_2$, 0.9 mM EGTA, 3 mM ATP, 5 mM $MgCl_2$, 0.6 to 0.7 mg of vesicle protein/ml and either 20 mM

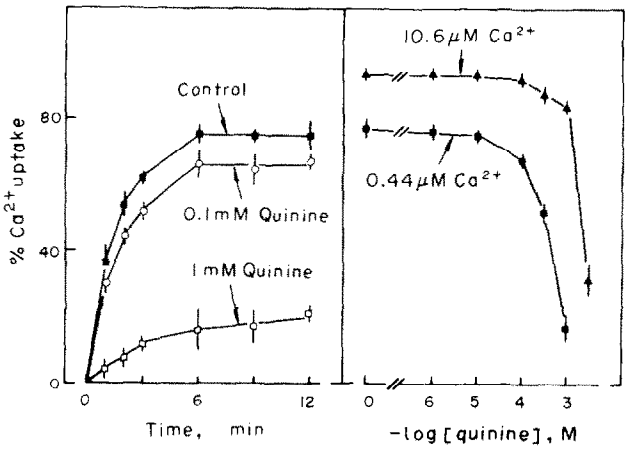


Fig. 1. Effect of quinine on the kinetics of Ca^{2+} uptake by SR vesicles. Left panel: the assay medium contained 2 mM ATP, 2 mM $MgCl_2$, 4 mM potassium oxalate, 20 mM Tris-maleate buffer (pH 7.0), 2 mM EGTA, 0.2 mM $^{45}CaCl_2$ and no (■), 0.1 mM (○) or 1.0 mM quinine (□). Each point represents the mean ± S. E. of three experiments. Right panel: same assay medium except that the EGTA concentration was 0.26 mM (▲) or 2 mM (■). The calculated initial free Ca^{2+} concentrations were, respectively, 10.6 and 0.44 μM. The incubation time was 12 min. The values represent the average ± S. E. of three experiments.

P_i or 4 mM potassium oxalate for 10 min. After centrifugation at 18,000 *g* for 20 min, the pellet was suspended in 0.1 mM KCl and used immediately. Two kinds of efflux can be distinguished in sarcoplasmic reticulum vesicles. The passive efflux is that measured in the absence of ADP and the active efflux that which is activated by the simultaneous addition of ADP and P_i [1, 8–10].

ATP synthesis. ATP synthesis was assayed by measuring the formation of glucose 6-^{32}P from $^{32}P_i$. The excess $^{32}P_i$ was extracted from the medium as phosphomolybdate with isobutyl alcohol–benzene [4, 32].

$^{32}P_i$. $^{32}P_i$ obtained from the Brazilian Institute of Atomic Energy was purified by extraction as the phosphomolybdate with isobutyl alcohol–benzene, re-extraction to the aqueous phase with ammonium hydroxide and precipitated as $MgNH_4PO_4$ [33].

[γ-^{32}P]ATP. [γ-^{32}P]ATP was prepared as previously described [19].

RESULTS

Ca^{2+} uptake and release. Quinine decreased both the initial rate of Ca^{2+} uptake and the amount of Ca^{2+} removed from the medium by the vesicles after a prolonged incubation (Fig. 1, left panel). This inhibitory effect of quinine was found to vary with the Ca^{2+} concentration of the assay medium (Fig. 1, right panel), the inhibition being much greater at lower initial Ca^{2+} concentration of the medium. In these experiments, the free Ca^{2+} concentration of the medium was calculated using the value of 3.95×10^{-6} M for the Ca–EGTA dissociation constant [34].

Addition of quinine to the assay medium after the vesicles had removed most of the Ca^{2+} from the medium resulted in a slow Ca^{2+} efflux (Fig. 2, left panel). The amount of Ca^{2+} released by the vesicles varied with the Ca^{2+} concentration of the medium. When the Ca^{2+} concentration was maintained below 10^{-6} M by the inclusion of EGTA in the incubation medium, more than 50 per cent of the calcium accumulated by the vesicles was slowly released (Fig. 2, left panel). In this condition, the free Ca^{2+} concentration in the medium increased from 0.08 to only about 0.25 μM. When EGTA was omitted, although a smaller percentage of calcium was released (Fig. 2, right panel), the Ca^{2+} concentration of the medium increased from 0.5 to about 2 to 4 μM.

