Nucleotide Specificity of Cardiac Sarcoplasmic Reticulum

INHIBITION OF GTPase ACTIVITY BY ATP ANALOGUE IN FLUORESCIN ISOTHIOCYANATE-MODIFIED CALCIUM ATPase

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Unlike skeletal muscle sarcoplasmic reticulum, canine cardiac sarcoplasmic reticulum hydrolyzes GTP in ways that are similar and different from ATP hydrolysis. Also, ATP and ATP analogues inhibit GTPase activity noncompetitively with a $K_i$ of 2-7 μM instead of competitively with 100 μM AMP-P(NH)P as reported previously (C. F. Tate et al., 1983; Tate et al., 1985). However, enzyme perturbation with submicellar amounts of detergent induces a qualitatively distinct mechanism, or perhaps GTP may enter the reaction pathway at separate nucleotide-binding sites toward low affinity. Thereafter, additional conformational changes occur in the enzyme allowing the release of inorganic phosphate from the terminal phosphate of the bound ATP to aspartyl residues on the enzyme. The bound calcium then is sequestered to the cytosol by the internal lumen of the vesicles following a change in the affinity of the calcium-binding sites toward low affinity. Therefore, additional conformational changes occur in the enzyme allowing the release of inorganic phosphate to the external medium (c.f. Inesi et al., 1990).

The SR isolated from skeletal muscle can utilize other nucleotides besides ATP for the calcium transport process. These nucleotides are hydrolyzed by the well-described classic reaction scheme outlined above (Van Winkle et al., 1981). Unlike skeletal muscle SR, however, we (c.f. Tate et al., 1989) demonstrated that the SR isolated from canine cardiac muscle hydrolyzes GTP by a qualitatively different mechanism from ATP in that: (a) GTP hydrolysis has minor or no calcium sensitivity in native cardiac SR; (b) GTP hydrolysis does not involve a calcium-sensitive phosphorylated intermediate; and (c) GTP hydrolysis is not stimulated by oxalate or ionophore. We (Van Winkle et al., 1981; Bick et al., 1983; Tate et al., 1985) hypothesized, however, that GTP and ATP are hydrolyzed by the same enzyme, the CaATPase, because: (a) both enzyme activities are similar and nonadditive; (b) the nonlinear kinetics as well as the kinetic constants of NTP hydrolysis are similar; and (c) GTP hydrolysis induces calcium accumulation into a compartment that is identical to the ATP-dependent calcium accumulation in its sensitivity to pH and ionophore even though GTP hydrolysis supports minor, if any, oxalate and calcium cotransport under the conditions of the assay (Van Winkle et al., 1981). However, enzyme perturbation with submicellar amounts of detergent induces a qualitatively distinct calcium-sensitive GTPase activity, compared to calcium-dependent ATPase activity, with a similar high affinity for calcium but with an apparent low affinity for GTP (Tate et al., 1989). In addition to these kinetic differences, ATP and the nonhydrolyzable analogue AMP-P(NH)P inhibit GTP hydrolysis in a noncompetitive fashion with a $K_i$ compatible with the high affinity nucleotide-binding site, suggesting that ATP and GTP may enter into the hydrolytic pathway of the CaATPase by alternative substrate entry sites (Bick et al., 1983; Tate et al., 1985). GTP and GTP analogues do not inhibit ATPase activity (Bick et al., 1983).

Based on the above information, we reasoned that GTP may be hydrolyzed by the canine cardiac CaATPase by a qualitatively distinct mechanism, or perhaps GTP may enter into the classic hydrolysis cycle of the CaATPase at a more "distal" site. We also reasoned that the substrate-binding sites of the two nucleotides are probably distinct because of the noncom-

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The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid; AMP-P(NH)P, adenylyl-5′-yl imidodiphosphate; FITC, fluorescein isothiocyanate; GMP-P(NH)P, guanylyl-5′-yl imidodiphosphate; NTP, nucleotide triphosphate; SDS, sodium dodecyl sulfate.
petitive nature of the ATP inhibition of GTPase activity. In this study, we utilized the lysine-directed fluorescent reagent FITC, which was reported to prevent ATP binding to the CaATPase protein, to examine the possibility of distinct binding sites for ATP and GTP. In the early 1980s, Pick and Karlish (1980), Pick and Bassillian (1981), and Pick (1981) demonstrated that the modification of the CaATPase protein of skeletal muscle SR by the incorporation of FITC inhibits ATP-dependent calcium transport and ATP-linked reactions. This inhibition of the function of the CaATPase is prevented by the inclusion of ATP in the incorporation medium. We therefore hypothesized that the incorporation of FITC into the cardiac enzyme would stop the ATP-dependent inhibition of GTPase activity by preventing high affinity ATP binding, but that FITC incorporation would not inhibit GTPase activity since GTP appears to bind at a different site than ATP, and that a GTP analogue would not prevent FITC incorporation.

