Comparison of Endogenous and Exogenous Sources of ATP in Fueling Ca^{2+} Uptake in Smooth Muscle Plasma Membrane Vesicles

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ABSTRACT A smooth muscle plasma membrane vesicular fraction (PMV) purified for the (Ca^{2+}/Mg^{2+})-ATPase has endogenous glycolytic enzyme activity. In the presence of glycolytic substrate (fructose 1,6-diphosphate) and cofactors, PMV produced ATP and lactate and supported calcium uptake. The endogenous glycolytic cascade supports calcium uptake independent of bath [ATP]. A 10-fold dilution of PMV, with the resultant 10-fold dilution of glycolytically produced bath [ATP] did not change glycolytically fueled calcium uptake (nanomoles per milligram protein). Furthermore, the calcium uptake fueled by the endogenous glycolytic cascade persisted in the presence of a hexokinase-based ATP trap which eliminated calcium uptake fueled by exogenously added ATP. Thus, it appears that the endogenous glycolytic cascade fuels calcium uptake in PMV via a membrane-associated pool of ATP and not via an exchange of ATP with the bulk solution. To determine whether ATP produced endogenously was utilized preferentially by the calcium pump, the ATP production rates of the endogenous creatine kinase and pyruvate kinase were matched to that of glycolysis and the calcium uptake fueled by the endogenous sources was compared with that fueled by exogenous ATP added at the same rate. The rate of calcium uptake fueled by endogenous sources of ATP was approximately twice that supported by exogenously added ATP, indicating that the calcium pump preferentially utilizes ATP produced by membrane-bound enzymes.

INTRODUCTION

The enzymes of the Embden-Meyerhoff pathway have traditionally been viewed as existing uniformly within the cytosol with the intermediate metabolites able to mix freely. Even with findings that glycolytic enzymes are localized to cytoskeletal structures (Clarke and Masters, 1975; Brady and Lasek, 1981; Pagliaro and Taylor, 1988) and intracellular membranes (Pierce and Philipson, 1985; Daum, Keller, and Lange, 1988), glycolysis is frequently considered to behave as a homogeneous cascade within the cytosol. Since the 1960s, a growing body of evidence suggests that

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compartmentation of the intermediates of glycolysis and glycogenolysis occurs in a variety of tissues (Shaw and Stadie, 1959; Threlfall and Heath, 1968; Dully, Bocek, and Beatty, 1969; Lynch and Paul, 1983). The functional significance of this compartmentation of glycolytic intermediates has remained unclear, though there is now evidence indicating that the localized glycolytic enzymes support different functions within the same cell (Parker and Hoffman, 1967; Mercer and Dunham, 1981; Balaban and Bader, 1984; Lynch and Balaban, 1987).

Smooth muscle is particularly well suited for the study of glycolytic compartmentation as it is characterized by a relatively high rate of aerobic lactate production, despite the fact that the largest fraction of its ATP is derived from oxidative phosphorylation. This aerobic glycolysis is not attributable to anoxia or mitochondrial insufficiency (Paul, 1980; Lynch and Paul, 1987), and accounts for approximately one-fifth of its ATP synthesis (Paul, 1980). Interestingly, oxidative metabolism and aerobic lactate production can vary independently and often in opposite directions (for review, see Paul, 1989). These distinct components of smooth muscle metabolism appear to be associated with different cellular functions under normal conditions. We have reported for porcine carotid and coronary arteries that the rate of oxygen consumption is nearly universally correlated with isometric force, and the rate of aerobic lactate production is strongly correlated with the activity of the Na pump. In guinea pig taenia coli, the time course of lactate production was correlated with K^+ recovery in potassium-depleted tissues under anaerobic conditions (Casteels and Wuytack, 1975). Under aerobic conditions in potassium-depleted hog carotid arteries, lactate production and potassium accumulation varied linearly with external potassium concentration with a K_m for either process between 2 and 3 mM (Campbell, Agubosim, and Paul, 1988; Campbell and Paul, 1991). In addition, at external potassium concentrations below K_m, the ratio of potassium uptake to ATP produced per lactate produced was consistent with the theoretical sodium pump stoichiometry of 2K:ATP. We proposed that oxidative metabolism supports the requirements of the actomyosin ATPase, whereas glycolysis is linked to membrane ion pump function (Paul, Bauer, and Pease, 1979; Lynch and Paul, 1983). This hypothesis is further supported by the observation that, in porcine carotid artery, the sole source of aerobic lactate production is exogenous glucose despite a substantial breakdown of tissue glycogen stores (Lynch and Paul, 1983). Thus, in smooth muscle there appears to be a compartmentation of glycolytic intermediates as well as a functional separation of oxidative and glycolytic metabolism. The strong evidence for compartmentation of carbohydrate metabolism in smooth muscle makes this tissue an ideal candidate for further studies into the mechanisms of glycolytic compartmentation.

