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ATP regulation of calcium transport in back-inhibited sarcoplasmic reticulum vesicles

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At high concentrations of ATP, ATP hydrolysis and Ca^{2+} transport by the ($Ca^{2+} + Mg^{2+}$)-ATPase of intact sarcoplasmic reticulum vesicles exhibit a secondary activation that varies with the extent of back-inhibition by Ca^{2+} accumulated within the vesicles. When the internal ionized Ca^{2+} is clamped at low and intermediate levels by the use of Ca-precipitating anions, the apparent K_m values for activation by ATP are lower than in fully back-inhibited vesicles (high internal Ca^{2+}). In leaky vesicles unable to accumulate Ca^{2+}, raising Ca^{2+} in the assay medium from 20–30 μM to 5 mM abolishes the activation of hydrolysis by high concentrations of ATP. The level of [^{32}P]phosphoenzyme formed during ATP hydrolysis from [^{32}P]phosphate added to the medium also varies with the extent of back-inhibition; it is highest when Ca^{2+} is raised to a level that saturates the internal, low-affinity Ca^{2+} binding sites. In intact vesicles, increasing the ATP concentration from 10 to 400 μM competitively inhibits the reaction of inorganic phosphate with the enzyme but does not change the rate of hydrolysis. In a previous report (De Meis, L., Gomez-Puyou, M.T. and Gomez-Puyou, A. (1988) Eur. J. Biochem. 171, 343–349), it has been shown that the hydrophobic molecules trifluoperazine and iron bathophenanthroline compete for the catalytic site of the P_i-reactive form of the enzyme. Here it is shown that inhibition of ATP hydrolysis by these compounds is reduced or abolished when Ca^{2+} binds to the low-affinity Ca^{2+} binding sites of the enzyme. Since inhibition by these agents is indifferent to activation of hydrolysis by high concentrations of ATP, it is suggested that the second K_m for ATP and the inhibition by hydrophobic molecules involve two different Ca-free forms of the enzyme.

Introduction

Sarcoplasmic reticulum membranes isolated from skeletal muscle form tightly closed vesicles that retain an active ($Ca^{2+} + Mg^{2+}$)-dependent, membrane-bound ATPase. This enzyme catalyzes the accumulation of Ca^{2+} into the vesicles' interior in a process that is coupled to the hydrolysis of ATP. An outstanding characteristic of the transport-coupled ATP hydrolysis is its complex ATP concentration-dependence [1–6]. Hydrolysis rates increase to a plateau as the ATP concentration is raised from 0.1 μM to 10 and 100 μM, and a second increase occurs in the millimolar range. Fast kinetic studies of calcium transport and steady-state rates of Ca^{2+} accumulation in the presence of oxalate also show an increase with millimolar ATP [7,8], whereas the steady-state level of phosphoenzyme formed from ATP remains constant after the first plateau [8–11]. These data are consistent with the postulate that the ATPase possesses two classes of binding site for ATP: a high-affinity, catalytic site; and a second, low-affinity site, where binding of ATP allosterically activates turnover at the catalytic site [11–13]. Recently, other signs of regulation by ATP have been detected, and more than one point in the ATPase reaction cycle has been suggested as a target [9,14–19]. In addition, it has been proposed that catalysis and its regulation involve only a single ATP binding site, which alternates between high and low affinities during the reaction cycle [9,14,17,20–22].

A second prominent feature of transport-coupled ATP hydrolysis is its inhibition by the accumulation of Ca^{2+} in the lumen of the sarcoplasmic reticulum [23–25].

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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Calcium at the external surface of the vesicles activates the ATPase when it binds to a high-affinity site ($K_{0.5}$ = 0.1–10 μM), and it is transported into the vesicles. 'Back-inhibition' is attributed to saturation of internally oriented, low-affinity sites by Ca^{2+} accumulated in the interior; persistence of this Ca_2-phosphoenzyme complex slows the release of P_i [26–28]. During net uptake of Ca^{2+}, initial rates of ATP hydrolysis therefore rapidly decline.