ATP hydrolysis. Leaky vesicles were used for these experiments in order to prevent the inhibition of the Ca^{2+}-activated ATPase activity caused by the accumulation of Ca^{2+} inside the vesicles [1–4, 7, 31, 32, 35]. In the presence of quinine, the

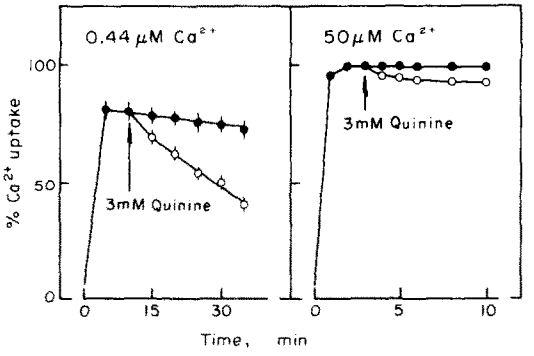


Fig. 2. Effect of quinine on the release of Ca^{2+} from vesicles. Left panel: vesicles were incubated in an assay medium identical to that described in Fig. 1. The initial free Ca^{2+} concentration was 0.44 μM. After a 10-min incubation 3 mM quinine was added to the medium. Each value represents the average ± S. E. of four experiments. Right panel: vesicles (0.3 mg protein/ml) were incubated in a medium containing 20 mM Tris-maleate buffer (pH 7.0), 15 mM $MgCl_2$, 10 mM P_i, 5 mM ATP and 50 μM $^{45}CaCl_2$. After a 3-min incubation 3 mM quinine was added to the medium. Each point is the average ± S. E. of three experiments. Key: (●) control; (○) quinine, to a final concentration of 3 mM, was added as indicated by an arrow.

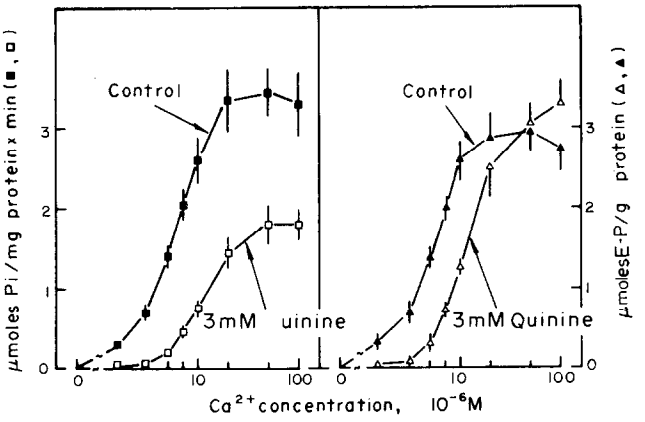


Fig. 3. Ca^{2+}-dependent inhibitory effect of quinine on ATPase activity and E-P formation. The assay medium contained 20 mM Tris–maleate buffer (pH 7.0), 5 mM $MgCl_2$, 1.0 mM [γ-^{32}P]ATP, 0.66 mg of leaky vesicle protein/ml, 0.2 mM $CaCl_2$ and a different EGTA concentration (1.0 to 0.1 mM) to obtain the indicated free Ca^{2+} concentrations. Zero Ca^{2+} refers to the addition of 1 mM EGTA and no $CaCl_2$. Key: control, without quinine (▲, ■); plus 3 mM quinine (△, □). Incubation time was 10 sec at 30°. Each value represents the average ± S. E. of five experiments.

