Interaction of an Aromatic Dibromoisothiouronium Derivative with the Ca^{2+}-ATPase of Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: Isothiouronium compounds [Hoving, S., Bar-Shimon, M., Tijmes, J. J., Goldshleger, R., Tal, D. M., and Karlish, S. J. (1995) *J. Biol. Chem. 270,* 29788−29793] act as high-affinity competitive antagonists for Na^+ and K^+ (Rb^+) on the renal Na^+/K^+-ATPase where they favor the E1 conformation. We have now characterized the effects of 1,3-dibromo-2,4,6-tris(methylisothiouronium)benzene (Br_2-TITU) on the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum. Br_2-TITU inhibited the Ca^{2+}-ATPase, both transport and catalytic activity, with a $K_{0.5}$ of 5−15 μM. Maximum inhibition was at 10 min with $t_{0.5}$ of 3−5 min. Br_2-TITU, 100 μM, quenched Trp autofluorescence by 80%, but the residual signal still responded to Ca^{2+} binding. Maximum quenching of fluorescence was at pH 9.0. Total E-P levels, during the steady state of turnover of the Ca^{2+}-ATPase, were increased from 0.5 to 5.8 nmol·mg^{-1} by Br_2-TITU at pH 6.8. Trinitrophenyl-ATP (TNP-ATP) superfluorescence, which monitors hydrophobicity of the ATP site, was increased 3−4-fold, suggesting that Br_2-TITU favors an "E2"-like state. Fluorescence was also increased 3−5-fold when E-P was induced with P_i plus EGTA. Br_2-TITU increased the rate constants of induction of superfluorescence with ATP plus Ca^{2+} from 0.32 to 0.69 s^{-1} and with P_i plus EGTA from 0.84 to 7.45 s^{-1}. Br_2-TITU also decreased rate constants for "off" reactions from 2.9 to 0.66 s^{-1} and from 10.9 to 0.73 s^{-1} for the ATP and P_i reactions, respectively. Br_2-TITU, which competitively inhibits the Na^+/K^+-ATPase, has a novel effect on the Ca^{2+}-ATPase. It promotes accumulation of E2-P species due to increased rate of formation and decreased rate of hydrolysis and quenches tryptophan autofluorescence. Br_2-TITU could be a useful inhibitor to probe intermediate reactions of the Ca^{2+}-ATPase that link catalysis with Ca^{2+} translocation.

P-type cation pumps belong to a large family of prokaryotic and eukaryotic ATPases that transport cations and include an intermediate phosphorylation step (*1*−*3*). Members of the family are divided into type I that transport heavy metals and type II that transport H^+, Na^+, K^+, and Ca^{2+} (*4*). These pumps have many features in common that give confidence to models of energy transduction, based on common properties. Understanding of mechanisms of transport has been facilitated by availability of specific probes, fluorescent and radioactive. In particular, ouabain and thapsigargin (*5*) have been used as high-affinity specific inhibitors of the Na^+/K^+-ATPase and Ca^{2+}-ATPase, respectively. In contrast to their catalytic cycles, where there is general consensus regarding reaction intermediates, relatively little is known about the topography of binding sites and the discrete steps that participate in unidirectionally coupled cation movements across the membrane.

A simplified reaction cycle for the Ca^{2+}-ATPase is shown in Scheme 1. Two global conformations, E1 and E2,[1] alternate between high Ca^{2+} affinity with cytosolic orientation and low Ca^{2+} affinity lumenal sites, respectively. The ATP binding site undergoes switching from hydrophilic to hydrophobic properties during the catalytic cycle (*2*) and is a key event in coupled turnover of the Ca^{2+} pump (*6*). The ATP binding pocket in intermediates E1-P·2Ca and E2-P·2Ca are ADP-sensitive and -insensitive, respectively. The ATP analogue, TNP-ATP, binds tightly to the Ca^{2+}-ATPase and is influenced by the state of hydration of the ATP binding site. This site is unique among cation pumps and becomes hydrophobic, causing the phenomenon of "superfluorescence" when the Ca^{2+}-ATPase is in the E2-P state (*7*−*9*) with severalfold increase in fluorescence. Autofluorescence

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[1] Abbreviations: Br_2-TITU, 1,3-dibromo-2,4,6-tris(methylisothiouronium)benzene; $[Ca^{2+}]_{lim}$, limiting concentration of medium or cytosolic free calcium ions; DMSO, dimethyl sulfoxide; E-P, total phosphorylated forms of the Ca^{2+}-ATPase; E1, conformation of the Ca^{2+}-ATPase with high-affinity Ca^{2+} sites facing the medium; E2, conformation of the Ca^{2+}-ATPase with low-affinity Ca^{2+} sites facing the lumen; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2-aminomethylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; F_{lim}, fluorescence of fluophore, Fluo-3, at limiting $[Ca^{2+}]$; F_{min} and F_{max}, fluorescence of Fluo-3 with excess EGTA and with saturating $[Ca^{2+}]$; NEM, *N*-ethylmaleimide; CAPS, 1-(cyclohexylamino)propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperzine-*N*′-3-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum.

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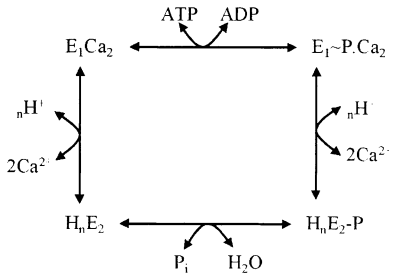
Scheme 1: Ca^{2+}-ATPase Catalytic Cycle



of tryptophans of the Ca^{2+}-ATPase is used to monitor E1 and E2 intermediates. Binding and release of Ca^{2+} causes a 5% increase and decrease, respectively.

There is ample evidence that H^+ is cotransported in the opposite direction to that of Ca^{2+} (*10*, *11*). Release of Ca^{2+} from E1-P·2Ca is followed by binding of H^+ from the lumenal surface, and binding of $2Ca^{2+}$ to E1 is linked to release of H^+ to the cytosolic surface (see Scheme 1). Charge translocation, following an ATP jump, is electrogenic, with $n < 4$ (*12*). Several analogues have been used as competitors of alkali metal cation binding. For example, ethylenediamine is a competitive analogue of monovalent cation binding by the Na^+/K^+-ATPase, with a dissociation constant of 50 μM (*13*). More recently, alkyl and aryl bisguanidinium derivatives have been shown to competitively block Na^+ and K^+ binding and occlusion (*14*). On the basis of the structure of amyloride, a widely used K^+-sparing diuretic for the treatment of hypertension, Karlish et al. (*15*) have synthesized a number of isothiouronium derivitives, which have so far been found to be the most potent competitive inhibitors with K_i values down to 0.32 μM. This study concerns possible effects of 1,3-dibromo-2,4,6-tris(methylisothiouronium)benzene (Br_2-TITU) on another member of type II cation pumps, the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum. The effects of this monocationic competitive inhibitor on transport and ATPase activity have been compared with its effects on quenching of ATPase tryptophans. Hydrophobicity of the ATP site has been monitored by TNP-ATP superfluorescence. No effects on cation binding were noted, but Br_2-TITU has unique effects related to contacts within the membrane phase as well as on solvent access to the cytoplasmically oriented ATP binding site. Br_2-TITU is a fairly specific inhibitor of transport and ATPase activity and quenches tryptophan fluorescence. Inhibition of catalysis is associated with increased superfluorescence. Generally, Br_2-TITU increases the rate of formation of the superfluorescent species and decreases its rate of decay. The site of Br_2-TITU inhibition is consistent with a block at the step involving hydrolysis of either E2-P·2Ca or E2-P.

MATERIALS AND METHODS

Materials. The sources of materials were as follows: ATP was from Sigma and amylase from Boehringer Mannheim. Standardized 100 mM $CaCl_2$ solution was prepared from Analar $CaCO_3$, adjusted to pH 5.6 with 1 M HCl. The pentaammonium salt of Fluo-3, lot number 2641-4, was obtained from Molecular Probes, Inc. (Eugene, OR). A 1 mM stock solution was made up in DMSO and kept at −20 °C in the dark. Br_2-TITU was synthesized according to Tal and Karlish (*16*). TNP-ATP was synthesized according to the method of Hiratsuka and Uchida (*17*) and standardized using $\epsilon_{408} = 26.4\ mM^{-1}\ cm^{-1}$ at pH 8.

Preparation of Skeletal Muscle Sarcoplasmic Reticulum Vesicles. Isolated sarcoplasmic reticulum vesicles were prepared from the back and hind leg muscle of white rabbits by the method of Champeil et al. (*18*). Amylase, 3 μg/mL, was added to the initial homogenate in order to decrease glycogen content and phosphorylase contamination to less than 5%, as determined by SDS−PAGE (*19*). Protein concentrations were determined from the optical absorbance at 280 nm in 50 mM sodium phosphate, pH 7.0, and 1% (w/v) sodium dodecyl sulfate (SDS) (*18*). Stock suspensions of SR vesicles, 35−40 mg/mL, were stored at −70 °C.

