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Demonstration of the simultaneous activation of Ca^{2+}-independent and Ca^{2+}-dependent ATPases from rat skeletal muscle microsomes

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The activation of the Ca^{2+}-independent (basal) ATPase from rat skeletal muscle microsomes is demonstrated in the presence of enough Ca^{2+} to provide the simultaneous activation of the (Ca^{2+} + Mg^{2+})-ATPase. It was achieved taking advantage of the delayed inorganic phosphate (P_i) release due to the formation of a phosphoenzyme complex during the Ca^{2+}-dependent enzymatic cycle, which is evidenced in fast experiments. The microsomes were immobilized on a filter and perfused at constant flow with an incubation medium which was briefly interrupted with a pulse of appropriate reactants to activate the ATPases, at 2°C. Successive samples were collected after passing through the filter, at approx. 0.1 s intervals. The P_i effluent profile coincides with the pattern of the pulse when it activates only the Ca^{2+}-independent ATPase, it appears delayed when the pulse activates only extra P_i production by the (Ca^{2+} + Mg^{2+})-ATPase, and it includes a rapid and a delayed component when both Ca^{2+}-independent and Ca^{2+}-dependent ATPases are activated simultaneously by the pulse.

Introduction

Native skeletal muscle microsomes display a Ca^{2+}-independent and Mg^{2+}-dependent ATPase activity, in addition to the well-studied (Ca^{2+} + Mg^{2+})-dependent ATPase activity, unequivocally related to Ca^{2+} transport through the sarcoplasmic reticulum membranes. Most authors assume that the Ca^{2+}-independent, or 'basal' ATPase, is not related to the Ca^{2+}-transport-coupled ATPase. Consequently, the Ca^{2+}-dependent ATPase activity is expressed by the difference between results obtained in the presence and absence of Ca^{2+}. It implies the assumption that the basal ATPase is unaffected by Ca^{2+}. This hypothesis has not been directly tested because both activities require Mg^{2+}. Several arguments favor the above assumptions: both activities have different temperature coefficients, pH profiles and substrate affinities. While the basal activity is impaired by treatments with detergents, the Ca^{2+}-dependent, or 'extra' ATPase activity is enhanced. Thiol reagents inhibit the extra ATPase and have no effect on the basal activity [1,2].

On the other hand, it has been suggested that both activities are different manifestations of a unique enzyme [3–5]. It would operate by different paths, depending on the experimental conditions. The basal ATPase activity would contribute in variable proportions to the total activity in the presence of Ca^{2+} [3].

In this paper, we show that the basal ATPase operates in the presence of Ca^{2+}, simultaneously with the (Ca^{2+} + Mg^{2+})-ATPase. We use a rapid

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perfusion technique [5,6], and take advantage of the delay in the appearance of P_i, due to the transient formation of phosphoenzyme intermediates of the $(Ca^{2+} + Mg^{2+})$-ATPase.

Materials and Methods

The microsomal fraction from rat skeletal muscles was obtained by differential centrifugation, as described previously [7]. Rat skeletal muscle microsomes are less stable than those isolated from rabbits [8]. The microsomal suspensions were stored at 0° C and used within 48 h after preparation. No significant changes in ATPase activity were observed during this period.

The microsomes were deposited on a Millipore filter, and successively perfused with three different solutions, at constant flow ranging from 0.4 to 1.3 ml/s in different experiments. 50-μl samples were successively collected after passing through the preparation. Details of the method are given elsewhere [5,6]. The volume of the solution secondly perfused was very small, so that the microsomes were briefly exposed to their reactants. The perfusion media contained [γ-^{32}P]ATP and other reactants, as specified under Results. Radioactive inorganic phosphate ([^{32}P]P_i) was measured in the effluent samples after extraction as a phosphomolybdate complex in isobutanol. The radioactivity remaining in the aqueous phases after the extractions was also measured and taken as an index of the amount of nonhydrolyzed ATP. The Cerenkov radiation from both phases was measured in a liquid scintillation counter.

In some experiments, tritiated water was included in one of the successively perfused solutions, as a marker of their mixing. The 50-μl effluent samples were diluted and aliquots were taken for the determination of [^{32}P]P_i and [^{32}P]ATP as described above, and for ^{3}H radioactivity measurement in Bray's solution.