To test our hypothesis that glycolysis is linked to ion transport in smooth muscle, we utilized a plasma membrane vesicular (PMV) fraction purified for the (Ca^{2+}/Mg^{2+})-ATPase as a model system representing the intact plasma membrane of smooth muscle. This PMV preparation has been well characterized (Raeymaekers, Wuytack, and Casteels, 1985) and is capable of ATP-supported calcium uptake into inside-out vesicles. The isolated membrane fraction enabled us to examine this glycolytic compartment, apparently associated with ion pump function, in relative isolation from metabolic machinery not associated with the plasma membrane.

Previous studies from our laboratory have shown that the glycolytic enzymes are associated with this plasma membrane fraction and that glycolytic substrates and cofactors can fuel the calcium pump (Paul, Hardin, Raeymaekers, Wuytack, and Casteels, 1989). To further study the nature of the coupling between glycolytic enzymes associated with the plasma membrane and the plasmalemmal calcium pump, we compared ATP synthesis by several membrane-associated enzymes with exogenous ATP in the support of calcium pump activity.

MATERIALS AND METHODS

Preparation of Plasmalemmal Fraction

Preparation of crude microsomal fraction. The PMV fraction derived from pig antrum was prepared as previously described (Raeymaekers et al., 1985). Briefly, the antral parts of pig stomachs were obtained at the slaughterhouse and transported to the laboratory covered with the mucosal side resting on ice. The mucosal side was removed with scissors and the remaining muscle was cut into strips ~1 cm wide. 250 g of tissue was passed through a French pressure cell press (American Instrument Company, Silver Spring, MD) at 10,000–20,000 psi. This material was then homogenized in 3 vol of a solution of 250 mM sucrose, 0.67 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol in a blender for 75 s, during which a hand-held homogenizer (BioSpec Products Inc., Bartlesville, OK) was inserted for three 10-s intervals. The final volume was adjusted with sucrose solution to 1.25 liters. The homogenate was centrifuged in a J2-21 centrifuge (Beckman Instruments, Inc., Fullerton, CA) at 14,000 *g* for 30 min at 4°C. This supernatant was designated the crude microsomal fraction.

Sucrose density centrifugation. The crude microsomal fraction was twice filtered through cheesecloth. Digitonin (120 mg in 10 ml 95% ethanol) was added to 500 ml of microsomal fraction and sucrose was added to bring the solution to a 51% sucrose density. This solution was loaded into a Ti-15 (Beckman Instruments, Inc.) zonal rotor beneath 250 ml of a 15–45% sucrose gradient and centrifuged in a Beckman L5-65 ultracentrifuge at 4°C for 20 h. Fixed volume fractions were collected and the sucrose density was measured using a Fisher hand-held refractometer. Selected fractions, typically of 31–37% sucrose density, were diluted 1:5 in water and centrifuged in a SW28 rotor (Beckman Instruments, Inc.) at 105,000 *g* for 1 h at 4°C. The pellet was resuspended in an equivalent volume of 250 mM sucrose and 1 mM DTT in a teflon homogenizer.

Characterization of the PMV fraction. The PMV fraction has been extensively characterized using the plasma membrane markers of Mg-ATPase, Na/K-ATPase, 5′-nucleotidase, and calmodulin binding (Raeymaekers et al., 1985; Wuytack, Raeymaekers, and Casteels, 1985). Furthermore, the endoplasmic reticular markers of rotenone-insensitive NADH and NADPH cytochrome *c* reductase as well as oxalate-stimulated calcium uptake were used. A single preparation, obtained from six porcine stomachs, yields ~40 mg of protein, which is at least 75–80% pure sarcolemma as estimated by quantitation of the above enzyme markers.

Measurements of Enzymes, ATP, and NADH

Enzyme activities (pyruvate kinase and creatine kinase) were measured fluorometrically using standard enzyme-linked assays and quantifying the appearance or disappearance of NADPH and NADH, respectively. Enzymes were obtained from Sigma Chemical Co. (St. Louis, MO), and unless otherwise specified received no further purification. NADH and NADPH were measured using a 650-10S fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, CT) with excitation 340 nm and emission 460 nm with both slit widths of 5 nm. 10 μl rotenone

(1 mM in 95% ethanol) was added to inhibit any NADHase activity due to potential mitochondrial contamination of the membrane fraction. Controls were performed to verify that only the enzyme to be measured was rate limiting and to correct for background activity contaminating the exogenously added enzymes.

Creatine kinase activity was measured using a linked enzyme protocol where net ATP production from creatine kinase is coupled to the production of NADPH via hexokinase (2 U/ml) and glucose-6-phosphate dehydrogenase (2 U/ml) in the presence of 2.5 mM glucose and 0.3 mM NADP. The reaction was started with 2 mM phosphocreatine in the presence of 0.2 mM ADP.

Pyruvate kinase activity was measured using a linked enzyme assay by measuring the rate of NADH disappearance in the presence of 1 mM phosphoenolpyruvate and lactate dehydrogenase (at a non–rate-limiting activity which varied from assay to assay).

Measurement of the bath ATP concentration generated glycolytically was measured fluorometrically using the same enzyme-linked assay as used for assay of creatine kinase activity, but using 2 U/ml of creatine kinase instead. Incubations utilized the same substrates and cofactors used for measurement of calcium uptake (below). Incubations were carried out at 30°C and stopped at various time intervals by immersion of the tubes in boiling water for 5 min. Incubates were then either analyzed immediately or frozen for future analysis.