Determination of Steady-State Levels of Extravesicular $[Ca^{2+}]_{free}$ and of Ca^{2+} Uptake. The kinetics of calcium uptake, and of steady-state levels of extravesicular free $[Ca^{2+}]$, were monitored under standard conditions at 25 °C in medium containing 20 mM MOPS/Tris, pH 6.8, 5 mM $MgCl_2$, 5 mM sodium oxalate, and 20 nM Fluo-3. The final concentration of SR vesicles was maintained at 0.25 mg of protein/mL for all experiments. Fluorescence was recorded in a 1 cm cuvette with continuous magnetic stirring, using a SPEX Fluoromax spectrofluorometer, with excitation at 509 nm and emission at 535 nm. Maximum fluorescence, F_{max}, was established by preincubation with 20 μM $CaCl_2$, prior to addition of 2 mM ATP, unless otherwise stated. F_{min} was determined with standard buffer using 1 mM EGTA instead of Ca^{2+}. Free $[Ca^{2+}]$ was calculated from the observed fluorescence, F, according to the equation:

$$[Ca^{2+}]_{free} = K_d(F - F_{min})/(F_{max} - F) \qquad (1)$$

assuming a K_d for Ca^{2+} binding of 450 nM at 25 °C (*20*, *21*).

V_{max} of Ca^{2+} transport was measured by monitoring $[Ca^{2+}]_{free}$ following rapid injection of 100 μM $CaCl_2$ into the cuvette. The reciprocal of the time taken for $[Ca^{2+}]_{free}$ to return to the midpoint between F_{max} and F_{min} was used to calculate V_{max}, since during the major fraction of the timed period $[Ca^{2+}]_{free}$ would be near to saturation of high- affinity Ca^{2+} binding by the Ca^{2+}-ATPase.

Determination of Rates of Hydrolysis of ATP by the Ca^{2+}-ATPase. Rates of Ca^{2+}-dependent ATPase activity were determined by the NADH-coupled method. Medium, including 5 mM oxalate, was identical with that used for assay of Ca^{2+} transport. Owing to the relatively high rates of activity with 0.25 mg/mL ATPase, 0.5 mM NADH was used instead of 0.1 mM NADH, and the light path was reduced to 0.2 cm.

The coupled assay included 1 mM phosphoenolpyruvate and 5 units/mL each pyruvate kinase and lactate dehydrogenase. The reaction was started by addition of 200 μM ATP, following which "basal" levels were measured. Note that this is not truly basal, which is the observed rate with excess EGTA, but represents the rate at $[Ca^{2+}]_{lim}$ of 30−50 nM (*22*). Total ATPase activity was measured following addition of 20 or 100 μM Ca^{2+}. Slopes of the traces were measured at maximum reaction rate in the steady state.

Autofluorescence of Tryptophan Residues of the Ca^{2+}-ATPase. Autofluorescence of the Ca^{2+}-ATPase was determined under the same conditions as those for Ca^{2+} uptake, except that Fluo-3 was omitted, and excitation and emission

were set at 275 and 333 nm. Inner filter effects were minimal (*23*). Absorbance of free Br_2-TITU in the absence of SR was negligible at 333 nm, while at 275 nm it was 6000 M^{-1} cm^{-1}.

Determination of E-P Levels. E-P levels were determined at the steady state of hydrolysis of ATP under conditions identical to those for Ca^{2+}-ATPase activity, except that 2 mM ATP was substituted by 200 μM [γ-^{32}P]ATP (1000−1500 cpm/nmol). Following incubation at 25 °C for 100 s, aliquots, 0.5 mL, of incubation medium were quenched with 5 mL of 4% TCA and 4 mM H_3PO_4. Following incubation for 30 min on ice, solutions were filtered through Whatman GF/F fiberglass filters and washed with 3.5% TCA and 2 mM H_3PO_4. Radioactivity on the filters was assayed by liquid scintillation counting. Maximum E-P levels were determined as described previously (*19*) in 50 mM TES, pH 7.55, 50 mM KCl, and 5 mM $CaCl_2$. Blanks were included in which 1 mM EGTA was substituted for $CaCl_2$.

Superfluorescence of TNP-ATP by the Ca^{2+}-ATPase. Superfluorescence of TNP-ATP was recorded in the same medium that was used for Ca^{2+} transport assays, except that Fluo-3 was omitted and 2.5 μM TNP-ATP was included. Excitation was at 408 nm and emission at 520 nm. The sequence of additions was SR, 0.25 mg/mL, 20 μM Ca^{2+}, 2.5 μM TNP-ATP, and 200 μM ATP, when phosphorylation was induced in the forward reaction. Phosphorylation and superfluorescence from P_i were induced by additions of SR vesicles, 0.25 mg/mL, 1 mM EGTA, 2.5 μM TNP-ATP, and P_i in the range 0.1−10 mM in the final solution. Fluorescence was quenched with 5 mM Ca^{2+}.

Stopped-Flow Analysis of the Kinetics of TNP-ATP Superfluorescence Formation and Its Decay. Determination of the kinetics of superfluorescence of TNP-ATP, and of its induction and decay, was carried out in an Applied Photophysics spectrophotofluorometer, with excitation at 408 nm and slit widths of 2 mm. Emitted light was filtered with a GG 495 cutoff filter. Sequential stopped flow was performed, in which, following 1:1 mixing and programmed delay, EGTA was added from a third syringe and the mixture flushed into the flow cuvette. Fitting of data was initially monoexponential. Biexponential fits were applied if there was a systematic deviation of actual versus fitted values.

RESULTS

Inhibition of Ca^{2+} Transport and ATP Hydrolysis Activity of the Ca^{2+}-ATPase by Br_2-TITU. The effects of Br_2-TITU on Ca^{2+} transport by the Ca^{2+}-ATPase of SR were determined by pulsed additions of Ca^{2+} while in the $[Ca^{2+}]_{lim}$ state. Free $[Ca^{2+}]$ was followed by the Ca^{2+} fluophore, Fluo-3, which has a $K_{0.5}$ of approximately 500 nM that is similar to the $K_{0.5}$ of binding of Ca^{2+} to the transport sites, which has a $K_{0.5}$ of 1.6 μM. Rates of transport, in the presence of oxalate to precipitate intravesicular calcium oxalate, are conveniently monitored as the time for the fluophore fluorescence to reach 500 nM following pulsed additions of Ca^{2+} (20 or 100 μM). This method assumes that for >90% of the timed period rates are close to V_{max} of transport (*22*). It is especially precise at low inhibited rates of Ca^{2+} transport. Early experiments showed that micromolar concentrations of Br_2-TITU had a significant effect on peak widths (W), indicating inhibition of transport. A typical trace is shown in Figure 1A, where V_{max} was decreased by 80%. Transport rates (1/W) decreased