In this report, we present data supporting a portion of this hypothesis. Although FITC incorporation inhibited the cardiac-dependent ATPase activity, the GTPase activity was not inhibited in native SR. In contrast to the protective effects of AMP-P(NH)P, GMP-P(NH)P did not protect against FITC-induced inhibition. However, AMP-P(NH)P (K < 3-7 μM) inhibited GTPase activity despite the incorporation of FITC. Finally, despite the protective effect of including cardionic SR with 0.2 μM [α-32P]ATP supports the notion that FITC incorporation does not prevent the binding of ATP or GTP to the CaATPase protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were purchased as reagent-grade. Non-labeled nucleotides and FITC were from Sigma. C12E8 (octaethylene glycol monododecyl ether) was purchased from Nihko Chemical (Tokyo). α-32P- and γ-32P-labeled ATP and GTP were obtained from Du Pont-New England Nuclear. Sodium dodecyl sulfate (SDS) was from Bio-Rad.

**Isolation Procedures**—Cardiac SR was isolated from canine hearts as previously described (Tate et al., 1985). The final cardiac SR pellet was suspended in 10 mM Tris maleate (pH 6.8). Protein was estimated by a biuret procedure. The amount of contaminating mitochondrial fragments was suspended in 10 mM Tris maleate (pH 6.8). Protein was estimated by the inclusion of ATP in the incorporation medium. We therefore hypothesized that the incorporation of FITC into the cardiac enzyme would stop the ATP-dependent inhibition of GTPase activity by preventing high affinity ATP binding, but that FITC incorporation would not inhibit GTPase activity since GTP appears to bind at a different site than ATP, and that a GTP analogue would not prevent FITC incorporation.

**RESULTS**

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**Inhibition of FITC—**FITC was incorporated into the SR following a modified procedure of Pick and Bassillian (1981). The SR (2.4 mg/ml) was treated with different concentrations of FITC by incubating the SR under mild conditions in 0.2 mM sucrose, 50 mM Tris-Cl (pH 7.0), 100 mM KCl, and 100 μM EGTA for 30 min at 30°C in the dark. The control (no FITC, plus dimethyl sulfoxide as carrier) was incubated under similar conditions. FITC was made up daily in dimethyl sulfoxide (carrier), which did not exceed 0.5% in the incorporation medium. The excess unlabeled FITC was removed either by using Sephadex G-50 columns or by diluting 100-fold directly in the reaction medium. The results were quantitatively similar.

**Photofluorometry**—Isolated membrane fractions (20–40 μg of protein) were preincubated for 2 min at 30°C in a total volume of 37.5 μl consisting of 40 mM Tris maleate (pH 7.0), 100 mM KCl, 10 mM Na3VO4, 2 mM diithothreitol, 1 mM EDTA, and 0.2 μM ω-Ala-ω-32P-labeled nucleotide (6 × 106 cpm, 1000 Ci/mmol). In separate reactions, the reaction mixture also included either 10 μM ATP or 10 μM GTP. The samples were placed on a sheet of Parafilm stretched over a block of ice and subjected to light exposure by exposing the samples to UV light (1010 quanta/s) for 2 min in a Rayonet photochemical reactor (Southern New England Ultraviolet Co.). In separate reactions, the reaction was not subjected to the UV light exposure as a control for nontoxic labeling. A 30-μl aliquot of each sample was subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 5–15% gradient gels as described by Laemmli (1970). Analysis of photolabeled proteins was accomplished by autoradiography of the dried gels at –80°C on Cronex 4 x-ray film with Quanta III intensifying screens (Du Pont-New England Nuclear).

