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Functional evidence of a transmembrane channel within the Ca^{2+} transport ATPase of sarcoplasmic reticulum

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Ca^{2+} efflux can be studied conveniently following dilution of sarcoplasmic reticulum (SR) vesicles preloaded with $^{45}Ca^{2+}$ by active transport. The rates of efflux are highly dependent on ATPase substrates and cofactors (P_i, Mg^{2+}, Ca^{2+} and ADP) in the efflux medium. On the other hand, phenothiazines stimulate efflux through a passive permeability channel with no coupled catalytic events. Efflux activation by manipulation of catalytically active ATPase ligands, as well as by the catalytically inactive phenothiazines, can be prevented by thapsigargin, which is a highly specific inhibitor of the Ca^{2+}-ATPase. This demonstrates that the passive channel activated by phenothiazines is an integral part of the ATPase, and can operate either uncoupled or coupled to catalytic events.

Ca^{2+} efflux; ATPase channel; Sarcoplasmic reticulum; Thapsigargin; Phenothiazine

1. INTRODUCTION

The structural model [1] derived from the amino acid sequence of the Ca^{2+} transport ATPase of sarcoplasmic reticulum (SR) includes ten helical segments partitioning within the SR membrane as five hairpins. Four of these helices contain residues which are involved in Ca^{2+} binding functions [2], and are likely to cluster forming a channel for Ca^{2+} translocation across the membrane by active transport [3]. From the functional standpoint, an experimentally convenient means to test a channel presence in this system is the measurement of Ca^{2+} efflux from SR vesicles preloaded by active transport and diluted with media of various composition. It is then found that Ca^{2+} efflux can occur concomitant with, but also independent of, Ca^{2+} pump reversal [4–6]. In fact, the rates of Ca^{2+} efflux can be modulated by ATPase-specific ligands such as P_i and Mg^{2+}, but also by agents such as phenothiazines which are not coupled to catalytic events [7]. While it is apparent that the uncoupled efflux occurs through a passive channel, it has been difficult to demonstrate that such a channel is the same device used for Ca^{2+} translocation by the Ca^{2+} pump in its forward and reverse operation. Taking advantage of the highly specific interaction of thapsigargin (TG) with the SR ATPase [8,9], and its interference with coupled and uncoupled Ca^{2+} fluxes, we can now show that an ATPase transmembrane channel mediates Ca^{2+} efflux under conditions favoring or precluding coupling with catalytic activity.

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2. MATERIALS AND METHODS

Vesicular fragments of longitudinal SR were isolated from rabbit skeletal muscle according to Eletr and Inesi [10]. Thapsigargin (TG) was purchased from LC Service Corp. (Woburn, MA). Chlorpromazine was obtained from Rhodia, S.P. All other chemicals were purchased from Sigma.

SR vesicles (35–45 μg protein/ml) were preloaded with Ca^{2+} in a reaction mixture containing 50 mM MOPS–TRIS, pH 7.0, 10 mM $MgCl_2$, 20 mM P_i–TRIS, 0.3 mM $CaCl_2$ (in the presence or in the absence of radioactive ^{45}Ca tracer) and 5 mM ITP. Following 40 min incubation at 37°C, 5.0 ml aliquots were centrifuged in a refrigerated centrifuge and each sediment was resuspended in 0.45 ml of ice-cold water. 50 μl (or larger) aliquots of this suspension were diluted in 1.5 ml (or more) of efflux medium containing 50 mM MOPS–TRIS, pH 7.0, either 10.0 or 0.1 mM $MgCl_2$, either 4.0 or 0.1 mM P_i–TRIS (in the presence or in the absence of ^{32}P radioactive tracer), 2.0 mM EGTA, 10 mM glucose and 10 μg hexokinase/ml, in the presence or in the absence of 0.2 mM ADP, at 37°C. Serial samples were then filtered and washed with $LaCl_3$ for determination of ^{45}Ca remaining with the vesicles. Alternatively, the samples were quenched with an equal volume of 1 M PCA plus 4 mM P_i, and processed for determination of ATP [11].

3. RESULTS AND DISCUSSION

When loaded SR vesicles are suspended in a medium containing no Ca^{2+} (presence of EGTA), and very low P_i and Mg^{2+} concentrations (well below their ATPase dissociation constants), Ca^{2+} efflux proceeds with an initial rate of 200 nmol/mg protein/min at 37°C (Fig. 1A). The efflux is inhibited by 40 micromolar Ca^{2+} (saturating the high affinity sites on the outer surface of the