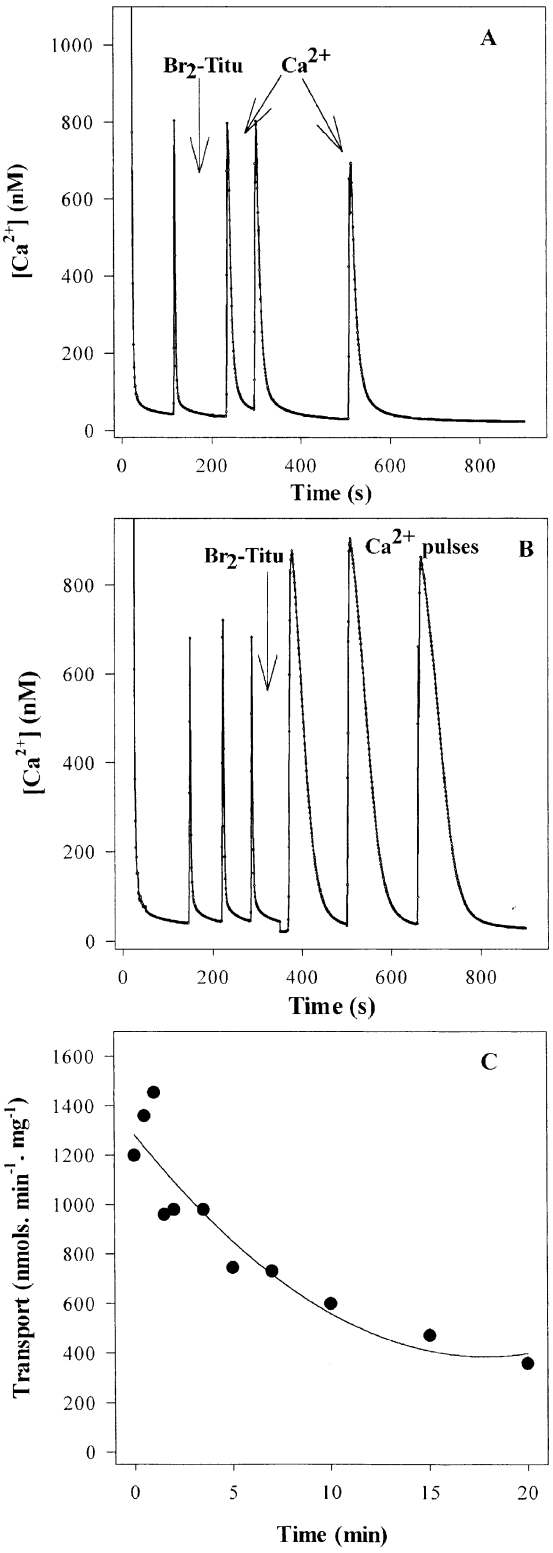


FIGURE 1: Effects of Br_2-TITU on Ca^{2+} transport by isolated SR vesicles. In (A) SR vesicles, 0.25 mg/mL, were incubated at 25 °C with medium containing 5 mM oxalate and 20 nM Fluo-3. ATP, 2 mM, was added at 10 s after which a pulse of 20 μM Ca^{2+} was added and then another three Ca^{2+} pulses (↓) after addition of 50 μM Br_2-TITU. Effects of preincubation with Br_2- TITU on transport are shown in (B). This was as described for panel A, except that after the first three Ca^{2+} pulses 50 μM Br_2-TITU was added, followed by a delay of 20 min, before the second three Ca^{2+} pulses were added. The effects of varying time of preincubation on inhibition of Ca^{2+} transport are shown in (C). Transport rates were calculated from Ca^{2+} pulse widths as described in Materials and Methods.

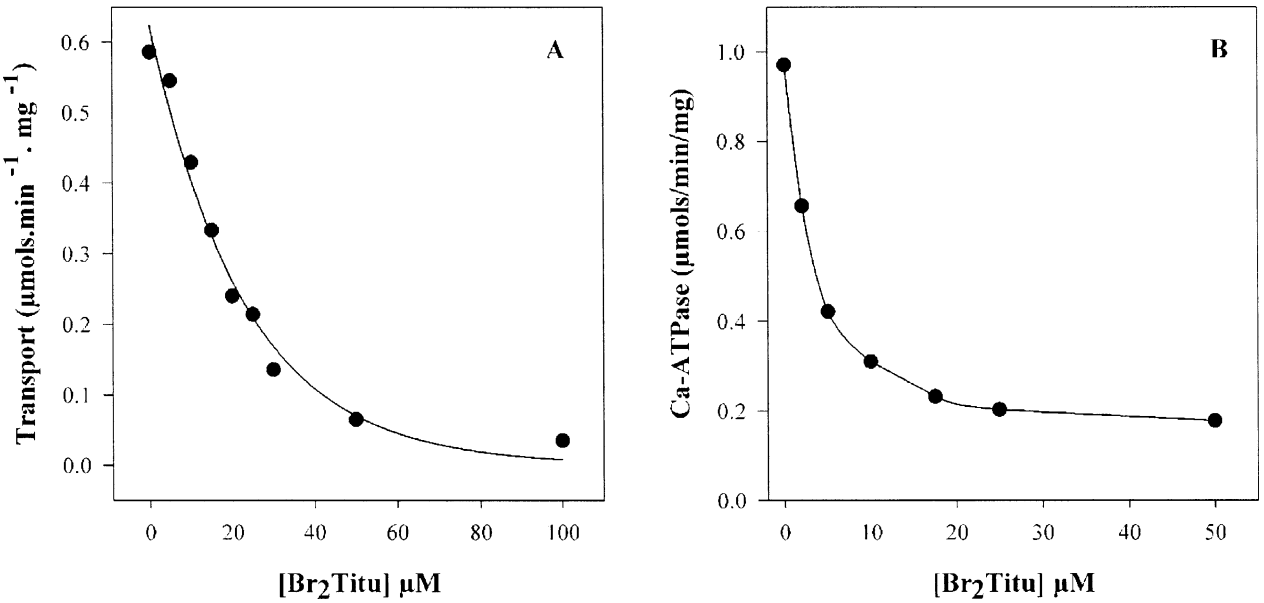


FIGURE 2: Concentration dependence of Br_2-TITU inhibition of Ca^{2+} transport and ATPase activity. SR vesicles, incubated in standard medium, were preincubated with varying concentrations of Br_2-TITU for 10 min at 25 °C. Following addition of 20 nM Fluo-3, ATP, 2 mM, was added. After $[Ca^{2+}]_{lim}$ was established, three pulses of 20 μM Ca^{2+} were injected. V_{max} of transport was calculated from peak widths of the fluorescence signal (A) as described in Materials and Methods. The effect of Br_2-TITU on Ca^{2+}-ATPase activity is shown in (B). SR vesicles, 0.25 mg/mL, EGTA, 0.1 mM, and ATPase medium for the NADH-coupled reaction were incubated with varying concentrations of Br_2-ATP for 20 min at 25 °C, following which 2% (w/w) of the ionophore, A23187, was added. The reaction was started with 2 mM ATP, and basal ATPase activity or Mg^{2+}-ATPase activity was recorded. Total ATPase activity was measured after addition of 200 μM Ca^{2+}. Ca^{2+}-dependent ATPase was taken as total minus basal activity.

when multiple pulses of 20 μM Ca^{2+} were added immediately after Br_2-TITU. It was also apparent that the incubation period between times of addition of Br_2-TITU affected the degree of inhibition of transport. Inibition was greater with little time dependence when Br_2-TITU was preincubated for 10 min before pulses of Ca^{2+} were added (Figure 1B). The effects of preincubation time with Br_2-TITU on inhibition of Ca^{2+} transport are shown in Figure 1C. Maximum inhibition of transport occurred at 10−20 min preincubation of Ca^{2+}-ATPase with inhibitor. Half-maximum inhibition occurred after approximately 3 min.

The concentration dependence of Br_2-TITU on transport is shown in Figure 2A, where $K_{0.5}$ for inhibition was approximately 15 μM. Inhibition by Br_2-TITU could be a V_{max} effect or be competitive with Ca^{2+} or substrate ATP. Increasing [ATP] from 2 to 10 mM had no effect on inhibition. Similarly, excess Ca^{2+} up to 1000 μM had no effect. These findings confirm that inhibition of transport is not due to changes in affinities for Ca^{2+} or ATP binding.

Ca^{2+}-dependent ATP hydrolysis was determined by the NADH-coupled method. The conditions for ATPase activity were identical with those used for transport, except that the Fluo-3 was omitted, as well as EGTA, 1 mM, and [ATP] was 200 μM instead of 2 mM. Initial rates were recorded following addition of ATP. This corresponds to the state $[Ca^{2+}]_{lim}$ of approximately 50 nM. Addition of Ca^{2+} caused a slow increase in NADH oxidation, which was not related to accumulation of ADP, since its exogenous addition resulted in a rapid decline in absorption (data not shown). Linear rates of ATP hydrolysis occurred within 3−5 min. Ca^{2+}-dependent ATPase activity was calculated as the difference between $[Ca^{2+}]_{lim}$-ATPase and total ATPase. The effects of increasing concentrations of Br_2-TITU on Ca^{2+}-dependent ATP hydrolysis are shown in Figure 2B. Significant inhibition was observed at 1−2 μM inhibitor, with $K_{0.5}$ of approximately 5 μM, while maximum effects were above 25 μM. Note that inhibition curves for transport and ATPase activity are not comparable, since the latter was measured at its maximum, by addition of the Ca^{2+} ionophore A23187. For the same reasons, coupling ratios, Ca^{2+}/ATP, cannot be calculated. Ionophore, 2% w/w, was added to minimize reversal of the pump by high levels of lumenal Ca^{2+}.

Effect of Br_2-TITU on Autofluorescence of the Ca^{2+}-ATPase. Autofluorescence of the Ca^{2+}-ATPase of SR is due to the presence of multiple tryptophan residues and has been used in many studies relating binding of ligands, particularly Ca^{2+} (*25*). The response to EGTA was a 5.0% fall in signal, which was almost completely reversed by excess Ca^{2+} (Figure 3A). EGTA showed a similar fraction of 4.0% of the fluorescence quench when the total signal was quenched by up to 500 μM Br_2-TITU. Stepwise increase of Br_2-TITU quenched Trp autofluorescence (Figure 3B) that was rapid. KBr, up to 2.3 mM, had no effect on either Ca^{2+}-ATPase or Ca^{2+} transport (data not shown) or on autofluorescence (Figure 4), indicating that the Br atom needs to be in organic form to bind and to be able to influence autofluorescence, as well as of other properties of the Ca^{2+}-ATPase (*25*). Increasing concentrations caused a rapid fall in signal that was apparently stable. Quenching of autofluorescence in native SR vesicles appears to be biphasic with high- and low-affinity sites with approximately equal amplitudes. The origins of this behavior are not obvious, but it may represent two classes of Trp residues. Excess Ca^{2+} appears to abolish this biphasic response.

The pH dependence of quenching of tryptophan fluorescence may give an indication of the mechanism of the reaction. Maximum effects were at pH 9.0 for the Ca^{2+}-ATPase (data not shown). The $K_{0.5}$ is approximately 30 μM.