Ca^{2+} concentration required for half-maximal activation of both the initial rate of ATP hydrolysis and membrane phosphorylation by ATP (steady state level) increased from 5 to 10 μM (Fig. 3). In presence of 50–100 μM $CaCl_2$, the inhibition of the membrane phosphorylation was abolished, but the Ca^{2+}-activated ATPase activity remained impaired.

Table 1 shows that quinine also inhibits the Mg^{2+}-dependent ATPase activity.

Ca^{2+} efflux and ATP synthesis. When vesicles loaded with either calcium oxalate or calcium phosphate are incubated in a medium containing EGTA, a steady efflux of Ca^{2+} can be measured [1, 10]. This represents a passive efflux and its rate is determined by the free Ca^{2+} concentration inside the vesicles. In the pH range of 6.0 to 7.0, the solubility of calcium phosphate is higher than that of calcium oxalate [9]. This could account for the different rates of passive Ca^{2+} efflux shown in Table 2. Upon addition of ADP and P_i to the medium, the rate of Ca^{2+} efflux is increased. Makinose and Hasselbach [1, 9, 10] have shown that the increment of Ca^{2+} efflux is coupled with the synthesis of ATP. Table 2 shows that quinine decreased both the Ca^{2+} efflux and ATP synthesis measured in the presence of ADP and P_i.

Membrane phosphorylation by P_i. ATP synthesis is initiated by the phosphorylation of the transport ATPase by orthophosphate [12, 14, 36, 37]. This reaction is inhibited by the addition of Ca^{2+} to the assay medium [12, 14, 18, 36, 37]. Quinine was found to in-

Table 1. Effect of quinine on Mg^{2+}-dependent ATPase activity*

Additions to assay medium	P_i (μmoles/mg protein/min)
None	0.061 ± 0.008 (9)
Quinine (3 mM)	0.033 ± 0.002 (9)

* The assay medium contained 20 mM Tris–maleate buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 1 mM EGTA and 0.66 mg of leaky vesicle protein/ml (five experiments) or alternatively 20 mM Tris–maleate buffer (pH 7.0), 15 mM $MgCl_2$, 5 mM [γ-^{32}P]ATP, 10 mM P_i, 1 mM EGTA and 0.3 mg of leaky vesicle protein/ml (four experiments). Incubation time was 10 sec in the first case and 5 min in the second one. Essentially the same results were obtained with the use of these two different incubation media. Therefore, all the values were pooled and are given as the mean ± S. E. of nine experiments. Other experimental conditions were as described in Materials and Methods.

Table 2. Effect of quinine on Ca^{2+} efflux and ATP synthesis*

Precipitating anion	Addition to assay medium	Ca^{2+} efflux (nmoles/mg protein/min)		ATP synthesis (nmoles/mg protein/min)
		Without ADP	With ADP (0.2 mM)	
Oxalate (4 mM)	None	20 ± 2	122 ± 31	42 ± 4
	Quinine (3 mM)	16 ± 1	53 ± 19	18 ± 5
Phosphate (20 mM)	None	36 ± 1	480 ± 80	
	Quinine (3 mM)	51 ± 3	288 ± 35	

* SR vesicles were preloaded as described in Materials and Methods using P_i or oxalate as the precipitating anion. Preloaded vesicles were added to a medium containing 20 mM Tris–maleate buffer (pH 7.0), 20 mM $MgCl_2$, 10 mM P_i, 10 mM AMP, 100 mM glucose, 15 mM EGTA, 6 units hexokinase/ml with or without 0.2 mM ADP. Final protein concentration was 0.3 to 0.4 mg/ml. For Ca^{2+} release, $^{45}CaCl_2$ and non-radioactive P_i were used. For ATP synthesis, $^{32}P_i$ and non-radioactive $CaCl_2$ were used. The synthesis of ATP (in the presence of ADP) was measured as described in Materials and Methods. Excess of AMP was included in the medium in order to inhibit the formation of ATP catalyzed by traces of adenylate kinase, usually contaminants of the vesicle preparation. Each value is the average of four experiments.