The relationship between back-inhibition by internal Ca^{2+}, which depresses ATP hydrolysis, and high concentrations of ATP, which activate it, is not clear. In the different preparations of intact and leaky vesicles and solubilized enzyme, values that have been reported for the second K_m vary over a wide range [3,11,20, 29–33]. In some cases, only the high-affinity K_m was found. These changes in ATP-dependence are also not fully understood. In this study, we make use of different experimental maneuvers to vary the extent of back-inhibition in intact and leaky vesicles. It is shown that the second K_m for ATP depends critically on the extent of saturation of the internal, low-affinity Ca^{2+} binding sites, and that back-inhibition alters the response to inhibitors that bind to the form of the enzyme that is phosphorylated by P_i [34].

Materials and Methods

Sarcoplasmic reticulum vesicles (intact). These were prepared from rabbit white skeletal muscle [35]. Leaky vesicles were prepared from intact vesicles according to Method 2 of Meissner et al. [36] and stored in liquid nitrogen. In this preparation, the vesicles retain their membranous profiles and have the same ratio of lipid to protein as native membranes, but they do not accumulate Ca^{2+} [36].

Assay mixtures. All mixtures used for hydrolysis, Ca^{2+} uptake and phosphorylation contained 50 mM Mops-Tris or Mops-Na, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate (tricyclohexylammonium salt), 28 μg or 10 U/ml pyruvate kinase (EC 2.7.1.40, Sigma Type II), 80 mM KCl, and concentrations of ATP (Na-Tris salt) ranging from 1 μM to 4 mM, at 25°C and pH 6.8–6.9. The use of excess Mg^{2+} ensured that most of the ATP present was in the form of $MgATP^{2-}$, the true substrate of the reaction [37]. For ionized Ca^{2+} concentrations in the micromolar range (20–30 μM), 0.12 mM $CaCl_2$ and 0.10 mM Tris-EGTA were added. For Ca^{2+} concentrations in the millimolar range, EGTA was omitted and 1–5 mM $CaCl_2$ was added.

Ca^{2+}-activated ATP hydrolysis. For the hydrolysis, assays were started by the addition of vesicle protein (0.05 mg/ml leaky vesicles or 0.2 mg/ml intact vesicles), and stopped by the addition of molybdovanadate reagent for the determination of inorganic phosphate [38]. In experiments with leaky vesicles, Mg^{2+}-dependent, basal ATP hydrolysis [39] in the absence of Ca^{2+} (EGTA added) was negligible. When intact vesicles were used, basal ATPase activity was measured in parallel experiments at each ATP concentration and subtracted from the activity in the presence of Ca^{2+}. For Ca^{2+} uptake, $^{45}CaCl_2$ and in some experiments, 4 mM Tris phosphate or Tris oxalate were present, at pH 6.6–6.9. Assays were started by the addition of intact vesicles (0.2 mg/ml) and stopped by Millipore filtration, or by quenching with a cold solution of 20 mM $LaCl_3$, 80 mM KCl and 20 mM Mops (pH 7.0) [40].

Enzyme phosphorylation from P_i. [^{32}P]Phosphate and protein concentrations of 0.3–0.4 mg/ml were used. Reactions were started by the addition of protein and stopped after 1 min at 25°C by addition of an equal volume of ice-cold, 0.25 M perchloric acid containing P_i, as previously described [41,42].

Reagents. Phosphoenolpyruvate, vanadium-free ATP, Mops, Tris, EGTA, pyruvate kinase, trifluoperazine, HCl and bathophenanthroline were obtained from Sigma Chemical Company (St. Louis, MO). The latter two compounds were prepared for use as described elsewhere [34]. ^{45}Ca was obtained from New England Nuclear (Wilmington, DE) and ^{32}P from the Brazilian Institute of Atomic Energy (São Paulo, Brazil). ^{32}P was purified as previously described [43] and stored in dilute HCl. Other reagents were of analytical grade.