*Additional Characterization of Cardiac SR—*Previously, we showed that our cardiac SR preparation is essentially devoid of sarcosommal and mitochondrial contamination, as shown by marker enzymes and the unresponsiveness of ATP-dependent calcium accumulation to high sodium, which causes release of calcium from calcium-loaded sarcosommal vesicles (Tate et al., 1985). Moreover, the enzymatic activity of the GTPase is not inactivated with time, as is the magnesium ATPase derived from the transverse tubules (Tate et al., 1989). Germane to this work, when the crude SR was subjected to centrifugation through a 15–40% linear sucrose density gradient, the bulk (75–80%) of the SR was harvested from the 36% sucrose layer. The protein profile, as determined by one-dimensional SDS-polyacrylamide gel electrophoresis, was identical for the crude and the fractionated SR (Fig. 1, upper). As shown by two-dimensional electrophoresis (Fig. 1, lower), there was only one protein found in the 96,000–100,000-Da range with a negative pI. This protein was identified as the CaATPase using immunoblotting techniques and a monoclonal antibody to the cardiac CaATPase (data not shown).

**Inhibition of Calcium-dependent ATPase Activity by FITC—**When FITC was incorporated at pH 7.0 and 30°C for 30 min, the GTPase activity was not inhibited, although the calcium-dependent ATPase activity was 100% inhibited at 60 μM (Fig. 2). The I₅₀ for FITC inhibition of calcium-dependent ATPase activity was 15 ± 2 μM (n = 3). This is a higher concentration than the I₅₀ value of 8 μM when the incorpora-
crude SR and Fraction C are similar and that a single protein exists

Coomassie Blue; SDS-polyacrylamide

cation; the SR was then diluted 100 times into the reaction medium,
and the reaction was started 5 min later with the addition of 1 mM
NTP. The GTPase activity was measured in the presence of calcium;
identical findings were observed in the absence of calcium. The
for the inhibition of calcium-dependent ATPase activity was 15 ± 2
μM (n = 3).

tion medium was pH 9; pH 7.0 was selected to preserve all
calcium-dependent reactions in cardiac SR, which are alkaline
pH-labile. Highsmith (1984) also noted that a higher concen-
tration of FITC is required for inhibition of the skeletal
muscle enzyme at neutral pH.

Protection against Inhibition by FITC—Full protection
against FITC-induced inactivation of calcium-dependent
ATPase activity was obtained by the inclusion of 2–5 mM
AMP-P(NH)P in the incorporation medium (Fig. 3), sup-
porting the work of others (Pick and Bassilian, 1981; High-
smith, 1984). We found that at least 100 μM AMP-P(NH)P
was required to protect against inactivation by FITC incor-
poration (Fig. 3). Our study indicates that FITC was bound
primarily (>95%) to the CaATPase protein and that this
binding was inhibited by AMP-P(NH)P (Fig. 4). The GTP
analogue GMP-P(NH)P (0.025–2 mM) did not protect against

enzyme inactivation or decrease the fluorescence intensity
(data not shown).

Inhibition of GTPase Activity by AMP-P(NH)P—As shown
previously (c.f. Tate et al., 1989), ATP and ATP analogues
inhibit GTPase activity in a noncompetitive fashion with a Kᵢ
compatible with the catalytic site (Kᵢ = 6–7 μM). The noncom-
petitive inhibition with a similar Kᵢ is observed in the presence
and absence of calcium (Tate et al., 1985) and in the presence
of both calcium and 2 μM ionomycin (data not shown). We
reasoned that if FITC incorporation prevents ATP binding
as postulated by Pick (1981), then AMP-P(NH)P should not
inhibit the GTPase activity in the FITC-modified enzyme.
This was not the case, however, as shown in Fig. 5. AMP-
P(NH)P inhibited the GTPase activity with a Kᵢ
compatible with the catalytic site (3–7 μM) despite FITC incorporation.
The type of inhibition, however, changed from noncompeti-
tive (no FITC) to competitive (plus FITC). The mechanism(s)
underlying this apparent shift in the type of inhibition is
unknown and cannot be resolved by the data in this paper.

Photosaffinity Labeling of SR by NTP—The inhibition
of GTPase activity by 1–5 μM AMP-P(NH)P suggested, but did
not prove, that the incorporation of FITC did not prevent
ATP binding to the CaATPase protein. To test directly this
hypothesis, [α-32P]ATP and [α-32P]GTP at 0.2 μM were co-

FIG. 1. Protein profiles of cardiac SR. Upper, one-dimensional SDS-polyacrylamide (10%) gels of crude SR and Fraction C (sedimenting at 96,000 Da) region. Similar findings were observed in three other experiments.