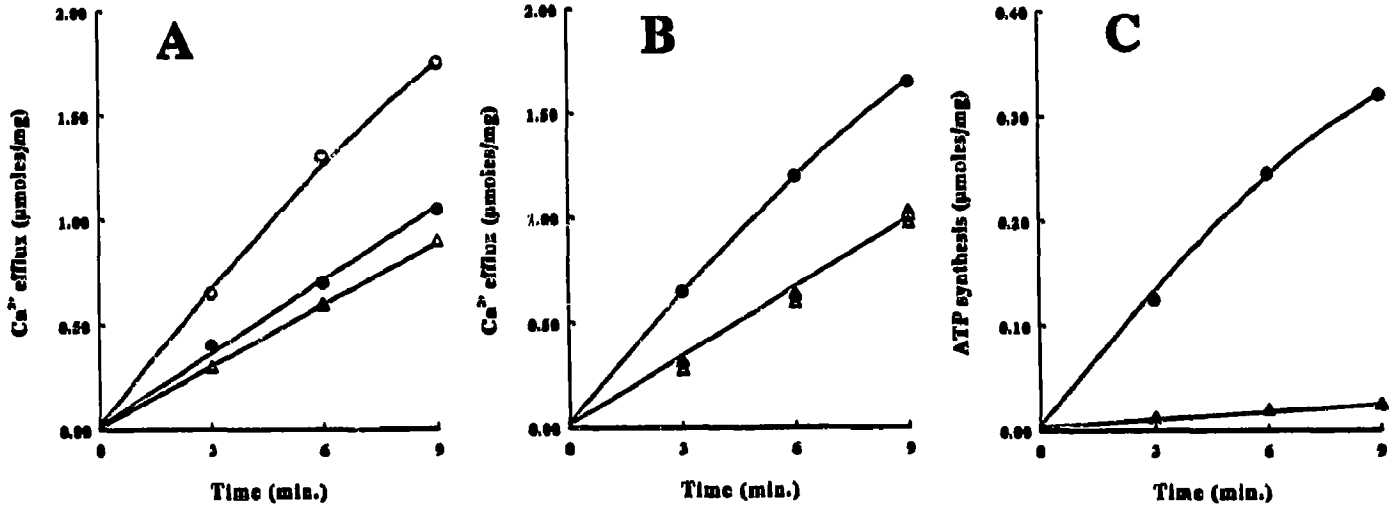

Fig. 1. Effect of substrate (P_i, ADP) and cofactor (Ca^{2+}, Mg^{2+}) manipulations on Ca^{2+} efflux: inhibition by TG. Preloaded SR vesicles were diluted with the following media. (A) 50.0 mM MOPS–TRIS, pH 7.0, 0.1 mM P_i, 0.1 mM $MgCl_2$, with either 2.0 mM EGTA (○), or 40 μM $CaCl_2$ (●), or 15 nmol TG/mg SR protein (△). (B and C) 4 mM P_i, 10 mM $MgCl_2$, 2 mM EGTA, and either no addition (○), or 0.2 mM ADP (●), or 15 nmol TG/mg SR protein (△), or 0.2 mM ADP plus 15 nmol TG/mg SR protein (▲).

vesicles), or (compare open circles in Fig. 1A and B) by millimolar Mg^{2+} and P_i (sustaining formation of phosphorylated enzyme intermediate through reversal of the catalytic cycle). If the enzyme is phosphorylated by P_i, the efflux can be reactivated by addition of ADP which allows cycling of the enzyme in the reverse direction, resulting in ATP synthesis, and coupled Ca^{2+} efflux can be regulated by specific substrates and cofactors of catalytic activity [4].

It was recently reported that the Ca^{2+}-ATPase of SR vesicles is inhibited by very low concentrations of TG [8], owing to stoichiometric and strong interaction of the inhibitor with the enzyme. Consistent with these findings, we find that the ADP-dependent Ca^{2+} efflux from loaded vesicles, as well as the coupled ATP synthesis, are totally inhibited by TG (Fig. 1B and C). Furthermore, even the efflux activation produced by lowering the P_i and Mg^{2+} concentrations is prevented by TG (Fig. 1A). Since ADP, P_i and Mg^{2+} are specific ATPase ligands, the experiments shown in Fig. 1 demonstrate that TG interferes with Ca^{2+} efflux from loaded vesicles through its interaction with the membrane-bound ATPase.

In addition to efflux activation by manipulations of ATPase substrates and cofactors, a large increase in Ca^{2+} efflux (Fig. 2) from loaded vesicles is produced by addition of phenothiazines such as trifluoperazine and chlorpromazine [7]. Owing to its uncoupling from catalytic activity, this efflux was interpreted as a passive flux of Ca^{2+} through a permeability channel opened by the