NADH production of PMV from 1 mM fructose 1,6-diphosphate was measured fluorometrically as described above in the same solution used for measurement of calcium uptake but in the presence of the glycolytic cofactors of 4 mM NAD, 10 mM potassium phosphate, and 0.2 mM ADP.

Measurement of Ca Uptake

^{45}Ca uptake was measured in an incubation mixture (pH 7.0) consisting of (final concentration in mM): 33 MOPS, 5 NaN_3, 100 KCl, 2 EGTA, 10 or 50 potassium phosphate, and 0.1 myokinase inhibitor P^1,P^5-di(adenosine-5′)pentaphosphate, with $MgCl_2$ added to attain free Mg^{2+} of 1 mM, and $CaCl_2$ added to attain a pCa of 5.0. The reaction was carried out in a shaker bath at 37°C and initiated by addition of either ATP or other substrates as indicated. At various time intervals aliquots were filtered under vacuum through filters with 0.45 μm pore size (Millipore Corp., Bedford, MA). [PMV] was typically 0.1–0.2 mg/ml protein unless otherwise noted. $^{45}CaCl_2$ was obtained from New England Nuclear (Boston, MA) and typically used at a final activity of 0.04 mCi/ml. ^{45}Ca on the filters was quantified using liquid scintillation counting. Volume changes due to additions to the incubation mixture, including ATP infusion, were accounted for in all calculations. Calculation of Mg^{2+}, Ca^{2+}, and other important ionic species was done with the computer program "bathe" (Godt and Maughan, 1988), which uses the binding constants published by Fabiato and Fabiato (1979).

Exogenous ATP Regenerating System

An ATP regenerating system, used as an exogenous source of ATP, consisted of 10 U/ml creatine kinase and 10 mM phosphocreatine. The bath ATP levels were varied by varying the concentration of ADP added to initiate the reaction. ATP concentration in the bath was separately measured as described above.

Matching ATP Production Rates

The initial rate of ATP production of the endogenous glycolytic cascade was estimated by measurements of the initial rate of glycolytic NADH production since glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase function sequentially. Two measures of endogenous glycolytic ATP production were made, an initial rate and a rate averaged over 30

min of incubation. The initial rate was measured well before sufficient pyruvate could accumulate to support flux through lactate dehydrogenase; therefore, in the measurement of the initial rate of NADH production, the consumption of NADH via lactate dehydrogenase did not occur. Since the initial rate calculations were clearly an upper bound, we also included the 30-min average NADH production rate as an alternative estimate of the ATP production rate over this time period. The rate of ATP production from the endogenous creatine kinase was measured by linking ATP production with consumption of ATP by hexokinase (2 U/ml) and glucose (2.5 mM) to the production of NADPH by glucose-6-phosphate dehydrogenase. Production of ATP by the endogenous pyruvate kinase was measured by NADH consumption through lactate dehydrogenase. Measurement of NADH production and consumption by PMV was carried out in solutions identical to those used for measurement of ^{45}Ca uptake. Since the activities of both creatine kinase and pyruvate kinase were higher than that of glycolysis in PMV, the rate of ATP production by pyruvate kinase and creatine kinase could be limited by reducing ADP levels. The [ADP] was adjusted until the rates of ATP production by pyruvate kinase and creatine kinase were matched (within an average of ±6%) to the rate of glycolytic ATP production in each preparation of PMV over a period of 30 min. Since the myokinase inhibitor P^1,P^5-di(adenosine-5′)pentaphosphate was included in all incubations and the concentration of ADP was well below the K_m of myokinase for ADP, none of the ATP production was likely to result from contaminating myokinase activity. This was further supported by control experiments demonstrating that ADP in the presence of P^1,P^5-di(adenosine-5′)pentaphosphate did not support calcium uptake. ATP could also be added exogenously by infusion at the appropriate rate via a programmable infusion pump (Harvard Apparatus, South Natick, MA) through polyethylene tubing (PE 30). ATP (~1 mM) was added to 0.3–0.5 ml of incubation mixture at a rate initially at ~1 μl/min. The pump rate was decreased accordingly, immediately after an aliquot was sampled so that the rate of ATP delivery per unit volume of incubation mixture remained constant. This is taken to be an unambiguous source of exogenous ATP.

ATP Trap Experiments

Hexokinase covalently attached to agarose was obtained from Sigma Chemical Co., washed three times, and resuspended in a solution, pH 7.0, of (in mM): 33 MOPS, 100 KCl, and 5 NaN_3. When desired, agarose-bound hexokinase was added to the ^{45}Ca uptake tubes at a final activity of 5 U/ml with 2.5 mM glucose.

Treatment of Data

All data are treated by standard parametric statistical techniques. All comparisons are by Student's t test with $\alpha = 0.05$. R values are from a linear least-squares regression of the data. Error bars on graphs are ±SD when results from one PMV preparation are shown, or ±SEM when multiple PMV preparations are used. N indicates the number of PMV preparations used, whereas n indicates the number of experiments.