Results

Calcium accumulation

Internal free Ca^{2+} concentrations, and thus the extent of back-inhibition, can be clamped at different levels in intact vesicles by the use of anions that have different solubility products for precipitation of their Ca salts [44]. In the presence of either 4 mM P_i or 4 mM oxalate, the rate of Ca^{2+} uptake increased from a low level (at 1–2 μM ATP) to a plateau value (extending from 20 to 400 μM ATP). A further increase occurred with ATP in the millimolar range, up to the maximum concentration that could be tested (data not shown). At any given ATP concentration, the rate of Ca^{2+} uptake by intact vesicles was much slower in the presence of P_i (one-third to one-sixth of the rate with oxalate). It is likely that the steady-state rate of Ca^{2+} uptake in the presence of 4 mM P_i reflects a partially back-inhibited condition due to the higher internal Ca^{2+} concentration, whereas in the presence of 4 mM oxalate, Ca^{2+} is lower and back-inhibition is much less evident [24]. Accordingly, the K_m value for ATP at the low-affinity site was 2 mM in the presence of P_i and 0.6 mM in the presence of oxalate (Table I). There was no change in the K_m at the high-affinity site (Table I).

ATP hydrolysis

In vesicles made permeable to Ca^{2+} by treatment with detergents [36], the level of back-inhibition is di-

TABLE I

ATP concentration-dependence of hydrolysis and Ca^{2+} uptake with different degrees of back-inhibition

Ca^{2+} uptake was measured using intact vesicles (0.2 mg/ml), 0.12 mM $^{45}CaCl_2$, 0.1 mM EGTA and either 4 mM P_i or 4 mM oxalate, with an ATP-regenerating system and 14 concentrations of ATP between 1 μM and 4 mM. Other components are given in Methods. Reactions were stopped by Millipore filtration after 1 min (for P_i) or 10 s (for oxalate). K_m values are based on Eadie-Hofstee plots. For ATP hydrolysis, the anions were omitted and concentrations of non-radioactive $CaCl_2$ ranging from 0.12 (with EGTA) to 5 mM (without EGTA) were used. Data with 20–30 μM and 5 mM Ca^{2+} are from Fig. 1. Reaction times were 1–4 min for leaky vesicles (0.05 mg/ml) and 2–4 min for intact vesicles (0.2 mg/ml). *n* represents the number of measurements made at each ATP concentration.

Vesicles	Activity	*n*	Ca^{2+} in medium	K_m for ATP (μM)
Intact vesicles plus 4 mM oxalate	Ca^{2+} uptake	4–6	20–30 μM	5.7 and 565
Intact vesicles plus 4 mM P_i	Ca^{2+} uptake	2–10	20–30 μM	3.2 and 2070
Leaky vesicles	ATPase	3	20–30 μM	7.8 and 111
		1	1 mM	9.2 and 247
		1	2 mM	6.8 and 333
		3	5 mM	6.3 and > 4000
Intact vesicles	ATPase	4	20–30 μM	4.5 and ≥ 2300

rectly dependent on the concentration of Ca^{2+} added to the assay medium. A comparison of ATP hydrolysis by intact and leaky vesicles under identical conditions reveals two distinct differences (Fig. 1, upper and middle curves). In both preparations, the medium contained sufficient Ca^{2+} to saturate the high-affinity Ca^{2+} binding sites (20–30 μM). With the intact vesicles, hydrolysis was measured after 2–4 min in the reaction mixture. This time is more than adequate for the Ca^{2+} content of intact vesicles to reach a steady value [25], and so the measured rate is characteristic of maximally back-inhibited vesicles. In agreement with other reports [11,20,25,45], the rate of hydrolysis by intact vesicles at any concentration of ATP tested (Fig. 1, middle curve) is only a fraction of that observed with leaky vesicles, where the concentration of Ca^{2+} is the same low value both inside and outside the vesicles (Fig. 2, upper curve). A second difference is seen in the upper range of ATP concentrations, where hydrolysis by leaky vesicles shows further activation. An Eadie-Hofstee plot of the data from 0.4 mM to 4 mM ATP (Fig. 1, inset) reveals a second apparent K_m of 0.11 mM for the leaky vesicles, whereas for intact vesicles the activation was detectable only above 2 mM. In this case, only a lower limit (2.3 mM) could be calculated for the K_m. The K_m values at the high-affinity sites, on the other hand, are similar for both preparations (Table I).