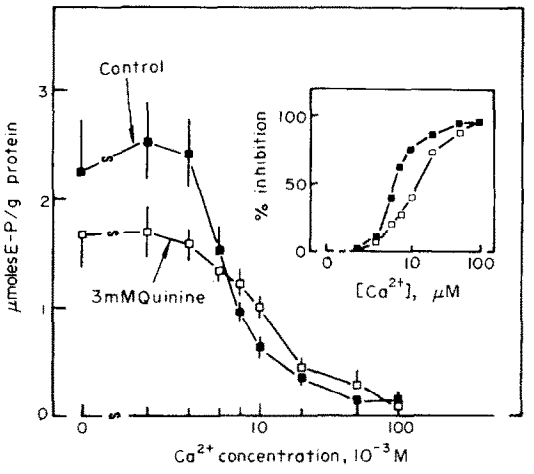


Fig. 4. Effect of quinine and Ca^{2+} on enzyme phosphorylation by P_i. The assay medium contained 20 mM Tris–maleate buffer (pH 7.0), 10 mM $MgCl_2$, 15 mM $^{32}P_i$, 0.66 mg of leaky vesicle protein, 0.2 mM $CaCl_2$ and different EGTA concentrations (0.1 to 1.0 mM) to obtain the indicated Ca^{2+} concentrations. Zero Ca^{2+} refers to the addition of 1 mM EGTA and no $CaCl_2$. Incubation time was 10 sec at 30°. Key: (■) control, without quinine; (□) plus 3 mM quinine. The insert shows the per cent of inhibition promoted by Ca^{2+}. For this calculation, the steady state level of phosphoenzyme measured in the absence of Ca^{2+} was computed as zero inhibition.

hibit the degree of membrane phosphorylation by P_i when measured in the presence of EGTA (Fig. 4). In the presence of quinine the membrane phosphorylation by P_i was less sensitive to Ca^{2+}. In the control experiment the Ca^{2+} concentration required to promote a 50 per cent decrease in the steady state level of phosphoenzyme was in the range of 5–6 μM, while in the presence of 3 mM quinine it was in the range of 10–15 μM (Fig. 4, inset).

ATP ⇌ P_i exchange. When sarcoplasmic reticulum vesicles were incubated in a medium containing ATP, Mg^{2+}, $^{32}P_i$ and Ca^{2+}, calcium phosphate is accumulated by the vesicles and a Ca^{2+} concentration gradient is built up until a steady state is reached in which a slow Ca^{2+} efflux is balanced by an ATP-driven influx. When this condition is reached, a steady rate of exchange between P_i and the γ-phosphate of ATP is observed [4, 8]. Figure 5 shows that quinine inhibits the rate of ATP ⇌ P_i exchange. When quinine was included in the assay medium before the addition of the vesicles, the maximal level of Ca^{2+} uptake was decreased and the rate of ATP ⇌ P_i exchange was sharply decreased (Fig. 5, left panel). When quinine was added after maximal Ca^{2+} accumulation had been reached, a small amount of the Ca^{2+} accumulated by the vesicles was released to the medium and the ATP ⇌ P_i exchange was inhibited (Fig. 5, right panel).

In previous studies [4, 32] it was shown that solubilized or leaky vesicles were still able to catalyze an ATP ⇌ P_i exchange provided that a Ca^{2+}-binding site of low affinity was saturated. The saturation of this site would concomitantly activate the rate of ATP ⇌ P_i exchange and inhibit the rate of ATP hydrolysis. This is shown in Fig. 6. No ATP ⇌ P_i exchange could be measured in the presence of 0.1 mM Ca^{2+} (Fig. 6, left panel). Raising the Ca^{2+} concentration of the medium resulted in a progressive activation of the ATP ⇌ P_i exchange reaction, half-maximal activation being attained at $CaCl_2$ concentrations in the range of 1.2 to 1.5 mM. Addition of quinine to the assay medium resulted in a modification of the Ca^{2+} dependence profile. The higher the quinine concentration, the higher the Ca^{2+} concentration required to activate the ATP ⇌ P_i exchange reaction. In the presence of 3 mM quinine, maximal activation was not reached even in the presence of 8 mM Ca^{2+}. Ca^{2+} concentrations higher than 8 mM were avoided in order to prevent the precipitation of calcium phosphate in the assay medium. Figure 6 (right panel) shows that the ATPase activity was progressively inhibited by raising the Ca^{2+} concentration from 0.5 to 8 mM. Quinine (3 mM) inhibited the ATPase activity, the inhibition being more pro-