RESULTS

Glycolytically Supported Calcium Uptake in Inside-out Vesicles

All the glycolytic enzymes responsible for catabolizing fructose 1,6-diphosphate to lactate were found to be associated with PMV (see Paul et al., 1989). Glycolytic enzyme activities ranged from 1.408 ± 0.290 U/mg protein for pyruvate kinase to 0.026 ± 0.005 U/mg protein for phosphoglycerate mutase (Paul et al., 1989). The

glycolytic enzymes endogenous to the smooth muscle PMV can work in a concerted fashion to produce ATP and lactate in the presence of 1 mM fructose 1,6-diphosphate, 4 mM NAD, 10 mM potassium phosphate, and 0.2 mM ADP (Paul et al., 1989). This glycolytically produced ATP is capable of supporting calcium uptake in the inside-out vesicles of PMV as shown in Fig. 1. Calcium uptake in the absence of an ATP source remains essentially constant during incubations. This is assumed to reflect calcium binding and is routinely measured and subtracted from the values of calcium uptake in the presence of an ATP source. Typically, fructose 1,6-diphosphate and ADP are excluded to measure calcium binding, although exclusion of any of the cofactors or substrates for glycolysis also abolishes glycolytically fueled ^{45}Ca uptake. Therefore the contribution of any contaminating myokinase to ATP production is negligible at these low concentrations of ADP in the presence of the myokinase inhibitor P^1,P^5-di(adenosine-5')pentaphosphate.

It is possible that the coupling between glycolysis and calcium uptake simply reflects the ability of the PMV to generate a significant bath ATP concentration,

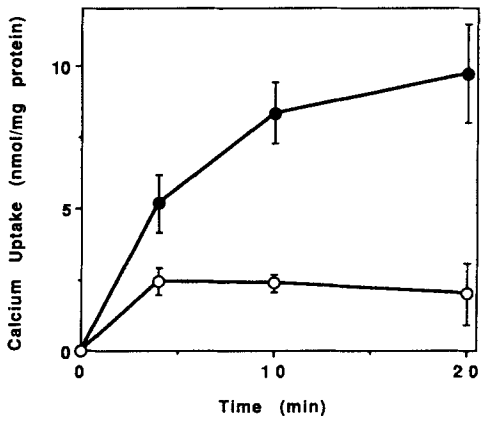

FIGURE 1. Typical experiment of ^{45}Ca uptake fueled solely by glycolytic substrates and cofactors (*filled circles*): 1 mM fructose 1,6 diphosphate, 0.2 mM ADP, 4 mM NAD, and 10 mM phosphate. Open circles represent ^{45}Ca associated with PMV in the absence of any metabolic substrates. This ^{45}Ca binding is routinely subtracted from the calcium uptake data presented (binding at each time point is subtracted from uptake at the same time point). Data points are triplicate determinations (±SD).

which in turn is sufficient to operate the calcium pump. Fig. 2 shows the time course of the bath ATP concentration and calcium uptake for a typical experiment. Under these conditions, glycolyzing PMV are capable of generating bath ATP concentrations of 10–30 μM. We measured the effects of ATP concentrations in this range on calcium uptake using exogenous creatine kinase and phosphocreatine (PCr) to serve as a source of controlled levels of ATP. Fig. 3 shows the dependence of calcium uptake on the nominal, buffered ATP concentration. Thus it is possible that calcium uptake is fueled by simple utilization of the low levels of bath ATP produced by glycolytic activity of the PMV.

Two observations permit this hypothesis to be tested. We observed that the level of ATP produced by glycolysis is linearly proportional to the concentration of PMV ($r = 0.975$, $n = 3$). Furthermore, the rate of calcium uptake (or total calcium accumulation at any point in time) was also observed to be linearly proportional to the bath ATP concentrations ($r = 0.952$, $n = 7$) (see Fig. 3). This would be expected as the conditions here were well within the linear range for the calcium pump, since

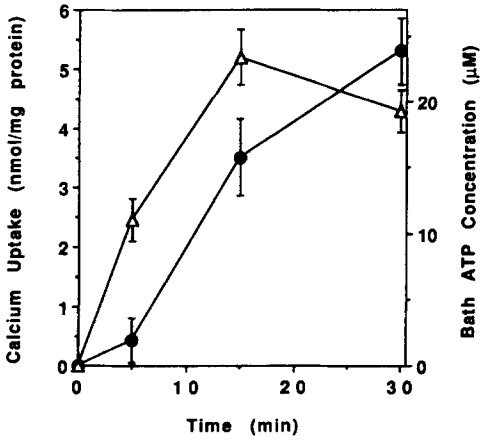

FIGURE 2. Time course of bath ATP concentration (in micromolar) (*triangles*) and ^{45}Ca uptake (*filled circles*) when PMV is provided with glycolytic substrates and cofactors. Measurements of [ATP] and ^{45}Ca uptake were done under identical conditions, except for the addition of isotope for uptake measurements, with the same PMV preparation. Data points are from one PMV preparation and are quadruplicate for [ATP] and triplicate for ^{45}Ca uptake (±SD).