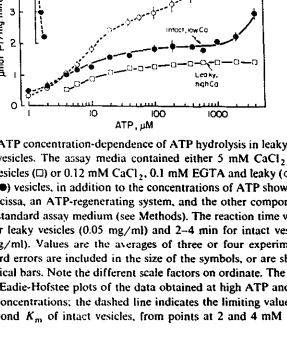


Fig. 1. ATP concentration-dependence of ATP hydrolysis in leaky and intact vesicles. The assay media contained either 5 mM $CaCl_2$ and leaky vesicles (□) or 0.12 mM $CaCl_2$, 0.1 mM EGTA and leaky (○) or intact (●) vesicles, in addition to the concentrations of ATP shown on the abscissa, an ATP-regenerating system, and the other components of the standard assay medium (see Methods). The reaction time was 1 min for leaky vesicles (0.05 mg/ml) and 2–4 min for intact vesicles (0.2 mg/ml). Values are the averages of three or four experiments. Standard errors are included in the size of the symbols, or are shown by vertical bars. Note the different scale factors on ordinate. The inset shows Eadie-Hofstee plots of the data obtained at high ATP and low Ca^{2+} concentrations; the dashed line indicates the limiting values for the second K_m of intact vesicles, from points at 2 and 4 mM ATP.

To explore the possibility that the higher Ca^{2+} concentration attained inside the intact vesicles might be responsible for shifting the K_m at the low-affinity ATP site, hydrolysis by leaky vesicles was measured with higher concentrations of $CaCl_2$ in the assay medium. With 5 mM $CaCl_2$, the second K_m could no longer be detected (Fig. 1, lower curve). With Ca^{2+} concentrations of 1 and 2 mM, intermediate K_m values were obtained (Table I). The K_m of the high-affinity site did

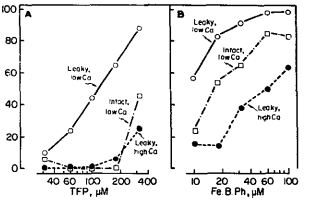


Fig. 2. Inhibition of ATP hydrolysis by trifluoperazine (TFP) and iron bathophenanthroline (Fe.B.Ph). Assay media contained 2 mM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 100 mM KCl, 50 mM Mops-Tris (pH 6.5), 5 mM P_i-Tris, and either 5 mM $CaCl_2$ (●) or 0.5 mM $CaCl_2$ + 0.5 mM EGTA (○, □). Reactions were started by adding 0.05 mg/ml of either leaky (circles) or intact (squares) vesicle protein, and terminated after 1 min at 35°C by the addition of activated charcoal in HCl [52]. After centrifugation, [^{32}P]P_i release was quantitated by counting the supernatant.

not change significantly with the increase in Ca^{2+} concentrations (Table I).

The experiments presented so far suggest that higher concentrations of ATP are needed to activate the enzyme when internal Ca^{2+} binding sites are occupied by Ca^{2+}. This might mean that the binding of Ca^{2+} to these sites reduces access to the regulatory site for ATP. Existing hypotheses for the nature of the ATP regulatory site include two alternatives: that ATP binds to the catalytic site itself, which can assume a low-affinity configuration, or that ATP binds with low affinity to a second site that is distinct from the catalytic site (Ref. 9, and references therein). The next experiments were designed to characterize occupancy of the catalytic site in the back-inhibited enzyme.

Effects of hydrophobic molecules

In previous reports, it has been shown that when the enzyme has a low affinity for Ca^{2+}, it can be phosphorylated readily by P_i [28,41,46]. Recently, the hydrophobic compounds trifluoperazine and iron bathophenanthroline were identified as molecules that interact with this P_i-reactive form of the enzyme [34]. Their ability to competitively inhibit ATP synthesis and phosphoenzyme formation from [^{32}P]P_i in the medium indicated that these two drugs block the entry of P_i into the catalytic site. Fig. 2 shows that both compounds inhibited ATPase activity of leaky vesicles in the presence of 2 mM ATP and a low Ca^{2+} concentration (no back-inhibition). However, inhibition by the drugs was reduced (Fig. 2B) or abolished (Fig. 2A) when the enzyme was back-inhibited. This was observed both in fully back-inhibited, leaky vesicles (5 mM $CaCl_2$ on both sides of the membrane), and in partially back-inhibited, intact vesicles that were allowed to accumulate Ca^{2+} in the presence of 5 mM P_i (lower and middle curves in Figs. 2A and 2B). In leaky vesicles at a low Ca^{2+} concentration, the use of different substrate conditions did not alter the profile of inhibition obtained with increasing concentrations of trifluoperazine or iron bathophenanthroline (data not shown). In these experiments, each drug tested was equally effective as an inhibitor, regardless of whether the substrate was 3 mM ATP (above the second K_m), 0.1 mM ATP (below the second K_m) or 1 mM ITP (which does not show the second K_m [47]).