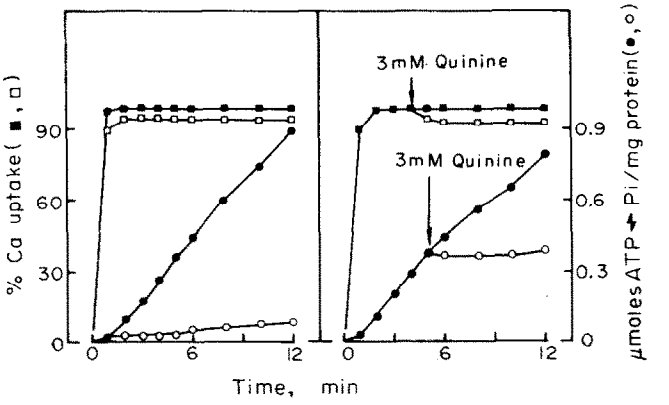


Fig. 5. Effect of quinine on Ca^{2+} uptake and ATP ⇌ P_i exchange. The incubation medium was the same described in Fig. 2, right panel. For Ca^{2+} uptake (■, □) $^{45}CaCl_2$ and non-radioactive P_i were used. For ATP ⇌ P_i exchange (●, ○), $^{32}P_i$ and non-radioactive $CaCl_2$ were used. Key: closed symbols (control, without quinine); open symbols (with quinine, final concn in the medium of 3 mM). The reactions were performed at 30°. The results show a typical experiment. Essentially the same results were obtained in three different vesicle preparations tested. Left panel: quinine included in the assay medium before the addition of the vesicles. Right panel: quinine added after Ca^{2+} accumulation as shown by arrow.

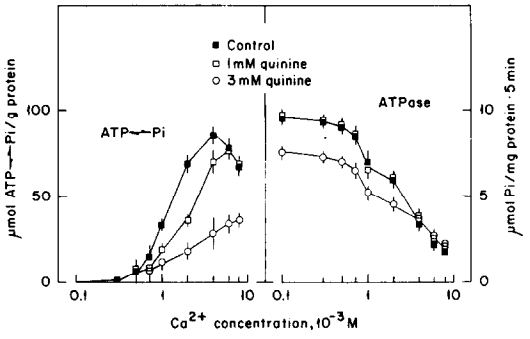


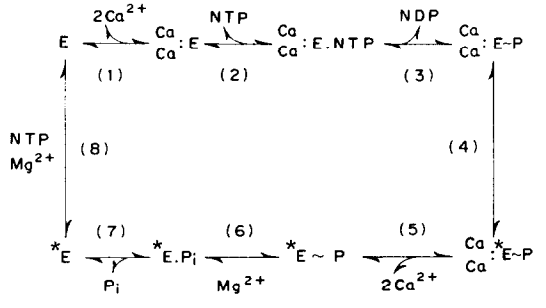
Fig. 6. Inhibition of the ATP $\rightleftharpoons$ P_i exchange and ATPase by quinine on leaky vesicles. The assay medium contained 20 mM Tris-maleate buffer (pH 7.0), 15 mM $MgCl_2$, 10 mM P_i, 5 mM ATP, 0.2 mg of leaky vesicle protein/ml and the specified $CaCl_2$ concentrations. The quinine concentrations were none (■), 1.5 mM (□) and 3.0 mM (○). Incubation time was 5 min at 30°. For the ATP $\rightleftharpoons$ P_i exchange (left panel), $^{32}P_i$ and non-radioactive ATP were used. For ATPase (right panel), [γ-^{32}P]ATP and non-radioactive P_i were used. Each value is the mean S. E. of five experiments.

nounced in the lower Ca^{2+} concentration range and abolished when the Ca^{2+} concentration of the medium was raised to the range of 4–8 mM.