FIG. 2. Inhibition of calcium-dependent ATPase activity after FITC incorporation. FITC was incorporated into the SR (see "Experimental Procedures") at pH 7.0 at the concentrations indicated; the SR was then diluted 100 times into the reaction medium, and the reaction was started 5 min later with the addition of 1 mM NTP. The GTPase activity was measured in the presence of calcium; identical findings were observed in the absence of calcium. The IC₅₀ for the inhibition of calcium-dependent ATPase activity was 15 ± 2 μM (n = 3).

FIG. 3. Protection of calcium-dependent ATPase activity from FITC modification by AMP-P(NH)P. The SR was incorporated with 60 μM FITC or carrier (control, no AMP-P(NH)P) for 30 min at 30 °C and pH 7.0 (see "Experimental Procedures"); AMP-P(NH)P (0.025–5 mM) was included in the incorporation medium. The SR then was diluted 100-fold into the reaction medium for ATPase activity; 5 min later, 1 mM was added to start the reaction. The control calcium-dependent ATPase activity (carrier control) was 0.23 μmol/mg of protein/min. The calcium-independent ATPase activity was 0.042 μmol/mg of protein/min; it was unaffected by FITC treatment. GMP-P(NH)P at 0.025–2 mM did not protect against FITC inhibition. Similar findings were observed in three other experiments.

FIG. 4. Fluorescent labeling of CaATPase. The SR was incorporated with 60 μM FITC (see "Experimental Procedures") in the presence and absence of AMP-P(NH)P. FITC primarily (>95%) labeled the CaATPase, although the two minor bands were observed at 50,000–60,000 Da. When 1 mM AMP-P(NH)P was included in the incorporation medium, the fluorescent intensity decreased, and the calcium-dependent ATPase activity was protected (~80% of control). Full protection with a complete absence of observed fluorescence occurred at 2 mM AMP-P(NH)P. See Fig. 3 legend for details.
Figure 5. Inhibition of GTPase activity by AMP-P(NH)P in FITC-modified CaATPase. In three separate experiments with three different preparations, we determined the apparent \( V_m \) and apparent \( K_m \) for GTPase activity (25–200 μM GTP) in the presence and absence of 0–5 μM AMP-P(NH)P with and without 60 μM FITC incorporation. The kinetic constants were derived via linear regression of the double-reciprocal plots and verified by direct linear plot analysis (Eisenhal and Cornish-Bowden, 1974). The resulting data were analyzed by analysis of variance with the 0.05 level established for significance. Upper (Control), the significant decrease in the apparent \( V_m \) with no change in the apparent \( K_m \) with AMP-P(NH)P indicates noncompetitive kinetics; analysis of the slopes determined a \( K_i \) of 3.8 ± 0.4 μM for these data. Lower (FITC), the significant decrease in the apparent \( V_m \) with no change in the apparent \( K_m \) with AMP-P(NH)P indicates competitive kinetics; analysis of the slopes determined a \( K_i \) of 7.4 ± 1 μM. The difference between the \( K_i \) for the control and the \( K_i \) for the FITC-treated SR was significant using nonparametric techniques.

Figure 6. Direct photoaffinity labeling of nucleotides. FITC (60 μM) was incorporated into the SR (see “Experimental Procedures”) and was centrifuged at 40,000 \( \times g \) for 30 min to remove the unbound FITC; the control (carrier only) was treated in the exact same manner. The resultant protein pellets were suspended in 40 mM Tris maleate (pH 6.8) and frozen at -80 °C. The thawed protein was included in the labeling medium as indicated. Shown is one out of three separate experiments; the labeling patterns were identical in both the control and FITC-modified SR. Note that several proteins besides the CaATPase were labeled with both ATP and GTP. Laser densitometric tracings of the autoradiographs revealed that the protein above the CaATPase has a molecular mass of ~130,000 Da; it apparently binds ATP and GTP at a common site. This protein is the only protein that was labeled with [γ-32P]GTP in the absence of UV radiation. The protein at ~50,000 Da binds ATP, but not GTP (to any degree); and the protein immediately below the CaATPase (~80,000 Da) binds GTP, but not ATP. Additionally, there are GTP-binding proteins in the 40,000-Da region; they are G-proteins as shown by previous work from this laboratory (Scherer et al., 1987).