Phosphorylation by P_i

Phosphorylation of the enzyme by P_i is inhibited by the binding of Ca^{2+} to the external, high-affinity sites [28,41,46]. However, this inhibition can be overcome by a low concentration of ATP: the ensuing reactions of ATP hydrolysis ensure a steady-state supply of enzyme in a form that reacts reversibly with P_i [47]. In the following experiments, the steady-state level of [^{32}P]phosphoenzyme formed during ATP hydrolysis

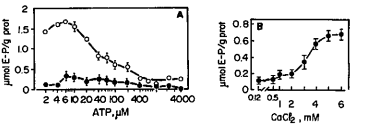


Fig. 3. Phosphorylation of intact and leaky vesicles by P_i. In (A), assay media contained 0.12 mM $CaCl_2$, 0.1 mM EGTA, 4 mM [^{32}P]phosphate, the concentrations of ATP shown on the abscissa and 0.4 mg/ml of either (○) intact or (●) leaky vesicle protein. In (B), assay media contained 4 mM [^{32}P]phosphate, 20 μM ATP, 0.35 mg/ml leaky vesicle protein, and the concentrations of $CaCl_2$ shown on the abscissa. At the lowest concentration of $CaCl_2$, 0.1 mM EGTA was also present. In both (A) and (B), an ATP-regenerating system was present. For other components, see Methods. Reactions were started by the addition of protein and quenched with cold perchloric acid after 1 min at 25°C. Average values (±S.E.) are from three experiments.

from [^{32}P]phosphate added to the medium was measured in order to evaluate the availability of P_i-reactive forms, with and without back-inhibition. The presence of an ATP-regenerating system ensured that overall reversal of the cycle through transfer of P_i from the phosphoenzyme to ADP was reduced to a minimum.

At low ATP concentrations, and when leaky vesicles were used so that the concentration of Ca^{2+} inside and outside the vesicles was sufficient to saturate only the high-affinity Ca^{2+} binding sites, very little [^{32}P]phosphoenzyme was formed from [^{32}P]phosphate (lower curve in Fig. 3A). In back-inhibited vesicles, on the other hand, [^{32}P]phosphoenzyme levels were 10–20-fold higher (upper curve in Fig. 3A). In these vesicles, increasing the ATP concentration from 10 to 400 μM caused [^{32}P]phosphoenzyme formation from [^{32}P]P_i to fall nearly to the level seen with low Ca^{2+} on both sides of the membrane. At a low ATP concentration (Fig. 3B), raising the concentration of Ca^{2+} in the medium from 20–30 μM to 5 mM eliminated about half of the difference between levels of [^{32}P]phosphoenzyme found in intact and leaky vesicles. These data indicate that back-inhibition involves accumulation of an enzyme

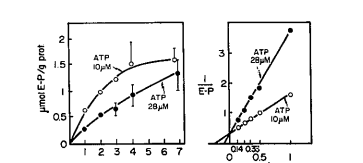


Fig. 4. ATP inhibition of phosphorylation by P_i. Reaction conditions as in Fig. 3 for intact vesicles, except that [^{32}P]phosphate was varied between 1 and 7 mM and ATP was either 10 μM (○) or 28 μM (●). The results shown are typical of three separate experiments.

form that is phosphorylated by P_i and that intermediate concentrations of ATP counteract the contribution of P_i to this phosphoenzyme pool.

The data of Fig. 4 show that ATP competitively inhibits phosphorylation by P_i. An increase in ATP concentration from 10 to 28 μM more than doubled the K_m for P_i, without affecting the calculated maximum steady-state level of phosphorylation for the enzyme saturated with P_i. These results, obtained during steady-state ATP hydrolysis (with Ca^{2+} present), are similar to those reported previously for equilibrium measurements (with EGTA in the medium) [48].