DISCUSSION

Several authors have already reported that quinine and its optical isomer quinidine inhibit the Ca^{2+} transport and ATPase activities of sarcoplasmic reticulum vesicles. Worsfold and Peter [26] and Balzer [25], using vesicles isolated from skeletal muscle, have presented evidence that quinine and quinidine are competitive inhibitors of Ca^{2+} transport. Pang and Briggs [27], using vesicles isolated from cardiac muscle, have shown that quinidine does not change the E-P level but inhibits the hydrolysis of the phosphoprotein formed by ATP. This paper shows that, depending on the experimental conditions used, these two effects of quinine can be detected. Balzer has shown that quinidine also inhibits the Ca^{2+} efflux coupled with ATP synthesis and further attenuates the inhibition of the ATPase activity promoted by excess of Ca^{2+}. In this paper these findings of Balzer were confirmed. In addition it was shown that quinine also inhibits the ATP $\rightleftharpoons$ P_i exchange reaction and the phosphorylation of the transport enzyme by P_i. The degree of inhibition of these activities depends on the Ca^{2+} concentration on each side of the membrane.

Reaction sequence. On the basis of accumulated evidence [4, 7, 12, 13, 18, 32, 37–41] the following reaction sequence was recently proposed [41].



In this reaction sequence the transport enzyme is represented in two different conformations, E and *E. In the E form, the site which translocates Ca^{2+} through the membrane faces outward from the membrane and has high affinity for Ca^{2+}; E can be phosphorylated by ATP (reaction 3) but not by P_i. In the *E conformation, the Ca^{2+}-binding site faces inward from the membrane and has low affinity for Ca^{2+}. The transport enzyme in this conformation is no longer phosphorylated by ATP but can be phosphorylated by P_i (reaction 6). ATP, besides phosphorylating the enzyme, can also activate to different extents the rate of interconversion of *E to E (reaction 8). According to this reaction sequence in the presence of suitable concentrations of P_i, ATP, ADP and Mg^{2+}, the synthesis or hydrolysis of ATP is regulated by the binding of Ca^{2+} to the enzyme. When Ca^{2+} binds only on the outer site of the membrane, the reaction sequence is directed from reaction 1 to reaction 7, leading to Ca^{2+} accumulation (reaction 5) and ATP hydrolysis. Under these conditions, although small amounts of the enzyme can be phosphorylated by P_i (reaction 6), the reaction sequence does not proceed backward because the Ca^{2+} concentration is not sufficient to saturate the site of low affinity (reaction 5). When vesicles previously loaded with Ca^{2+} are incubated in a medium containing EGTA (Table 2), Ca^{2+} binds only to the inner site of low affinity (reaction 5), the enzyme is not phosphorylated by ATP (reactions 1–3), and the cycle flows backward from reaction 7 to 1, leading to ATP synthesis and Ca^{2+} release. When both sites are saturated (Fig. 6), the transport enzyme will simultaneously catalyze the hydrolysis and the synthesis of ATP (ATP $\rightleftharpoons$ P_i exchange).

Effect of quinine on membrane phosphorylation and ATP synthesis or hydrolysis. According to this reaction sequence, quinine inhibits reaction 6 and decreases the apparent affinity of Ca^{2+} for both the site of high affinity and of low affinity. The binding of Ca^{2+} to the external site of high affinity (reaction 1) simultaneously activates the enzyme phosphorylation by ATP and inhibits its phosphorylation by P_i [12, 18, 37, 39]. In the absence of Ca^{2+} and ATP (Fig.