all the ATP concentrations are below the K_m for the calcium pump (100–300 μM in Hardin, Raeymaekers, Wuytack, Casteels, and Paul, 1987; and >50 μM MgATP required for half maximal activity in Grover and Samson, 1986). Thus if calcium uptake were dependent on the bath [ATP] generated by PMV, the total calcium uptake measured would show a quadratic dependence on the PMV concentration; i.e., $Ca_i^{2+} = \beta[ATP][PMV] = \beta[PMV]^2$, where β is a proportionality constant. However, if glycolytically supported calcium uptake was independent of bath [ATP], calcium uptake per vesicle would be independent of the concentration of the vesicles. For example, if only one vesicle were present, its capacity to raise bath [ATP] would be negligible but its calcium uptake should not be affected if membrane-associated

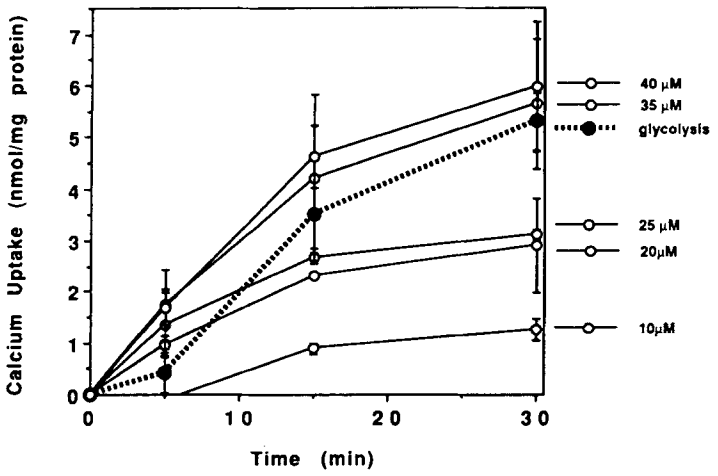

FIGURE 3. ^{45}Ca uptake fueled by either glycolysis (*dashed line*) or exogenously added phosphocreatine and creatine kinase (*solid lines*). In the presence of the exogenous creatine kinase ATP-regenerating system, the concentration of added ADP to the PMV suspension varies from 10 to 40 μM. Comparisons are from the same experiment using the same PMV preparation and are in triplicate (±SD).

glycolysis provides ATP directly to the calcium pump. In this case, the total calcium uptake would be linearly proportional to the PMV concentration.

This was tested by measuring the effects of PMV concentration on calcium uptake as shown in Fig. 4. As shown in Fig. 4, serial dilution of PMV leads to a linear, not quadratic dependence of calcium uptake on PMV concentration. At [PMV] where the bath [ATP] is in the micromolar range (1:10 dilution), calcium uptake in PMV per milligram protein remains at the same level as when glycolytically produced ATP in the bath is 10-fold higher. All dilutions were made with the same incubation solution such that the only difference was [PMV]. The reaction was started with glycolytic substrate and cofactors (1 mM fructose 1,6-diphosphate, 0.2 mM ADP, and 4 mM NAD). Thus the data are consistent with the hypothesis that glycolytically supported calcium uptake in PMV is independent of the bath [ATP] produced glycolytically and therefore may be mediated by some more direct coupling of glycolytic ATP production and calcium pump function.

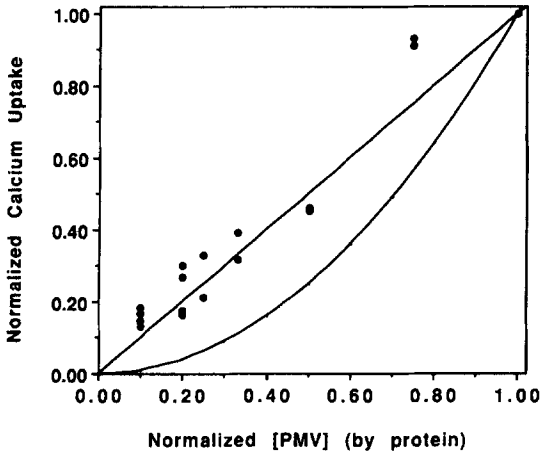

FIGURE 4. ^{45}Ca uptake measurements on three different preparations of PMV serially diluted over a 10-fold range. The data are normalized to the ^{45}Ca uptake measured at the highest [PMV] used in that experiment. The [PMV] used as the normalization point ranged in concentration from 0.13 to 0.21 mg protein/ml. Each data point represents triplicate measurements. Eight serial dilution experiments were performed with a total of three PMV preparations. A linear regression through the data points yields an r value of 0.988, a slope of 0.976, and a y intercept of 0.041. The dotted line depicts the parabolic relationship expected (see text) if Ca uptake depends on bath ATP concentration.