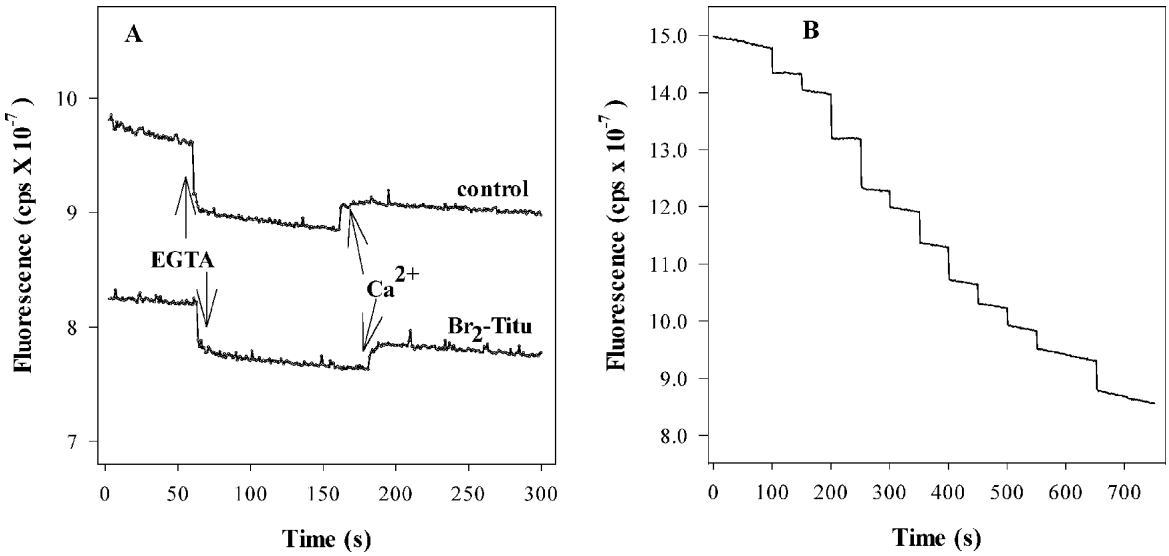


FIGURE 3: Effect of Br_2-TITU on Ca^{2+}-induced changes in autofluorescence of Ca^{2+}-ATPase tryptophans. EGTA, 50 μM, and Ca^{2+}, 100 μM, were added sequentially to SR vesicles that had been preincubated for 10 min in the absence (control) and presence of 50 μM Br_2-TITU (A). The Br_2-TITU trace has been shifted on the *Y*-axis for clarity. In (B) increasing amounts of Br_2-TITU were added to SR vesicles in standard medium with rapid stirring. The total amount of Br_2-TITU that has been added stepwise to the cuvette was 0, 0.5, 1.0, 2.0, 5.0, 10.0, 20, 50, 75, 100, 125, 150, and 200 μM.

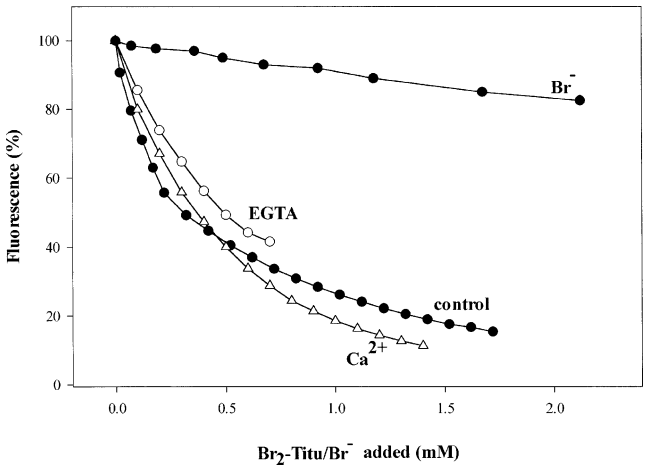


FIGURE 4: 4: Effects of Ca^{2+} and EGTA on quenching of autofluorescence induced by Br_2-TITU. Increasing amounts of Br_2-TITU or KBr were added to buffered medium, containing 0.25 mg/mL SR with either control, 100 μM Ca^{2+}, or 1 mM EGTA, with constant stirring. KBr was added to control vesicle, without added Ca^{2+} or EGTA.

Table 1: Effect of pH and Br_2-TITU on Steady-State Total E-P Levels[a]

pH	control (nmol/mg)	Br_2-TITU (nmol/mg)
6.80	0.52 ± 0.04	5.70 ± 0.55
8.50	3.50 ± 0.10	4.0 ± 0.09

[a] SR vesicles, 0.25 mg/mL, were preincubated at 25 °C for 20 min with and without 50 μM Br_2-TITU. [γ-^{32}P]ATP, 200 μM, was then added, and after 100 s further incubation, aliquots were taken and E-P levels were determined as described in Materials and Methods.

Effects of Br_2-TITU on E-P Levels from ATP or P_i. E-P levels at steady state were determined from [γ-^{32}P]ATP plus Ca^{2+}. Results are shown in Table 1. Maximum E-P level in controls was greater at pH 8.5 than at pH 6.8. Br_2-TITU increased steady-state E-P 10-fold at pH 6.8 but had less effect at pH 8.5, where levels were near maximum in controls (*10*). Br_2-TITU enhanced E-P at pH 8.5 by less than 10%.

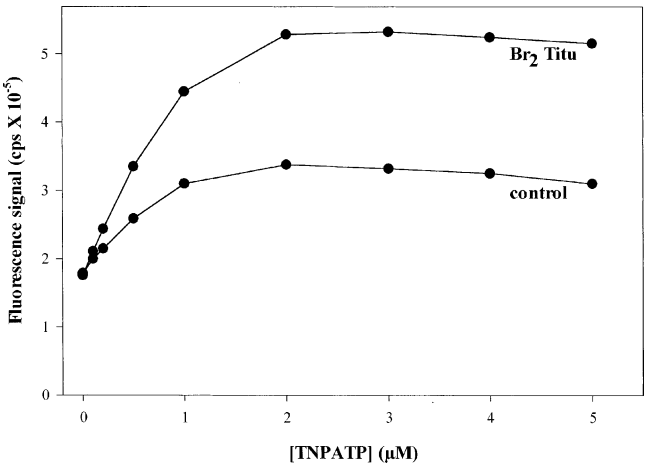


FIGURE 5: Effects of Br_2-TITU on TNP-ATP binding and fluorescence by the Ca^{2+}-ATPase. SR vesicles, 0.25 mg/mL, were preincubated without or with 50 μM Br_2-TITU for 20 min at 25 °C. TNP-ATP was added sequentially, with stirring, up to a total of 5 μM.

The theoretical maximum value is approximately 8 nmol/mg, assuming 90% purity of the sarcoplasmic reticulum preparation. In practice, values of 4−5 nmol/mg are the maximum observed. The reason for this lower stoichiometry will be included in the Discussion.

Effects of Br_2-TITU on TNP-ATP Binding and Superfluorescence by the Ca^{2+}-ATPase. The trinitrophenyl derivative of ATP, TNP-ATP, binds with high affinity to the Ca^{2+}-ATPase with an increase of approximately 2-fold of fluorescence (Figure 5). Passive high-affinity TNP-ATP binding to SR vesicles, in the absence of ATP or P_i, results in an increase in fluorescence signal. A single site may be titrated which saturates at 1.0 mol/mol of ATPase (*26*). Binding of TNP-ATP shows an inflection point at a 1:1 ratio of probe to Ca^{2+}-ATPase (Figure 5). In the presence of Br_2-TITU fluorescence is enhanced and also shows an inflection point at 4 nmol/mg of ATPase. Thus Br_2-TITU causes an increase in fluorescence quantum yield, but there is no increase in the number of interacting sites.

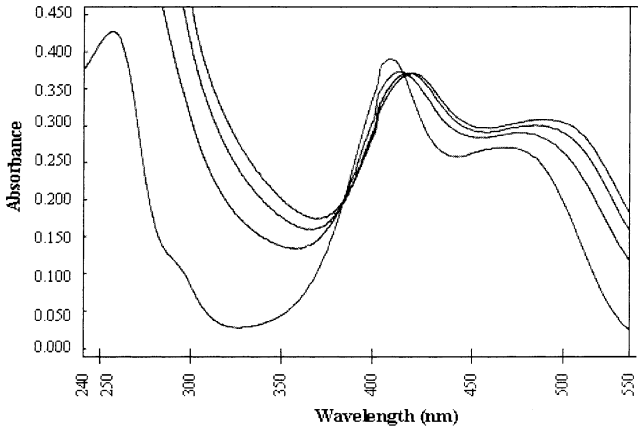


FIGURE 6: Effects of free Br_2-TITU on the absorbance spectrum of free TNP-ATP. Absorbance spectra of free TNP-ATP, 4 μM, in standard medium and with increasing concentrations of Br_2-TITU were measured in free solution in the absence of SR vesicles, The traces are identified by their increasing absorbance at 480 nm as comtrol, 25, 50, and 100 μM Br_2-TITU.