4) the reaction sequence is interrupted and the steady state level of enzyme phosphorylated by P_i will depend solely on the equilibrium of reactions 6–8. The addition of Ca^{2+} in the μM concentration range results in the formation of the enzymatic form 2Ca-E (reaction 1), which is not phosphorylated by P_i, leading to a decrease in the steady state level of phosphoenzyme [12, 39]. In accordance with this reasoning, Figs. 3 and 4 show that, upon the addition of quinine (3 mM), the Ca^{2+} concentration required for half-maximal enzyme phosphorylation by ATP increases from 5 to 10 μM and that the Ca^{2+} concentration required to promote half-maximal inhibition of membrane phosphorylation by P_i also increases from 6 to 12 μM.

In presence of 0.1 mM $CaCl_2$, quinine inhibits the ATPase activity (Figs. 3 and 6) but the level of phosphoenzyme formed by ATP is not modified (Fig. 3), indicating that at this Ca^{2+} concentration the effect of quinine on reaction 1 is overcome and the inhibition of the ATPase activity appears to be caused by a direct effect of quinine on the hydrolysis of the phosphoenzyme (reaction 6). In the reaction sequence proposed, the enzyme is phosphorylated by P_i through the reversal of reaction 6. Accordingly, Fig. 4 shows that, in the presence of EGTA, quinine also decreases the degree of enzyme phosphorylation by P_i.

The apparent affinity of the internal binding site (*E) for Ca^{2+} (reaction 5) cannot be measured in experiments similar to those described in Table 2 and Fig. 5 due to the difficulty in estimating the Ca^{2+} concentration inside the vesicles. This can, however, be indirectly estimated (Fig. 6) by using leaky vesicles for measuring both the activation of the $ATP \rightleftharpoons P_i$ exchange and the inhibition by excess Ca^{2+} of the Ca^{2+}-activated ATPase [4, 32]. In previous reports it was shown that for $ATP \rightleftharpoons P_i$ exchange to occur, the enzyme phosphorylated by P_i (reaction 6) is only able to transfer its phosphate to ADP when the inner Ca^{2+}-binding site is saturated [32, 37]. The saturation of this site, by driving reaction 5 to the right, will also inhibit the Ca^{2+}-activated ATPase. This experimental approach revealed that quinine also decreases the apparent affinity of the inner Ca^{2+}-binding site (Fig. 6). The lack of effect of quinine on the ATPase activity at higher Ca^{2+} concentrations (Fig. 6) is probably the result of the double effect of quinine in reactions 5 and 6. Although quinine impairs the hydrolysis of the phosphoenzyme, it also decreases the apparent affinity of the inner Ca^{2+}-binding site, thus reducing the inhibitory effect of higher Ca^{2+}.

The inhibition of Ca^{2+} efflux and ATP synthesis by quinine (Table 2) can be accounted for by the effect of quinine on both reaction 6, where it impairs enzyme phosphorylation by P_i in presence of EGTA (Fig. 4), and on reaction 5, where it decreases the apparent affinity of the inner Ca^{2+}-binding site, thus interfering with the transfer of the phosphate from the phosphoenzyme to ADP.

At present we do not know why quinine inhibits the Mg^{2+}-dependent ATPase.

Effect of quinine on Ca^{2+} *accumulation.* In the experimental conditions described in Figs. 1 and 2, the vesicles accumulate Ca^{2+} until the concentration of Ca^{2+} in the assay medium decreases to a level just sufficient to activate the transport of Ca^{2+} at a rate which equals the Ca^{2+} efflux resulting from passive diffusion. In the experiments of Fig. 2 the addition of quinine to the assay medium at equilibrium resulted in a net Ca^{2+} efflux due to the inhibition of the Ca^{2+} transport ATPase (Fig. 3). This persisted until the Ca^{2+} concentration in the assay medium increased to a level sufficiently high to activate the Ca^{2+} transport ATPase to a rate fast enough to equal the rate of Ca^{2+} efflux.

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