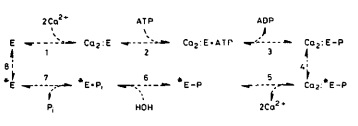
Discussion

The data presented in this paper show three pronounced effects of back-inhibition by Ca^{2+} in intact and leaky vesicles: (1) an increase in the steady-state level of [^{32}P]phosphoenzyme formed from [^{32}P]P_i added to the medium (Fig. 3); (2) a decrease in the interaction with hydrophobic drugs (Fig. 2); and (3) an increase in the second K_m for ATP (Table I). A number of our experiments were aimed at characterizing the occupancy of the catalytic site of the P_i-reactive enzyme forms that have a low affinity for Ca^{2+}, and the data suggest that access to one of these forms may be an important factor for regulation by ATP. To a considerable extent, this access will be altered by changes in the position of the rate-limiting step.

A number of studies from different laboratories indicates that the rate-limiting reaction at low internal Ca^{2+} concentrations occurs during or after hydrolysis of the phosphoenzyme [7,10,17,49]. One possible interpretation [47] localizes the rate-limiting step at the * E-to-E transition (step 8) in Scheme I.

The experimental evidence for this sequence has been discussed elsewhere [47,49,50]. In this scheme, * E can be phosphorylated by P_i and has low-affinity Ca^{2+} binding sites that face the vesicle interior, whereas E can bind ATP and has high-affinity Ca^{2+} sites facing outwards.

In leaky vesicles at low Ca^{2+} concentrations, most of the enzyme is phosphorylated by the nucleotide [12], and the level of phosphoenzyme from $^{32}P_i$ added to the medium is low (Fig. 3). In back-inhibited vesicles, however, the low-affinity Ca^{2+} binding sites are saturated and the release of Ca^{2+} into the interior of the vesicles



Scheme I.

becomes rate-limiting [28]. The enzyme form with Ca^{2+} bound to the low-affinity sites (Ca_2:* E-P) accumulates, and the steady-state level of phosphoenzyme from [^{32}P]P_i increases (Fig. 3).

The fact that back-inhibition leads to loss of activation by ATP as well as loss of inhibition by hydrophobic drugs indicates that both classes of compound require access to a form of the enzyme that does not have Ca^{2+} bound to the low-affinity sites. This might be the P_i-reactive form, * E, or the phosphoenzyme * E-P; both populations would be reduced by the accumulation of the forms Ca_2:* E-P in back-inhibited vesicles. The following observations are relevant to identification of the target for high concentrations of ATP: (1) The hydrophobic drugs competitively inhibit phosphorylation by P_i [34]; (2) Inhibition of hydrolysis by trifluoperazine is indifferent to the concentration of ATP, whether it is above or below the K_m for activation of turnover; (3) Inhibition by the hydrophobic drugs is equally effective with ITP, a substrate that does not activate turnover [47]. Thus, although both the second K_m and the hydrophobic drug effects are reduced or eliminated by back-inhibition, these data lead to the conclusion that different forms of the enzyme are involved in the two cases. Since the hydrophobic molecules appear to react with the catalytic site of * E [34], it may be that high concentrations of ATP react with * E-P. Intermediate concentrations of ATP would form an * E · ATP complex at the catalytic site, accelerating step 8, but the acceleration of turnover characterized by the second K_m would require binding of ATP to * E-P as well as to * E. In this view, a high concentration of ATP would accelerate both the rate of * E-P hydrolysis [7,17] and the rate of interconversion of * E and E [10,47,49]. This hypothesis is consistent with the competitive inhibition of phosphoenzyme formation from [^{32}P]P_i seen in Fig. 4. Competitive inhibition would also be observed if binding of ATP to * E-P accelerates * E-P hydrolysis without impeding the entry of P_i into the catalytic site in the reverse reaction – i.e., phosphorylation of * E by P_i. Binding of ATP to the phosphoenzyme * E-P just before release of P_i would be consistent with the proposal that the catalytic site of the ATPase is transformed into the regulatory site (low affinity) by phosphorylation [9,14,17,22,51]. The possibility that ATP also accelerates the forward reaction by binding to Ca_2:E-P [19] in back-inhibited vesicles is not excluded by these data.

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