Figure 7. Inhibition of calcium-dependent phosphoenzyme content in FITC-modified SR. The calcium-dependent phosphoenzyme formed from 100 μM ATP (see “Experimental Procedures”) was inhibited in FITC-modified SR (A); however, the calcium-independent phosphoenzyme from ATP (B) was unaffected by FITC modification. Similar findings were noted when the nucleotide concentration was 50 μM. The same pattern of phosphorylation (no FITC) for both 50 μM ATP and 50 μM GTP was observed when rapid chemical quenching techniques (Bick et al., 1983) were used. See text for additional details.
TABLE I

Inhibition of detergent-activated calcium-dependent GTPase activity by FITC incorporation.

The SR was incorporated with 60 μM FITC or carrier (control) for 30 min at pH 7.0 (see "Experimental Procedures") and then was diluted 100-fold into the reaction medium for GTPase activity (20 μg of protein/ml final concentration); 1 mM GTP started the reaction 5 min later. After 5 min, 5 μg/ml (final concentration) CaEG was added to the ongoing reaction; this procedure was repeated three more times with 5 min between each addition. Note that the detergent-activated calcium-stimulated GTPase activity was inhibited in the FITC-modified SR. The calcium-insensitive GTPase activity remained unaffected by FITC modification. Similar results were observed with five other experiments.

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<tr>
<th>CEG</th>
<th>Control</th>
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<td>μg/ml</td>
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DISCUSSION

We previously reported that ATP and ATP-like analogues noncompetitively inhibit the apparent hydrolysis of GTP by canine cardiac SR with a K compatible with the high affinity nucleotide-binding site (Bick et al., 1983; Tate et al., 1985). Because FITC treatment blocks high affinity ATP binding, as reported by others (c.f. Pick and Bassilian, 1981), we hypothesized that the incorporation of FITC would prevent the inhibition of GTPase activity by AMP-P(NH)P. However, this hypothesis was rejected because: 1) the inhibition of GTPase activity by 3–7 μM AMP-P(NH)P remained despite the incorporation of FITC, and 2) the results from direct photoaffinity labeling of ATP and GTP demonstrated that the incorporation of FITC did not prevent nucleotide binding at 0.2 μM. Furthermore, protection of calcium-dependent ATPase activity against FITC incorporation occurred over a much higher concentration range (>100 μM AMP-P(NH)P than that required to saturate the high affinity catalytic site, supporting the earlier work of Pick (1981) and Highsmith (1984), but not Murphy (1988). Because there is one ATP-binding site/CaATPase molecule (Meissner, 1973; Pang and Briggs, 1977; but see Coll and Murphy, 1991), we conclude that FITC incorporation into the cardiac CaATPase does not prevent high affinity nucleotide binding. During the course of this study, other studies appeared in the literature that suggested (but did not prove) that FITC incorporation may not interfere with low affinity ATP binding to the skeletal muscle CaATPase (Champel et al., 1988) and to the (Na+ + K+)-ATPase (Davies and Robinson, 1988). Ball and Friedman (1987), using immunological techniques, concluded that the FITC-binding site may not be the ATP-binding site of the (Na+ + K+)-ATPase. We agree with this latter conclusion with the CaATPase of cardiac SR.

Although nucleotide binding in cardiac SR is not prevented by FITC, the results of our experiments indicate that FITC incorporation may alter the calcium-dependent conformational changes necessary for calcium-sensitive nucleotide hydrolysis. This suggestion is supported by the experiments in which FITC incorporation abolished: 1) calcium-dependent ATPase activity in native cardiac SR and detergent-activated calcium-sensitive GTPase activity, 2) calcium-sensitive phospho-enzyme formation from ATP, and 3) calcium accumulation by both ATP and GTP. Although calcium-dependent reactions were inhibited by FITC incorporation, this cannot be construed necessarily to mean that FITC blocks calcium binding since fluorescent changes are noted after the addition of calcium to the FITC-labeled enzyme (Pick and Karlish, 1980). It is possible, however, that calcium binding to an essential domain necessary for calcium-stimulated nucleotide hydrolysis or some calcium-dependent partial reaction is prevented by FITC incorporation. In our previous work (Tate et al., 1989), we emphasized that, although they differed markedly regarding nucleotide specificity and sensitivity, both the calcium-dependent ATPase activity and the detergent-activated calcium-dependent GTPase activity are dependent on high affinity calcium binding and are blocked by micromolar vanadate levels. Thus, even though they are distinct, these two calcium-dependent nucleotidase mechanisms may share a common calcium-dependent partial reaction (Tate et al., 1989) that is in some manner altered by FITC incorporation.