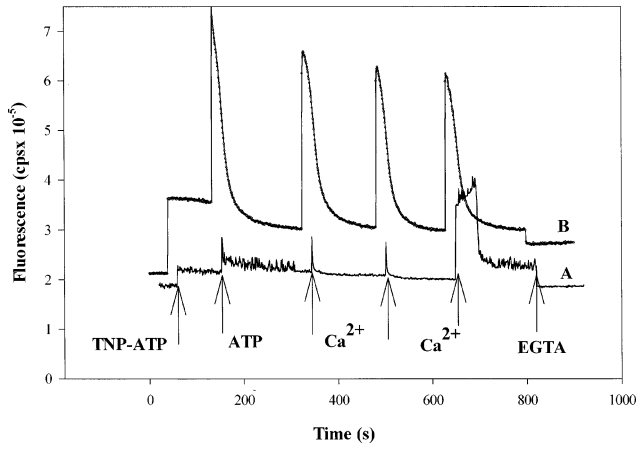


FIGURE 7: TNP-ATP superfluorescence induced by ATP plus Ca^{2+} and effects of Br_2-TITU in the presence of oxalate. SR vesicles were incubated in standard medium, following preincubation in the absence (A) and presence (B) of 20 μM Br_2-TITU for 20 min. After equilibration with continuous stirring, TNP-ATP was added together with 20 μM Ca^{2+}. Superfluorescence was initiated by addition of 200 μM ATP. After return to baseline fluorescence, three pulses of 20 μM Ca^{2+} were added. The last pulse of Ca^{2+} was 100 μM to control vesicles. The true baseline, due to light scattering, was assessed after addition of 1 mM EGTA.

Aromatic ring structures of Br_2-TITU and TNP-ATP suggested that they might interact. Absorbance spectra of TNP-ATP showed typical absorbance peaks at 259 nm due to the adenine ring and peaks at 408 and 435 nm due to the trinitrophenyl ring (Figure 6). Addition of increasing concentrations of Br_2-TITU to TNP-ATP in free solution showed consistent changes, including red shifts of the 408 and 435 nm peaks, with isosbestic points at 381 and 413 nm. This behavior is consistent with formation of at least one interactive soluble complex.

A typical trace of TNP-ATP fluorescence that follows forward cycling from ATP and Ca^{2+} is shown in a control experiment in Figure 7. Following passive binding of TNP-ATP to control SR, ATP induces an increase in fluorescence species, which declines rapidly, owing to sequestration of contaminating Ca^{2+} in the presence of oxalate that decreases to a limiting value, typically 50 nM, in the calcium limited state, $[Ca^{2+}]_{lim}$ (*22*). Addition of 20 μM pulses of Ca^{2+} caused a transient rise in fluorescence that declined to baseline within several seconds. Preincubation of SR with 100 μM Br_2-TITU for 10 min at 25 °C resulted in a characteristic increase in peak superfluorescence of more than 5-fold that declined to a baseline similar to that in the control (Figure 7). Increased width of the peaks indicates approximately 90% inhibition of Ca^{2+} transport.

Intermediates E1∼P·2Ca and E2-P·2Ca are ADP-sensitive and -insensitive, respectively. The effect of ADP on superfluorescence in the pseudo-steady-state during Ca^{2+} uptake showed that controls were quenched by 50% after addition of 1 mM ADP. Enhanced superfluorescence with Br_2-TITU was inhibited by 70%, from which it may be concluded that E1∼P and E2-P accumulate in the steady state. Another possibility is that ADP competes with TNP-ATP for the nucleotide binding site.

Further studies of superfluorescence were carried out in the absence of oxalate, representing the steady state. Following addition of ATP in the presence of Ca^{2+} there appeared a pseudo-steady state in which the signal was rapidly induced (termed phase 1) and declined within the next 5 min to 60% of its initial value (Figure 8A). Addition of EGTA rapidly quenched the signal (phase 2). Br_2-TITU increased maximal steady-state superfluorescence 3−5-fold. Superfluorescence, induced by P_i plus EGTA, showed a rapid increase to a plateau, following a small overshoot (Figure 8B). Br_2-TITU also enhanced the steady-state fluorescence from P_i by 3−5-fold (phase 3). Addition of Ca^{2+} rapidly quenched this signal (phase 4).

Previous studies on superfluorescence have been carried out in the pH range 6.5−7.0, based on the assumption that catalytic activity is maximal at neutral pH. The pH dependence of steady-state superfluorescence from ATP plus Ca^{2+} is shown in Figure 9. A typical bell-shaped curve of two overlapping processes, with $K_{0.5}$ values at pH of 7.5 and 10.5, and a maximum in the range 8.5−9.0, is shown in Figure 9. Fluorescence at pH 6.8 was 30% of maximum. Steady-state fluorescence intensity, monitored by convenional spectrofluoroimetry following manual mixing, is too slow to observe detailed kinetics. However, there was an indication that the quench of ATP-induced superfluorescence and of P_i-dependent signal was prolonged (Figures 7 and 8). We therefore analyzed the rapid kinetics of both induction and decay of TNP-ATP superfluorescence by means of stopped flow. These experiments were carried out so that the final concentrations of ligands, following 1:1 mixing, were the same as those used for steady-state measurements. Four sets of experiments, phases 1−4, were analyzed for superfluorescence from (1) ATP plus Ca^{2+}, (2) followed by EGTA quench, (3) P_i plus EGTA, (4) followed by Ca^{2+} quench. Two pH values were chosen, pH 6.8, being physiological, and pH 8.5, where superfluorescence was near maximum.

Stopped-flow traces of ATP-dependent superfluorescence and the effects of EGTA at pH 6.8 are shown in Figure 10. Full details of rate constants and amplitudes of fluorescence signals and the effects of Br_2-TITU are shown in Table 2. At pH 6.8, ATP "on" rate constants (phase 1) were doubled by Br_2-TITU (Figure 10 and Table 2). Following addition of ATP, in the presence of excess Ca^{2+}, fluorescence rapidly reached a maximum and decreased slowly over a period of 100−200 s to reach a new equilibrium. For this reason rapid kinetics of the EGTA quench were determined after sequen-

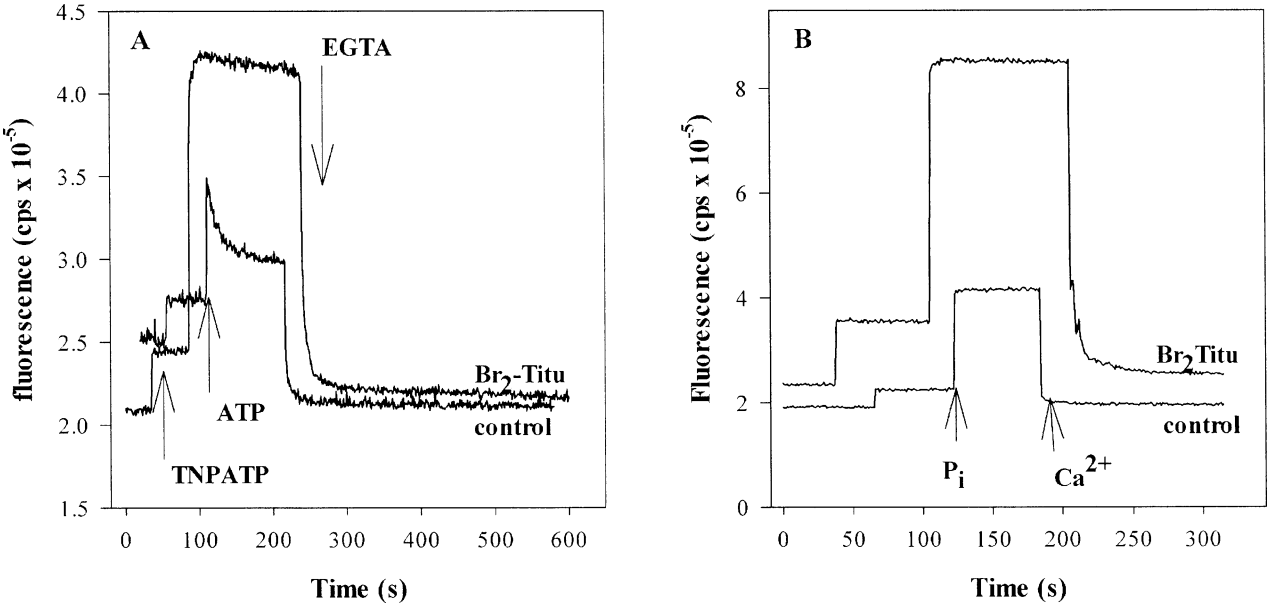


FIGURE 8: Induction of superfluorescence of TNP-ATP in the absence of oxalate. Conditions were as described in the legend to Figure 7, except that oxalate was omitted from the medium. Superfluorescence of TNP-ATP was induced in the forward direction by addition of 20 μM Ca^{2+} and 200 μM ATP (A) with a quench by 1 mM EGTA. Superfluorescence in the back reaction from 5 mM P_i and 0.1 mM EGTA (B) was quenched by 1 mM Ca^{2+}.