The experiments were performed at 2° C. Blanks were obtained by determination of [^{32}P]P_i present in the solutions before perfusions, and subtracted from the results.

[γ-^{32}P]ATP was prepared by the method of Glynn and Chapell [9]. ^{32}P was obtained from the Comisión Nacional de Energía Atómica de la República Argentina, as orthophosphoric acid. Disodium ATP and the enzymes and cofactors for [γ-^{32}P]ATP synthesis were obtained from Sigma, St Louis, MO. All other reagents were analytical grade. Protein concentration was measured by the method of Lowry et al. [10], using bovine serum albumin as standard.

Results

The microsomal preparation exhibited typical $(Ca^{2+} + Mg^{2+})$-dependent ATPase activity and Ca^{2+}-independent ATPase activity. When assayed in 3 mM ATP/3 mM $MgCl_2$/0.1 mM EGTA/100 mM KCl/50 mM Tris-maleate (pH 7.25), at 37° C, the addition of 0.1 mM $CaCl_2$ increases the ATPase activity 4–5-fold. Under these conditions, the basal ATPase activity is not significantly in-

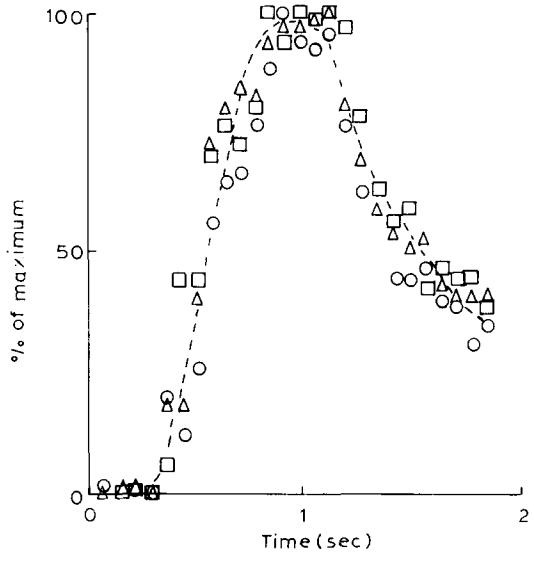

Fig. 1. A microsomal suspension containing 0.25 mg protein was deposited on a Millipore filter, of 0.45 μm average pore size and 13 mm diameter. The microsomes were successively perfused with three solutions at 0.7 ml/s. 50-μl effluent samples were collected at approx. 70-ms intervals. All perfused solutions contained 100 mM KCl/0.1 mM EGTA/0.05 mM NaH_2PO_4/50 mM Tris-maleate (pH 7.25). The second solution contained, in addition, 3 mM $MgCl_2$/14 μM [γ-^{32}P]ATP/^3H_2O, and amounted to 0.1 ml. The first and third solutions amounted to several ml. ^{3}H (□), [^{32}P]P_i (△), and [^{32}P]ATP (○) were measured in the effluent samples as described in the text. Results are expressed as percentages of the maximal values for each species, as a function of time. Only those obtained in the vicinity of the change in perfusing solutions are shown. Temperature, 2° C.

hibited by 0.5 mM sodium azide (data not shown).

Fig. 1 shows the results obtained after perfusion with a brief pulse of a solution containing [γ-^{32}P]ATP, $MgCl_2$ and ^3H_2O, which run through the microsomes between two solutions without these reactants. The three solutions contained similar concentrations of KCl, EGTA, NaH_2PO_4 and Tris-maleate. The effluent ^3H, [^{32}P]P_i, and [^{32}P]ATP concentrations are shown at the time of changing the solutions. The rise in the effluent ^3H activity indicates the arrival of the second solution, and the further decay indicates the replacement of the second by the third solution. The appearance of [^{32}P]P_i in the effluent samples indicate the activation of the basal ATPase, upon arrival of the pulse at the filter. Fig. 1 also shows the time-course of appearance of nonhydrolyzed [γ-^{32}P]ATP in the effluent samples.

The results are expressed as percentages of the maximal values for each species. The radioactivity in the P_i fraction averaged 25% of the total ^{32}P radioactivity in the effluent samples, indicating that 25% of the perfused ATP was hydrolyzed after passing through the enzyme. There is no appreciable delay between the appearance of ^3H, [^{32}P]P_i, and [^{32}P]ATP activity in the effluent, indicating that neither P_i nor ATP is retained by the microsomes in significant amounts and/or during an appreciable time.