Matching ATP Production Rates of Exogenous and Endogenous ATP Sources

Since ATP produced by the endogenous glycolytic cascade appears to fuel calcium uptake in PMV independently of the bath [ATP], we compared calcium uptake fueled by either ATP produced endogenously by PMV or ATP added exogenously to PMV. Glycolytic ATP production was estimated from the rate of glycolytic NADH production. As an exogenous source of ATP, we added ATP directly to the suspension of PMV via an infusion pump. Shown in Fig. 5, glycolytically fueled uptake is compared with uptake fueled by ATP infused at either the initial rate of glycolytic ATP production or the average rate of glycolytic ATP production over 30 min of incubation. Since the time course of glycolytic NADH production is approximately hyperbolic (see Paul et al., 1989), the linear rate of ATP infusion was matched to the

glycolytic NADH production over a discrete period of time (30 min) or to the measured initial rate. The accuracy of the match of the ATP infusion to the glycolytic NADH production was taken to be the same as the error of estimation of the glycolytic ATP production (< 5% error) plus the error of the accuracy of the infusion pump (< 1%). In this PMV preparation, glycolytically fueled calcium uptake was approximately twofold greater than that supported by ATP infused to match the initial rate of glycolytic ATP production and approximately fourfold greater than ATP infused to match the 30-min glycolytic rate. Thus, ATP produced by the endogenous glycolytic cascade appears to fuel calcium uptake in PMV to a greater extent than ATP added exogenously at the same rate. The glycolytically produced ATP does not appear to fuel calcium uptake via increases in bath [ATP] and may fuel calcium uptake in some more direct, or coupled, fashion.

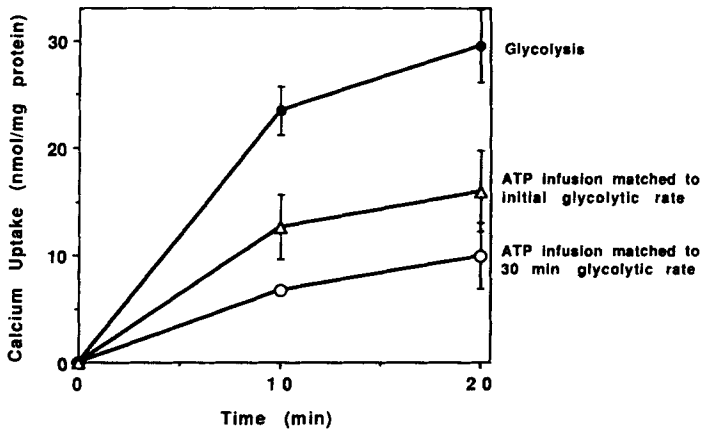

FIGURE 5. Comparison of ^{45}Ca uptake fueled by either glycolysis (*filled circle*) or ATP added exogenously via an infusion pump at rates matching the initial rate of glycolytic ATP production (0.271 ± 0.028 nmol/min; *open triangles*) or the 30 min rate of glycolytic ATP production (0.129 ± 0.002 nmol/min; *open circles*). Data shown are from a typical experiment using one preparation and are in duplicate for ATP and triplicate for glycolysis (±SD).

Comparison of Glycolysis to Other Endogenous Sources of ATP

To determine if there is a specific site of coupling between the endogenous glycolytic cascade and the calcium pump in PMV, we compared calcium uptake fueled by the entire glycolytic cascade (including phosphoglycerate kinase and pyruvate kinase) with calcium uptake fueled by pyruvate kinase alone using phosphoenolpyruvate as the substrate. ATP production rates for the two systems were matched over 30 min (average error of match was ±6% for each of the three preparations used). The average rate of ATP production for glycolysis over 30 min was 7.2 nmol/min · mg (±1.0 SEM, $N = 3$). Fig. 6 *A* shows calcium uptake in PMV fueled either by the entire glycolytic cascade or through pyruvate kinase alone. Although initially pyruvate kinase alone fuels calcium uptake better than glycolysis, by 20 min calcium uptake is similar in this preparation. Thus glycolysis supports calcium uptake in PMV at least

as well as pyruvate kinase alone. Creatine phosphokinase was also endogenous to PMV and we used the creatine kinase as yet another endogenous source of ATP for fueling calcium uptake. The ATP production rate of creatine kinase was matched to that of glycolysis and the calcium uptake fueled by these sources is compared in Fig. 6 *B*. The time course of calcium uptake fueled by creatine kinase is similar to that of pyruvate kinase and the magnitude of the calcium uptake is initially greater than for glycolysis. Since the single enzyme systems of pyruvate kinase and creatine kinase produce ATP with a different time course than that of glycolysis, even though the average ATP production rates are matched, the time course of Ca uptake of these systems varies accordingly as shown in Fig. 6, *A* and *B*.