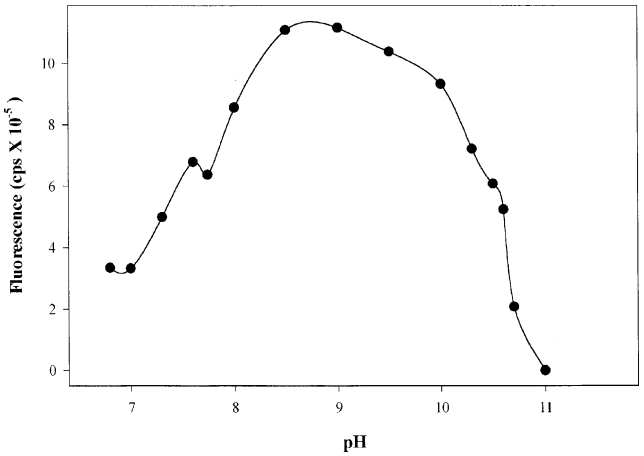


FIGURE 9: Effect of pH on Trp fluorescence of the Ca^{2+}-ATPase. SR vesicles, 0.25 mg/mL, were preincubated in buffers prepared for pH 6.8 and 7.0 (20 mM Tris/MOPS), pH 7.4−8.7 (20 mM EPPS), pH 9−10 (20 mM CHES), and pH 11 (20 mM CAPS) for 10 min in the presence of 50 μM Br_2-TITU.

tial stopped flow. In these experiments, following mixing of ATP with SR, there was a programmed delay of 20 s, after which EGTA was added (for details see legend to Figure 10). Total amplitudes were increased 3-fold by Br_2-TITU, and the ratios of slow and fast phases were 75/25 and 82/18 for control and inhibited curves. Significantly, rate constants for the fast phases were increased 2-fold, and the slow phase increased 5-fold. At pH 8.5, where superfluorescence is maximum, there was also a 7.5-fold decrease in rate constant of the rapid phase with little change in amplitude.

Superfluorescence, induced by P_i plus EGTA at pH 6.8, showed a single exponential increase in control SR (Figure 11 and Table 3). Br_2-TITU induced equal amplitudes of rapid and slow phases, with marked acceleration (7.4 vs 0.84 s^{-1}). The slower phase was similar to that in controls. It is concluded that Br_2-TITU accelerates the induction of superfluorescence from P_i in the absence of Ca^{2+}. Superfluorescence from P_i/EGTA was quenched by Ca^{2+} (Figure 11). Br_2-TITU caused a marked decrease in rate constants of the quench reaction being 15-fold and 60-fold slower for the fast and slow phases. Amplitudes were increased approximately 2-fold. Less marked decreases in rate constants were noted at pH 8.5 (Table 2) than at pH 6.8, with 1.7- and 2.0-fold decreases in the slow and fast phases, respectively. Note that the on-phase rate constant from ATP in control SR vesicles of 0.3 s^{-1} is slower than the turnover rate of approximately 10 s^{-1}. This may be due to inhibition of catalysis by TNP-ATP. It should be noted that the observed rate constants do not necessarily apply to individual intermediate reactions.

Specificity of Br_2-TITU Effects on SR Ca^{2+}-ATPase. Compounds related to Br_2-TITU have significant effects on the Na^+/K^+-ATPase (*4*, *15*). Ethylenediamine, and guanidinium, up to 10 mM, had no effect on Ca^{2+} transport or superfluorescence (data not shown). In summary, Br_2-TITU has significant effects on the induction and decay in superfluorescence, induced by either ATP/Ca^{2+} or P_i/EGTA. Two phases, slow and fast, were identified in several conditions and were equally affected by the inhibitor. There was an increase in rates of induction of superfluorescence, at both pH 6.8 and pH 8.5. The most striking and consistent finding was that Br_2-TITU decreased the "off" rate constants for the EGTA quench of ATP-induced superfluorescence, from 2.90 to 0.66 s^{-1} and from 0.75 to 0.15 s^{-1} for fast and slow components and from 10.9 to 0.73 s^{-1} and from 1.8 to 0.03 $^{-1}$ for the Ca^{2+}-quenched P_i-induced signal.

DISCUSSION

Previously, Br_2-TITU was shown to competitively inhibit K^+/Rb^+ binding to occlusion sites on the Na^+/K^+-ATPase (*15*, *27*) or "19 kDa membranes", in which cytoplasmic domains of the Na^+/K^+-ATPase are removed but transmembrane segments and monovalent cation occlusion sites are intact (*28*). The aim of the present study has been to determine whether Br_2-TITU has effects on another P-type

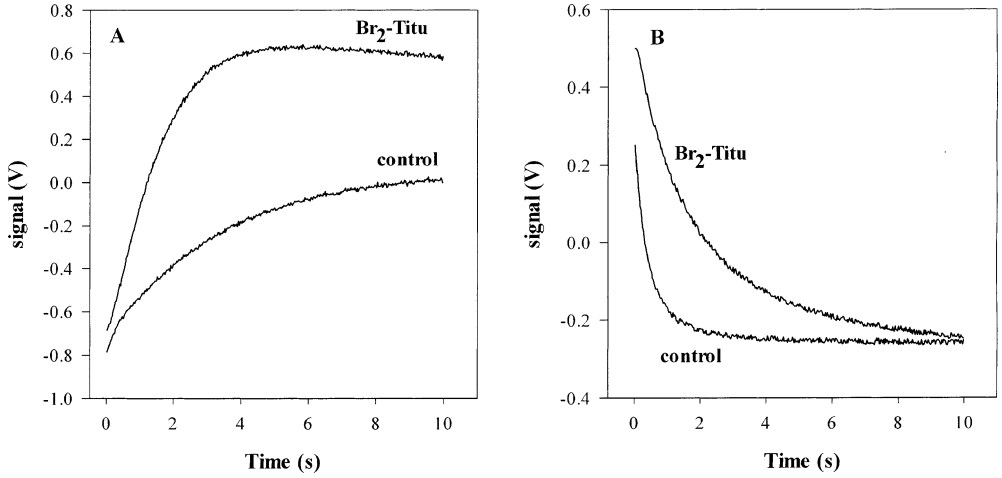


FIGURE 10: Kinetics of ATP plus Ca^{2+}-induced superfluorescence of TNP-ATP as determined by sequential stopped-flow analysis of reagents so that their final concentrations of buffers and reagents were equal to conditions used for pseudo-steady-state conditions. Buffering was with 20 mM Tris-HCl for pH 6.8 and 20 mM EPPS at pH 8.5. $MgCl_2$, 5 mM, was included, and oxalate was excluded. For the ATP-induced "on" reaction (phase 1) panel (A), syringe A contained, in addition, 0.5 mg/mL SR, 10 μM Ca^{2+}, and 2.5 μM TNP-ATP. Syringe B contained identical reagents, except that 400 μM ATP was included. Control and Br_2-TITU (50 μM) contents of syringe A were preincubated for 20 min at 25 °C. EGTA quench (phase 2) was recorded with the sequential stopped-flow method using a three-syringe mode. ATP and SR were mixed in syringes 1 and 2 and incubated for 20 s. The mixture was quenched with 5 mM EGTA from syringe C.

Table 2: Effects of Br_2-TITU on Kinetics of ATP-Dependent TNP-ATP Superfluorescence[a]

		ATP_{on}		ATP_{off} (EGTA quench)	
pH		control	Br_2-TITU	control	Br_2-TITU
6.80	k_1	0.32 ± 0.01	0.68 ± 0.007	2.90 ± 0.09	0.66 ± 0.03
	k_2		0.60 ± 0.008	0.75 ± 0.06	0.15 ± 0.02
	A_1	0.80 ± 0.004	1.10 ± 0.01	0.38 ± 0.01	1.26 ± 0.26
	A_2		0.43 ± 0.006	0.13 ± 0.01	0.27 ± 0.03
8.50	k_1	1.91 ± 0.02	0.23	0.38	0.05 ± 0.003
	k_2	0.31 ± 0.007	0.14 ± 0.005	0.13	
	A_1	0.95 ± 0.02	1.93 ± 0.11	1.27	1.90 ± 0.03
	A_2	0.94 ± 0.04	0.29 ± 0.09	0.66	

[a] Conditions were as described in Materials and Methods. Rate constants k_1 and k_2, fast and slow phases, and amplitudes A_1 and A_2 were determined.

ATPase, the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum, which has well-characterized structure and function. K^+ is known to increase turnover of the Ca^{2+} pump by promoting the hydrolysis of E2-P → E2·P_i with a $K_{0.5}$ of 20−50 mM (*29*). The cytoplasmic site of Br_2-TITU binding to the Na^+/K^+-ATPase has been suggested to be localized in the loop joining M6 and M7 (*30*). The Ca^{2+}-ATPase has been shown to have similar features (*31*, *32*).