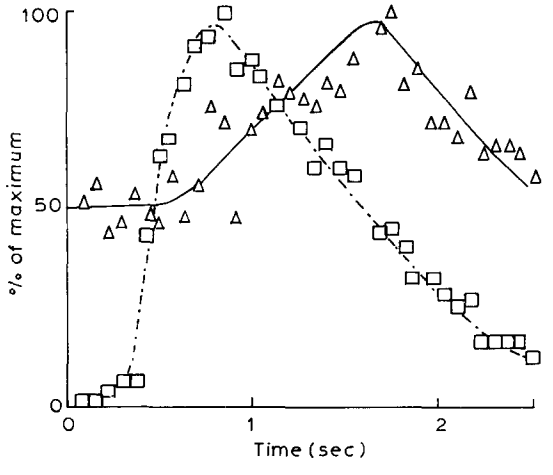

Fig. 2. The microsomal protein deposited on the filter amounted to 0.08 mg. The three successively perfused solutions contained: solution I, 100 mM KCl/0.1 mM EGTA/0.05 mM NaH_2PO_4/10 μM [γ-^{32}P]ATP/3 mM $MgCl_2$/50 mM Tris-maleate (pH 7.25); solution II, same as I, plus 0.5 mM $CaCl_2$ and ^3H_2O; solution III, same as I, but 1.0 mM EGTA. Solution II amounted to 0.2 ml. Perfusing flow, 0.6 ml/s. Symbols and other conditions are as in Fig. 1.

Fig. 2 shows the results of an experiment where the second solution contained $CaCl_2$ and ^3H_2O. The three solutions contained [γ-^{32}P]ATP, $MgCl_2$, KCl, EGTA, NaH_2PO_4 and Tris-maleate. Ca^{2+}-independent ATPase is activated by the first solution, at the extent indicated by [^{32}P]P_i values preceding the arrival of ^3H. The later increase in [^{32}P]P_i indicates the activation of the (Ca^{2+} + Mg^{2+})-ATPase by Ca^{2+} included in the second solution. Despite the large scattering of the [^{32}P]P_i data, it can be appreciated that both the increase and fall of [^{32}P]P_i effluent concentrations are delayed when compared with the ^3H curve. The delay is ascribed to transient phosphate retention by the enzyme as a phosphoenzyme complex. The formation of the well-studied acid-stable phosphoenzyme intermediate has been reported to be measurable with this technique [5]. [^{32}P]P_i collected in samples from the first perfused solution amounted to 9% of the total ^{32}P, indicating that Ca^{2+}-independent ATPase hydrolyzes 9% of the perfused ATP.

The large scattering in Fig. 2 is attributed to: (1) the percentage of hydrolyzed ATP was kept low by reducing the amount of enzyme deposited on the filter. It was necessary to avoid the dilution of the extra phosphate released by Ca^{2+} activation into phosphate produced by the basal activity, during mixing of the successively perfused media; (2) measurement of double-labeled samples introduce counting errors; double-labeled samples are subjected to additional preparative steps, as described under Materials and Methods.

Taking advantage of the similar profiles of the ^3H_2O and [^{32}P]ATP curves (Fig. 1), the nonhydrolyzed effluent ATP was used as a marker of the second solution in the experiment shown in Fig. 3. The second solution contained [γ-^{32}P]ATP, $CaCl_2$, $MgCl_2$, KCl, EGTA, NaH_2PO_4 and Tris-maleate, and was preceded and followed by solutions containing only KCl and Tris-maleate. In this experiment, both, the basal and the (Ca^{2+} + Mg^{2+})-dependent ATPases are activated simultaneously upon arrival of the second solution at the microsomes. Both the increased amount of enzyme deposited on the filter, which increased the per-

centage of ATP hydrolysis, and the absence of tritiated water reduced the experimental errors, and the data exhibit low scattering. The total radioactivity in the effluent P_i fraction amounted 77% of the total effluent radioactivity.