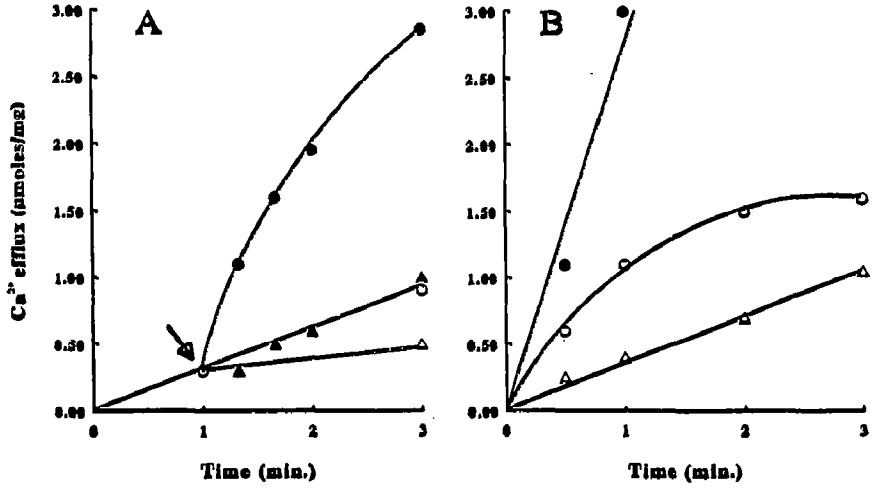

Fig. 2. Effect of phenothiazines on Ca^{2+} efflux: inhibition by TG. Preloaded SR vesicles were diluted with the following media. (A) 50 mM MOPS–TRIS, pH 7.0, 0.1 mM P_i, 0.1 mM $MgCl_2$, 2.0 mM EGTA, with either no addition (○), or 100 μM chlorpromazine added 1 min after dilution (●), or 1 μM TG (△), or 100 μM chlorpromazine and 1 μM TG added simultaneously 1 min after dilution (▲). (B) 0.1 mM P_i, 0.1 mM $MgCl_2$, 2.0 mM EGTA and 100 μM trifluoperazine (●), with either 4 μM TG (△), or 100 μM trifluoperazine plus 4 μM TG (○).

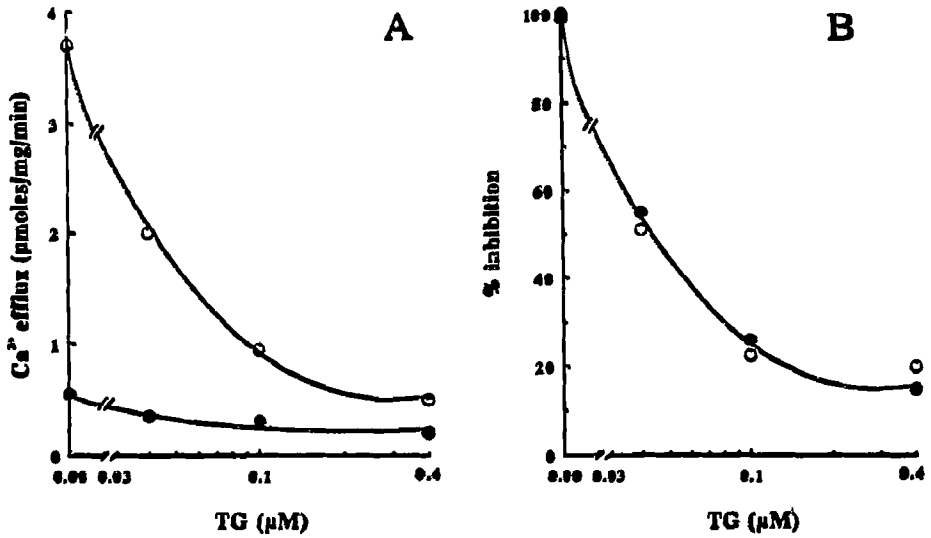

Fig. 3. Concentration dependence of TG inhibition of Ca^{2+} fluxes activated by substrate and cofactor manipulations, or by chlorpromazine. Preloaded SR vesicles were diluted in 50 mM MOPS–TRIS, pH 7.0, 0.1 mM P_i and 0.1 mM $MgCl_2$, with either no further addition (●), or 0.2 mM chlorpromazine (○).

phenothiazines. Such a channel could not be attributed unambiguously to the ATPase, since non-specific membrane perturbations by phenothiazines might also be involved. It was reported, however, that specific ATPase ligands prevent activation of Ca^{2+} efflux by phenothiazines without changing their membrane partition [7].

We now find that the Ca^{2+} efflux activation by phenothiazines can be prevented by TG (Fig. 2). It can be shown that efflux activation by ATPase substrate manipulations or by chlorpromazine, are prevented by TG within the same concentration range (Fig. 3), although the requirement for TG is increased if the phenothiazine concentration is raised higher. Since TG is a specific- and high-affinity inhibitor of the Ca^{2+}-ATPase, acting by stoichiometric interaction with the ATPase, its interference with the phenothiazine activation of Ca^{2+} fluxes must also be produced on the ATPase itself. We then conclude that the effect of phenothiazines on Ca^{2+} efflux is due to destabilization of the ATPase, independent of catalytic events. We also conclude that the destabilized domain corresponds to the transmembrane channel suggested by structural considerations, relatively distant from the catalytic domain. It is apparent that the ATPase channel can mediate Ca^{2+} fluxes in either a coupled or an uncoupled mode, with respect to catalytic events.

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