In the previous work with Na^+/K^+ATPase Hoving et al. (*15*) reported two additional phenomena, which cannot be explained by competititon of Br_2-TITU and monovalent cations at the cytoplasmic side. First, high concentrations of Br-TITU stabilized an E2 conformation (K_i = 10 μM), suggesting that the compound may bind at the extracellular surface. Second, incubation of renal Na^+/K^+ATPase with Br_2-TITU at pH 9 led to irreversible inactivation of Rb^+ occlusion with protection by Rb^+ or Na^+ ions. This phenomenon required much higher concentrations of Br_2-TITU than for the reversible competitive effects. Although initially it was thought that Br_2-TITU could act as a lysine- or cysteine-specific modifying reagent, no specific incorporation of ^{14}C-labeling into the protein was detected using [^{14}C]Br-TITU (^{14}C in the isothiourea moiety) (M. Bar Shimon, D. M. Tal, and S. J. Karlish, unpublished). As we now discuss, the primary effects of Br_2-TITU on the Ca^{2+}-ATPase include some which resemble these additional effects on Na^+/K^+-ATPase and may provide an explanation. The present study showed an irreversible time-dependent (minutes) inhibition of catalysis. Quenching of autofluorescence was rapid and of the order of seconds.

Inhibition of ATPase and Transport of SR Ca^{2+}-ATPase by Br_2-TITU. Active transport of Ca^{2+} and of Ca^{2+}-dependent ATPase activity was approximately equally inhibited by Br_2-TITU in the range 0−100 μM (Figures 1 and 2). A feature of this inhibition was its time dependence such that approximately 20% of total inhibition was evident by 30 s, while maximum inhibition was only attained at 10−20 min. Inhibition of both transport and ATPase activity appear to be noncompetitive for Ca^{2+} and ATP, since high concentrations of these ligands failed to overcome this inhibition. By analogy to its interaction with the Na^+/K^+-ATPase, where Br_2-TITU competitively inhibits Na^+ and Rb^+ binding, both NaCl and KCl did not reverse inhibition of the Ca^{2+}-ATPase (data not shown) Several possible mechanisms might be able to explain inhibition of the Ca^{2+} pump. Effects may be global and indirect through perturbation of the membrane phase with altered fluidity, as discussed by Shinitzsky (*33*). Another possible mechanism is that Br_2-TITU causes a local decrease in the hydrophobic thickness of the membrane, as suggested previously (*34*). The Ca^{2+}-ATPase appears not to be particularly sensitive to alterations in surrounding lipids, with which it interacts, since detergents of various chain lengths can substitute for natural phospholipids (*35*). This study suggests that Br_2-TITU has a more direct action on the membranous segments of the Ca^{2+}-ATPase. The kinetics of inhibition are not fast as expected if the Br_2-TITU were interacting with a cation binding site.

Characteristics of Quenching of Autofluorescence of Tryptophanyl Residues of the Ca^{2+}-ATPase. Several previous studies have described the interaction of brominated amphiphiles with tryptophan residues of the membranous

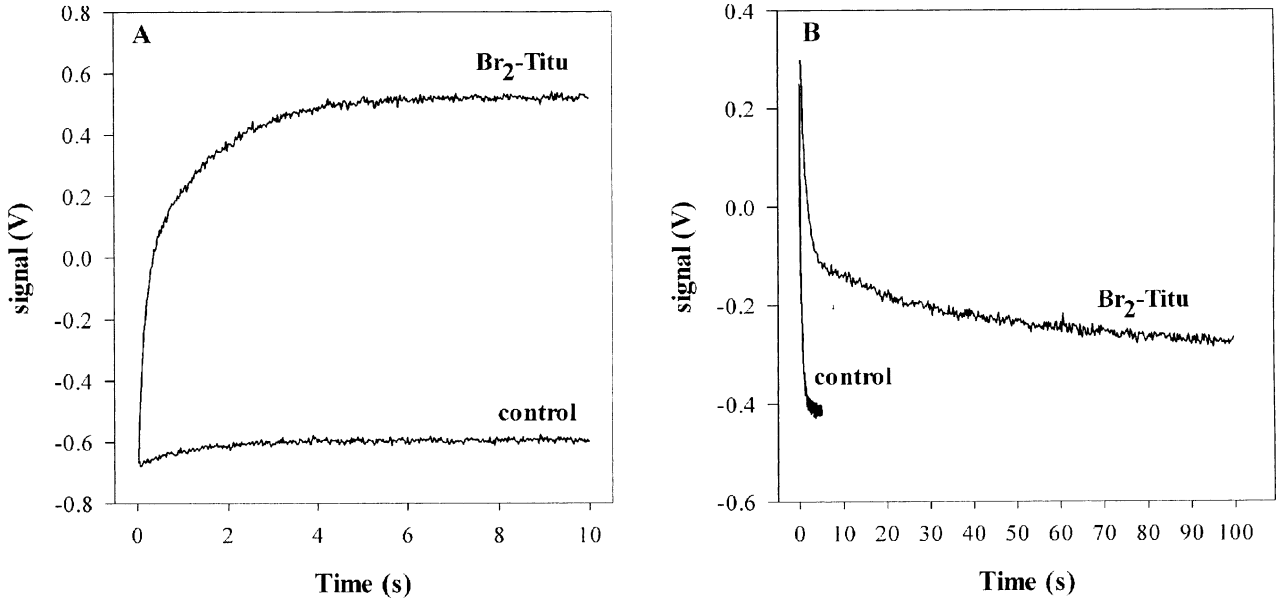


FIGURE 11: Kinetics of P_i-induced superfluorescence studied by stopped-flow analysis. A 1:1 mixture of reagents was designed to duplicate conditions used in manual mixing. Tris/HCl, 20 mM, was used for pH 6.8. Conditions and preincubations were as described for Figure 10, except that EGTA, 1 mM, replaced Ca^{2+} for the "on" (phase 3) reaction. SR was included in syringe A and 20 mM P_i in syringe B (A). For the "off" reaction (phase 4) syringe A and B contents were mixed and allowed to age for 60 s and then quenched with 5 mM Ca^{2+} from syringe C (B).

Table 3: Kinetics of P_i-Dependent TNP-ATP Superfluorescence and Effects of Br_2-TITU[a]

		P_i on		P_i off (Ca^{2+} quench)	
pH		control	Br_2-TITU	control	Br_2-TITU
6.80	k_1	0.84 ± 0.05	7.45 ± 0.13	10.90 ± 0.57	0.73 ± 0.02
	k_2		0.69 ± 0.006	1.79 ± 0.06	0.03 ± 0.001
	A_1	0.08 ± 0.007	0.72 ± 0.02	0.30 ± 0.01	0.53 ± 0.008
	A_2		0.61 ± 0.012	0.44 ± 0.01	0.18 ± 0.008
8.50	k_1	4.45 ± 0.18	0.25 ± 0.004	0.30 ± 0.01	0.52 ± 0.03
	k_2			0.04 ± 0.01	0.08 ± 0.001
	A_1	0.14 ± 0.01	0.25 ± 0.01	2.52 ± 0.15	2.44 ± 0.26
	A_2			0.29 ± 0.03	0.98 ± 0.10

[a] Conditions were as described in Materials and Methods and the legend of Table 2.

segments of the Ca^{2+}-ATPase. In general, brominated compounds, including phospholipids and detergents, have been used to study the mechanisms and topography of interacting Trp (*36*, *37*). By analogy, Br_2-TITU may be expected to have similar actions, in which there is orbital overlap of halogen (Br) with the chromophore (Trp). Such an interaction is short range (<1 nm) (*38*) heavy atom quenching (*23*, *39*). Of significance for the present study is that dibromo derivatives have a higher efficiency of quenching than monobromo analogues (*23*). In this model the polar headgroup of, e.g., brominated phospholipd, is anchored at the lipid−water interface, while the Br is located some distance into the membrane. Inorganic Br^- does not penetrate the membrane, and it can be concluded that only two Br atoms attached to the phenyl ring of Br_2-TITU are responsible for quenching.

Location of the 13 Trp in the ATPase are reasonably certain (*40*−*43*) and confirmed by the X-ray crystallographic model at 2.6 Å resolution, determined by Toyoshima et al. (*44*). One Trp is unambiguously assigned to the cytoplasmic headpiece, relatively far from the membrane domain. The remaining Trp are equally localized within cytoplasmic and lumenal leaflets of the membrane. Approximately eight residues occur at the protein−membrane interface. Kinetics of replacement of native phospholipids with detergent amphiphiles varies. In this study it is presumed that there is ready partition into the cytoplasmic leaflet, followed by "flip-flop" across to the other leaflet. Rates of incorporation vary widely; e.g., brominated $C_{12}E_8$ takes only seconds to reach equilibrium (*45*).

In agreement with partition of other amphiphiles into SR membranes (*46*), the present study is compatible with decreased autofluorescence, due to replacement of phospholipids by Br_2-TITU. It is also presumed that quenching is "static", as concluded previously for phospholipids (*47*). The mean residence time of Br_2-TITU on the hydrophobic surfaces of transmembrane segments is expected to be longer than the Trp fluorescence lifetime of 1−7 ns (*36*, *37*, *48*, *49*).