The rising phase of [^{32}P]P_i (Fig. 3) initially coincides with that of [^{32}P]ATP, as in Fig. 1. Therefore, [^{32}P]P_i released initially comes from the operation of the basal ATPase. A further and delayed increase in [^{32}P]P_i is ascribed to ATP hydrolysis by the (Ca^{2+} + Mg^{2+})-dependent ATPase. [^{32}P]P_i decay appears delayed, as in Fig. 2, indicating the operation of the (Ca^{2+} + Mg^{2+})-dependent ATPase. The [^{32}P]P_i effluent profile can be interpreted as the sum of a rapid component with a [^{32}P]ATP-like pattern, as in Fig. 1, caused by activation of the basal ATPase, and a delayed component reflecting the operation of the (Ca^{2+} + Mg^{2+})-dependent ATPase.

The above interpretation is supported by other experiments (Figs. 4 and 5). The experimental conditions in Fig. 4 are similar to those from Fig. 1, except that EGTA was included only in the second solution. Despite the absence of Ca^{2+} in the second solution, a delayed [^{32}P]P_i decay is observed. According to the above interpretation, it indicates the operation of the (Ca^{2+} + Mg^{2+})-ATPase, which can be activated by contaminating Ca^{2+} from the first solution. [γ-^{32}P]ATP arriving with the second solution interacts with (Ca^{2+} + Mg^{2+}-ATPase molecules with a certain degree of saturation of their high affinity Ca^{2+}-binding sites. Presumably, the operation of the (Ca^{2+} + Mg^{2+}-ATPase does not last more than one enzymatic cycle, because of the simultaneous arrival of EGTA, thus making the delay in [^{32}P]P_i decay less marked, and the total amount of P_i released not so large, as in Fig. 3.

The experimental conditions from Fig. 3 provide optimal activation of the (Ca^{2+} + Mg^{2+})-ATPase. In Fig. 5, the pulse reactants presumably activate this enzyme to a small extent. The pulse comprised 3 mM $CaCl_2$/10 μM [γ-^{32}P]ATP, and all solutions were devoid of Mg^{2+}. It has been reported that high Ca^{2+} concentrations inhibit (Ca^{2+} + Mg^{2+})-ATPase [11,12], that the basal

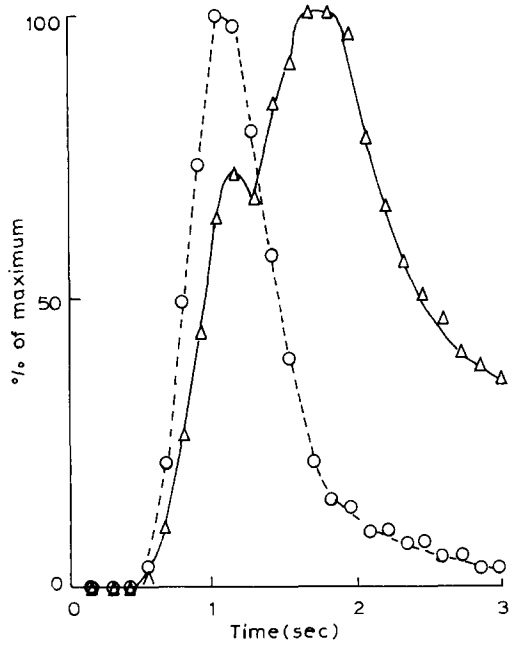

Fig. 3. The microsomes (0.35 mg protein) deposited on a Millipore filter were perfused at 0.4 ml/s with 0.2 ml of a solution containing 3.5 μM [γ-^{32}P]ATP/0.1 mM $CaCl_2$/3 mM $MgCl_2$/100 mM KCl/0.1 mM EGTA/0.05 mM NaH_2PO_4/50 mM Tris-maleate (pH 7.25), preceded and followed by solutions containing only KCl and Tris-maleate at similar concentrations. Symbols and other conditions are as in Fig. 1.

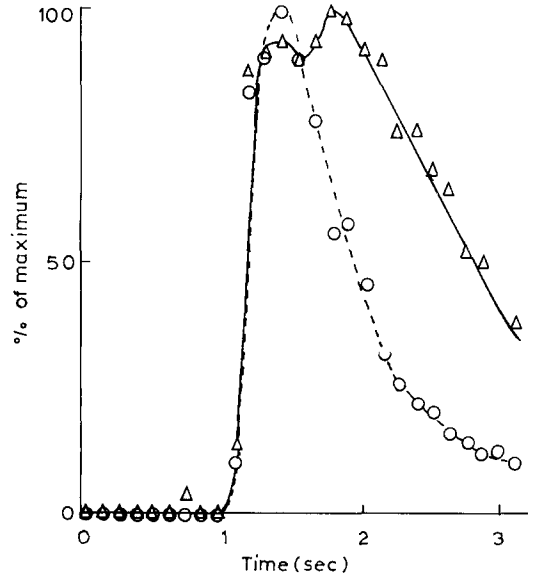

Fig. 4. The perfusing solutions are as in Fig. 1, except that EGTA was included only in the second solution. The symbols and other conditions are as in Fig. 1.