In keeping with the above hypothesis, the phosphoenzyme formed from 50–100 μM ATP or GTP in the absence of calcium was unaffected by FITC pretreatment. This phosphoenzyme was acid-stable and sensitive to hydroxylamine, indicating that the phosphorylated species is an acylphosphate. Others (Froehlich and Taylor, 1976; Carvalho-Alves and Scofano, 1987) also noted a magnesium-dependent calcium-insensitive acylphosphoenzyme in skeletal muscle CaATPase.

There is also the possibility in cardiac CaATPase that there may be more than one FITC reactive species (Kirley et al., 1986). In this context, Swoboda and Hasselbach (1985) demonstrated that FITC reacts with amino and thiol groups in the skeletal muscle CaATPase and that the fast reaction of the hyper-reactive lysine to FITC depends on intact sulfhy-
dryl groups in native vesicles. In detergent-modified vesicles, they found that FITC reacts preferentially with sulphydryl groups rather than amino groups. Therefore, it is possible that FITC may alter essential sulphydryl groups as well as amino groups in cardiac CaATPase that are necessary for calcium-linked conformational changes required for calcium-dependent NTP hydrolysis.

In our original study comparing and contrasting ATP and GTP hydrolysis by canine cardiac SR (Van Winkle et al., 1981), we suggested that GTP hydrolysis by the cardiac CaATPase occurs either by an alternative hydrolysis cycle or by entry via a different reaction into the classic nucleotide hydrolysis cycle. In subsequent studies (Bick et al., 1983; Tate et al., 1985), we presented additional evidence compatible with an alternative substrate entry site for these nucleotides in cardiac SR. Further data supporting this latter hypothesis is given in this report, where we show, using photoaffinity labeling techniques, substantial binding of an equivalent amount of ATP or GTP at 0.2 μM. The addition of a 50-fold excess of homologous nucleotide (10 μM) completely displaced either radiolabeled nucleotide, whereas heterologous nucleotide (10 μM) displaced relatively minor nucleotide (never >60%).

During the course of this study, a report from Ogurusu et al. (1989) appeared showing that, in their hands, a calcium-stimulated GTP hydrolysis is observed with canine cardiac SR. They presented data indicating that the mechanism of GTP hydrolysis goes through the same classic enzyme cycle as does ATP. They challenged us by stating that GTP hydrolysis is calcium-sensitive, involves a turnover of a calcium-sensitive acylphosphate, and supports oxalate-facilitated calcium uptake. Our disagreement, like Ogurusu et al., (1989), we also find a minor GTP-dependent oxalate-facilitated calcium uptake. Our disagreement, however, is that we find no evidence of a calcium-sensitive phosphoenzyme formed from 50 and 100 μM GTP (this report) or 1 mM GTP (Tate et al., 1989) under similar assay conditions even when a calcium-sensitive GTPase activity is observed. We consistently find that AMP-P(NH)P inhibits the GTPase activity noncompetitively in native cardiac SR, this inhibition was not addressed by Ogurusu et al. (1989).

With the data in this report as well as in our previous studies (Van Winkle et al., 1981; Bick et al., 1983; Tate et al., 1985, 1989; Entman et al., 1986), we suggest that the GTP hydrolysis, which has similarities and dissimilarities to ATP hydrolysis, may occur by a different, but related mechanism in the CaATPase of cardiac SR. The pathway(s) of the GTPase mechanism in the overall reaction cycle of the enzyme is unknown presently; however, the pathway of the GTPase shares with the ATPase a substrate that has a high affinity for calcium, a low affinity for nucleotide, and a high sensitivity to vanadate (c.f. Tate et al., 1989) and, as shown in this report, is completely inhibited by FITC incorporation. The possibilities include parallel pathways or entry by GTP at a more distal site in the classic enzyme cycle.

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