The fact that autofluorescence shows a similar fractional change of 4−5% on binding and release of Ca^{2+}, in control and as well as Br_2-TITU-quenched vesicles, indicates that the ATPase is still able to undergo the conformational transition by those Trp residues whose autofluorescence has not been quenched.

There appears to be at least two possible mechanisms of inhibition of Na^+/K^+-ATPase and Ca^{2+}-ATPase function, the main difference being whether a covalent complex of exposed Trp's is formed. Rapid binding of Br_2-TITU at the lipid−protein interface on the Ca^{2+}-ATPase quenches Trp autofluorescence by overlap of Br atoms and Trp residues. This is followed by a slow conformational change leading to inactivation of transport and ATPase activity (5−10 min). It has now been found for Na,K-ATPase that the conditions for irreversible inactivation by Br_2-TITU lead to partial quenching of the tryptophan fluorescence, and Rb^+ or Na^+ ions protect (M. Bar Shimon and S. Karlish, unpublished). Thus Br_2-TITU may bind rapidly to the ATPases and then covalently modify Trp residues at the lipid/protein interface,

where most of these residues are located. This reaction would be induced by a reactive benzylic methylene group in the Br_2-TITU molecule. It is similar to that produced with Koshland's reagent (*50*).

Effects of Br_2-TITU on Superfluorescence of Trinitrophenyl-ATP (TNP-ATP) Bound to the Ca^{2+}-ATPase of SR. TNP-ATP superfluorescence was originally examined in order to determine which "E2-like" state predominates in the Br_2-TITU-inhibited Ca^{2+} pump. An unexpected finding was that inhibition of catalysis caused enhanced superfluorescence (Figure 8), best evident from broadening of TNP-ATP fluorescence peaks that were approximately 5-fold enhanced in the forward mode of the catalytic cycle. From this we conclude that Br_2-TITU inhibition of the calcium pump favors an E2-like state and that the inhibition seems to be at the step or steps that result in the E2 $\rightarrow$ E1 transition, by release of Ca^{2+} and hydrolysis of E_2-P.

Binding of Br_2-TITU that results in quenching of Trp fluorescence is presumed to be adjacent to residues at or near to the lipid−medium interface. At the same time enhanced superfluorescence is related to distal events, including exclusion of H_2O from the ATP-binding cleft [for review, see McIntosh (*51*)]. On the basis of the observations described here, it may be concluded that long-range effects, which couple intramembranous sites to the cytoplasmic domain of the Ca^{2+}-ATPase, remain intact.

TNP-ATP, which contains a bulky fluorescent derivative, has a higher affinity than ATP for the nucleotide binding site that has been explained by structural flexibility of the ribose, necessary for catalysis (*52*). "Puckering" of the ring is essential for interaction between ATP binding sites of the Na^+/K^+-ATPase. There are some differences in interpretation of the nature of the TNP-ATP binding site and its relationship to the catalytic site. However, the general consensus is that TNP-ATP binds to the catalytic site following phosphorylation and release of ADP (*28*, *41*).

Thiol group modification by *N*-ethylmaleimide affects the cysteinyl group that is essential for phosphoenzyme decomposition (*53*), resulting in accumulation of enzyme species in the E2-P state. It was concluded that ADP-insensitive E2-P is the intermediate responsible for superfluorescence. Changes at the active site from a hydrophilic to hydrophobic environment appear to be a feature of active transport, since intramolecular uncoupling of the Ca^{2+}-ATPase is associated with a decrease in steady-state superfluorescence (*54*). However, Bishop et al. (*55*) have proposed that the fluorescence change is constant for all phosphorylated species, based on the effects of pH and KCl.

The origins of superfluorescence from P_i plus EGTA are less ambiguous than that from ATP plus Ca^{2+}. P_i only forms E2-P, whereas ATP gives rise to both E1$\sim$P and E2-P in the steady state (*56*, *57*). Decrease of the rate constant, *k*, for decay of E2-P superfluorescence means that Br_2-TITU blocks E2-P hydrolysis. It seems unlikely that Br_2-TITU also blocks the isomeric step E1$\sim$P$\cdot$2Ca $\rightleftharpoons$ E2-P. This finding supports the view that E2-P is the only superfluorescent species.

The primary observations reported in the present study are the effects of Br_2-TITU on accumulation of total phosphorylated species, from steady-state incorporation of ^{32}P from [γ-^{32}P]ATP into E-^{32}P and manyfold increase in superfluorescence from both "forward" and "back" reactions. Total E-^{32}P during steady-state turnover from ATP plus Ca^{2+} was increased 10-fold at pH 6.8 by Br_2-TITU (Table 1). E-P levels were maximum at pH 8.5, with little or no enhancement by Br_2-TITU. It appears from absorbance spectrophotometry that there is a direct interaction in free solution between TNP-ATP and Br_2-TITU, with the formation of a single unimolecular adduct, inferred from well-defined isosbestic points in the absorbance spectrum (Figure 6). The nature of the complex is unknown but it is reasonable to assume it to be a single species, stabilized by $\pi-\pi$ bond interaction.

Many reports indicate that maximum levels of E-P at equilibrium are in the range 4−5 nmol/mg (*19*), compared to the theoretical value of 8 nmol/mg. Explanations for this discrepancy include half-sites reactivity in a dimer and inactivation of the enzyme during preparation of sarcoplasmic reticulum vesicles.

In the present study passive binding fluorescence was enhanced 2−3-fold in the absence of phosphorylation and the presence of either saturating Ca^{2+} or EGTA. It is possible that the TNP-ATP/Br_2-TITU complex, noted in free solution, is the species binding passively at the active site and which has enhanced fluorescence as compared to free TNP-ATP.

The pH dependence of ATP-induced superfluorescence was shown to be maximal at pH 8.5−9.0. Comparison of the effects of Br_2-TITU at pH 6.8 and 8.5 showed in general that effects of Br_2-TITU at pH 6.8 were greater than those at pH 8.5. Partition of Br_2-TITU into octanol was 350:1 at pH 7 and 1:1 at pH 9 (Karlish, unpublished observations). This may explain increased effects at alkaline pH. Biexponential kinetics of both formation and decay of E-P species have been reported previously and have been proposed as evidence for oligomer formation (*58*, *59*). Mulriexponential behavior is abolished by detergent (*60*).

The findings of dual exponential activation and decay of superfluorescence are of some interest. Intermediate fluxes could be into two parallel paths, such as from an oligomer. Alternately, it could arise from a linear consecutive model, with an initial fast equilibrium, followed by a slow essentially irreversible reaction.

More than one mechanism may be involved to explain the markedly increased superfluorescence of TNP-ATP that is nonideal for E-P levels at steady state. (a) Total E-P species at steady state are increased approximately 10-fold by Br_2-TITU at pH 6.8. (b) TNP-ATP and Br_2-TITU form a complex in free solution and would have greater fluorescence when bound to the nucleotide binding site. (c) There is increased "on" rate of formation of the superfluorescence species, as determined by stopped flow and (d) decreased "off" rate of E_2-P, presumably due to decreased rate of hydrolysis.

In conclusion, we have characterized the effects of a water-soluble aryl-brominated thiouronium probe on the Ca^{2+}-ATPase. The inhibitor that binds competitively to the Na^+/K^+-ATPase had no effect on Ca^{2+} binding by the Ca^{2+}-ATPase. Micromolar concentrations of inhibitor did lead to rapid quenching of fluorescence of Trp residues, suggesting partition into the membrane lipid bilayer at or adjacent to protein−lipid interfaces. In addition, in the case of the Ca^{2+}-ATPase, membrane binding has relatively long ranging effects, leading to exclusion of water at the ATP binding site, and may explain enhanced TNP-ATP superfluorescence.

The effects on the two cation pumps differ in some and are similar in other respects. Low concentratations of Br_2-TITU bind to the Na^+/K^+-ATPase and favor an E1-like state. In contrast, enhanced superfluorescence of bound TNP-ATP in the Br_2-TITU-inhibited Ca^{2+}-ATPase indicates accumulation of E2-P. This may be similar to the effect of high concentrations of Br_2-TITU to stabilize the E2 form of the Na^+/K^+-ATPase.

Inhibition of the Ca^{2+}-ATPase by Br_2-TITU is a well-defined reaction and is unique in that it leads to near maximal levels of E-P at steady state under nonideal conditions that would normally be associated with low levels of E-P. This reagent is a useful addition for determination of total levels of active Ca^{2+}-ATPase during studies on intermediate species stoichiometry. Br_2-TITU favors passive binding and superfluorescence of TNP-ATP via a soluble interacting species. The ability to increase TNP-ATP binding could be useful in studies on labeling of active ATP catalytic sites in other systems. Br_2-TITU may be a useful reagent, like thapsigargin, to study intermediate reactions of the catalytic cycle and mechanisms of energy coupling.

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