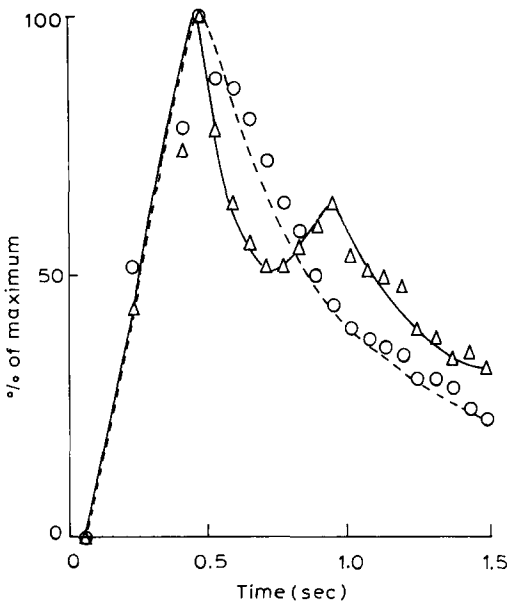

Fig. 5. The first perfused solution contains 100 mM KCl/0.1 mM EGTA/0.05 mM NaH_2PO_4/50 mM Tris-maleate (pH 7.25). The second solution (0.2 ml) contains, in addition, 14 μM [γ-^{32}P]ATP and 1 mM $CaCl_2$. The third solution differs from the first in its content of 10 mM EGTA. The microsomal protein deposited on the filter amounted 0.15 mg, and the perfusing flow was 1.3 ml/s. The symbols and other conditions are as in Fig. 1.

ATPase is activated either by Mg^{2+} or Ca^{2+}, with low affinity [12,13], and that phosphorylation of (Ca^{2+} + Mg^{2+})-ATPase can be achieved in the absence of added Mg^{2+} [14]. The results agree with these reports and with our previous interpretation of the present results, since most of the released [^{32}P]P_i follows the effluent pattern of nonhydrolyzed [^{32}P]ATP, and a small and delayed peak is observed in the falling phase of the [^{32}P]P_i curve, indicating a small degree of activation of the (Ca^{2+} + Mg^{2+})-ATPase.

Discussion

The continuous perfusion of sarcoplasmic reticulum fragments previously deposited on Millipore filters has been proved to be suitable for the demonstration of the retention and release of several ligands during the operation of the Ca^{2+}-transporting enzyme [5,6]. Upon perfusion of the membranes with [γ-^{32}P]ATP, two fractions of retained ^{32}P were distinguished: ^{32}P bound to the enzyme as an acid stable complex, and ^{32}P associated with binding of the nucleotide as the enzyme-substrate complex [5]. Those experiments were designed to measure the total amounts of bound ligands, and no inferences were made with regard to the kinetics of the reaction. Indeed, the time between the successively collected samples was large, in comparison with the rates of the partial reactions of the enzymatic cycle: the perfusing flow was low, and its amount large enough to allow equilibration between the enzyme and the perfused ligands.

Since, in the present experiments, the perfusing flow was larger, the time between the successively collected samples short enough as to be of the same order as the turnover of the enzymatic cycle [15,16], and since perfusion of the activating reactants was promptly interrupted, so that the system was far from reaching a steady state, any delay in the appearance of a ligand in the effluent samples can be attributed to its transient retention by the membranes.

The maximal ^3H_2O level in Fig. 1 is 12% of that in the secondly perfused solution, indicating that the system is far from reaching a steady state. The similarity of the effluent patterns of ^3H_2O and nonhydrolyzed [γ-^{32}P]ATP (Fig. 1) indicates that the turnover of the enzyme-substrate complex is fast enough to preclude the detection of bound substrate, under the present conditions. This applies both to the enzyme-substrate complex of the Ca^{2+}-independent and the Ca^{2+}-dependent ATPases, since ATP binding to the (Ca^{2+} + Mg^{2+})-ATPase does not require Ca^{2+} [17].