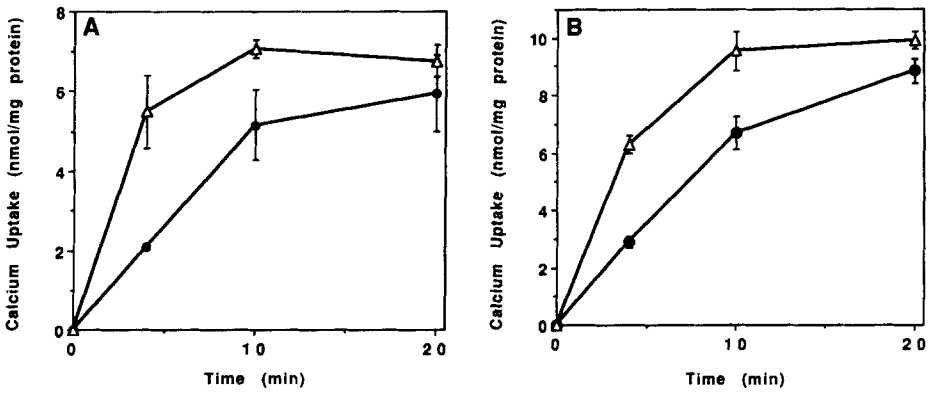

FIGURE 6. (*A*) Comparison of ^{45}Ca uptake fueled by either glycolysis (*filled circles*) or endogenous pyruvate kinase (*open triangles*). Data shown are from a typical experiment using one preparation and are in triplicate (±SD). The pyruvate kinase reaction was started by addition of 1 mM phosphoenolpyruvate and 17 μM ADP. The ATP production rate of pyruvate kinase was matched to that of glycolysis averaged over 30 min of incubation. (*B*) Comparison of ^{45}Ca uptake fueled by either glycolysis (*filled circles*) or endogenous creatine kinase (*open triangles*). The creatine kinase reaction was started by addition of 1 mM phosphocreatine and 15 μM ADP. The ATP production rate of creatine kinase was matched to that of glycolysis averaged over 30 min of incubation.

Fig. 7 shows the comparison of calcium uptake fueled by glycolysis to that fueled by pyruvate kinase alone, creatine kinase, or exogenous ATP. Only in the case of exogenously added ATP was the magnitude of calcium uptake lower than that of glycolysis. Thus all endogenous sources of ATP appear to fuel calcium uptake in PMV to a significantly greater extent than exogenous ATP.

Since all endogenous sources of ATP fuel calcium uptake comparably well, they may all fuel the calcium pump via similar mechanisms. It is unlikely that a direct coupling of all these different kinases can occur with the calcium pump; thus we hypothesized that these kinases may all contribute to a membrane-associated pool of ATP which is utilized preferentially by the calcium pump. To determine whether endogenously produced ATP mixes freely with ATP added exogenously in the bath, we utilized a combination of glucose and agarose-bound hexokinase to react with ATP and act as an ATP "trap." In the presence of this ATP trap, ATP added

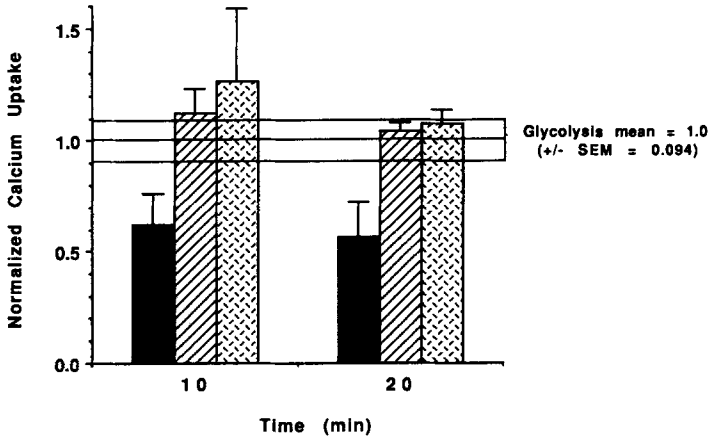

FIGURE 7. Summary of the comparisons of ^{45}Ca uptake fueled by various ATP sources to that fueled by glycolysis. Data are compiled from three preparations and are normalized to the glycolytically fueled ^{45}Ca uptake of that preparation at each time point. The data from the individual preparations were in at least triplicate and data are ±SEM of the normalized data. The ATP production rates of the various ATP sources were matched using the different PMV preparations individually (as described in text). The horizontal lines represent the mean and the average SEM of the normalized glycolytically fueled ^{45}Ca uptake. Calcium uptake supported by different ATP sources: ATP infusion (*solid bars*), creatine kinase (*hatched bars*), and pyruvate kinase (*stippled bars*).

exogenously at a rate matched to the initial glycolytic rate does not significantly support calcium uptake in PMV (Fig. 8). The initial rate of glycolysis averaged 13.6 nmol/min · mg (±2.9 SEM, $N = 3$). Thus the ATP trap has much greater access to the exogenously added ATP than does the calcium pump. However, all of the endogenous sources of ATP (with ATP production rates matched to that of glycolysis) can fuel calcium uptake in PMV despite the presence of the ATP trap. The abolition