The Ca^{2+}-independent (basal) ATPase activity cannot usually be assessed under conditions providing optimal activation of the (Ca^{2+} + Mg^{2+})-ATPase, since both activities require Mg^{2+}. Doubts have been raised as to whether both activities operate simultaneously in the presence of Ca^{2+}, or, depending on the presence or absence of Ca^{2+}, a single enzyme follows different paths [4]. With this methodology, and taking advantage of well-known facts [1,2] that the hydrolysis of the phosphoenzyme intermediate is the rate-limiting step of the (Ca^{2+} + Mg^{2+})-ATPase reaction and that a phosphoenzyme intermediate is not detected in the absence of Ca^{2+}, we attempted to demon-

strate, or discard, the activation of the basal ATPase in the presence of Ca^{2+}.

Different delays of the effluent $[^{32}P]P_i$ pulses are observed upon brief perfusions of reactants activating only the basal (Fig. 1) or the (Ca^{2+} + Mg^{2+})-dependent (Fig. 2) ATPases. On the other hand, the effluent $[^{32}P]P_i$ profile can be always interpreted as the sum of a rapid and a delayed production of P_i, under several conditions varying the relative activation of both ATPase activities (Figs. 3–5). In Fig. 3, the early appearance of effluent P_i and the presence of a slight discontinuity in its rising phase, coinciding with the maximal output of nonhydrolyzed ATP, and its delayed decay, all indicate that Ca^{2+}-dependent and Ca^{2+}-independent activities are operating simultaneously. Some degree of Ca^{2+} binding to the high-affinity site of the (Ca^{2+} + Mg^{2+})-ATPase is expected to occur in Fig. 4, due to contaminating Ca^{2+} in the first, EGTA-free, perfused solution. The delayed falling phase of the P_i curve indicates also the simultaneous activation of both catalytic reactions. (Ca^{2+} + Mg^{2+})-ATPase activity in Fig. 4 is lower than in Fig. 3. The conditions for activation of the (Ca^{2+} + Mg^{2+})-ATPase are still less favorable in Fig. 5 than in Figs. 3 and 4, but a small activation is evidenced by the discontinuity in the falling phase of the $[^{32}P]P_i$ curve, also indicating the simultaneous activation of both enzymes. The basal ATPase is activated by Mg^{2+} in Figs. 1–4, and by Ca^{2+} in Fig. 5.

In Figs. 2 and 5, the third solutions are Ca^{2+}-free and contain EGTA at larger concentrations than those of calcium in the preceding solutions. In these cases, calcium is depleted from the microsomal environment not only by introduction of EGTA at higher concentrations, but also by the continuous flow which mechanically removes calcium eventually released from the membranes to the media.

The ATP concentrations used in these experiments are far from optimal for activation of the basal ATPase: K_m values for ATP are larger for the basal ATPase than for the (Ca^{2+} + Mg^{2+})-ATPase [12,18]. Nevertheless, it has been reported [3] that the Ca^{2+}-independent ATPase activity is measurable, during the transient state of the reactions, at ATP concentrations as low as those used in the present experiments. Furthermore, the basal ATPase/total ATPase activity ratio increases upon the decrease of the temperature [4]. The use of low ATP concentrations was necessary to obtain a reasonable percentage of ATP hydrolysis, since, due to the large perfusing flow, the exposure of the enzyme to the substrate is extremely brief. The percentages of the perfused ^{32}P radioactivity found in the $[^{32}P]P_i$ effluent fraction were 25% in the absence of Ca^{2+} (Fig. 1) and 77% in the presence of Ca^{2+} (Fig. 3), but these figures cannot be directly compared because of differences in other experimental conditions.

Recently, Fernandez et al. [19] concluded that the basal ATPase of isolated sarcoplasmic reticulum fragments originates in mitochondrial and plasmalemmal contaminations of the preparations. We did not detect an inhibitory effect of sodium azide, an inhibitor of the mitochondrial ATPase, on our basal activity. We cannot discard its plasmalemmal origin. Our results are in line with that report, since they indicate that both activities operate simultaneously, but they did not ascertain whether both activities originate in the same or in different enzymes.

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