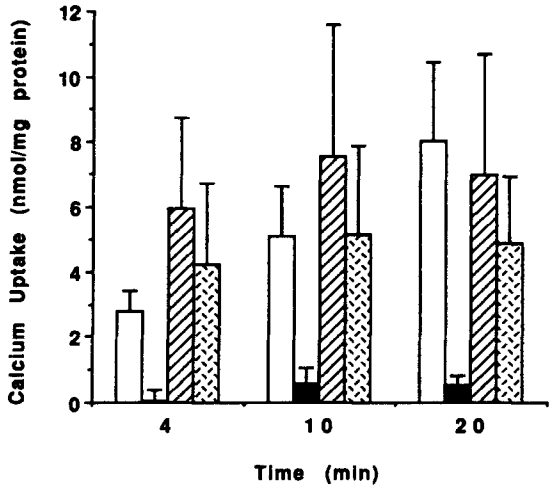

FIGURE 8. Summary of the comparisons of ^{45}Ca uptake fueled by various ATP sources to that fueled by glycolysis in the presence of the hexokinase-based ATP trap: ATP infusion (*solid bars*), creatine kinase (*hatched bars*), pyruvate kinase (*stippled bars*), and glycolysis (*open bars*). Data are compiled from three different preparations. The data from the individual preparations were in at least triplicate and are ±SEM.

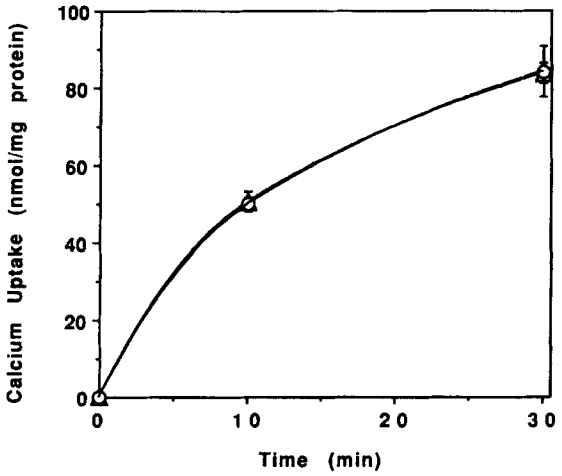

FIGURE 9. Calcium uptake fueled by 1 mM ATP in the presence of 50 mM Pi in the presence (*circles*) or absence (*triangles*) of hexokinase attached to beaded agarose. Glucose was not present in the experiment. Data are from one experiment and are in triplicate (±SD).

of calcium uptake fueled by exogenous ATP by hexokinase and glucose is not an artifact of the agarose-bound hexokinase. When agarose-bound hexokinase alone (without glucose) is added to PMV and 1 mM ATP, calcium uptake is unaltered (Fig. 9). In this experiment, 50 mM phosphate was added to increase the net uptake in order to amplify any differences between the two groups. Orthophosphate additions act to keep intravesicular Ca^{2+} concentrations low by formation of calcium phosphate deposits (see Raeymaekers, Agostini, and Hasselbach, 1981). Our data indicate that the calcium pump has greater access to the endogenously produced ATP than does the hexokinase in suspension, whereas the opposite is true for exogenous ATP.

Hexokinase in soluble (not bound to agarose) form in the presence of glucose is capable of eliminating calcium uptake fueled by either glycolysis or 2 mM ATP (Fig. 10). Again, this experiment was performed in the presence of 50 mM orthophosphate. Thus, soluble hexokinase appears to have access to both endogenously and

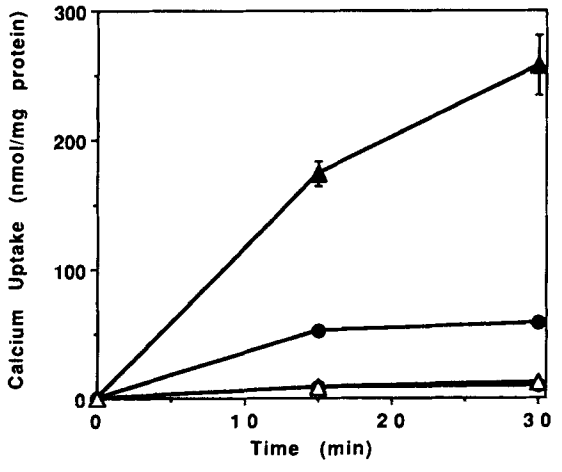

FIGURE 10. Calcium uptake fueled by either 2 mM ATP (*triangles*) or glycolysis (*circles*) in the presence (*open symbols*) or absence (*filled symbols*) of soluble hexokinase (7.5 U/ml) and glucose (5 mM). The open circles are obscured by the open triangles. 50 mM Pi was included in all treatments. Data are from one experiment and are in triplicate (±SD).

exogenously produced ATP, whereas agarose-bound hexokinase has limited access to endogenously produced ATP.

To what extent does the agarose-bound hexokinase–based ATP trap have access to the endogenously produced ATP? In Fig. 11, the percent of glycolytically fueled calcium uptake remaining in the presence of the trap compared with that remaining in the absence of the trap is plotted. At the 4-min point, ~90% of the calcium uptake remains in the presence of the ATP trap, with that value decreasing to ~40–50% between 10 and 30 min. Although there is variation in the absolute magnitude of ^{45}Ca uptake among different preparations of PMV, the percent reduction of the ^{45}Ca uptake by the ATP trap was very similar among preparations.

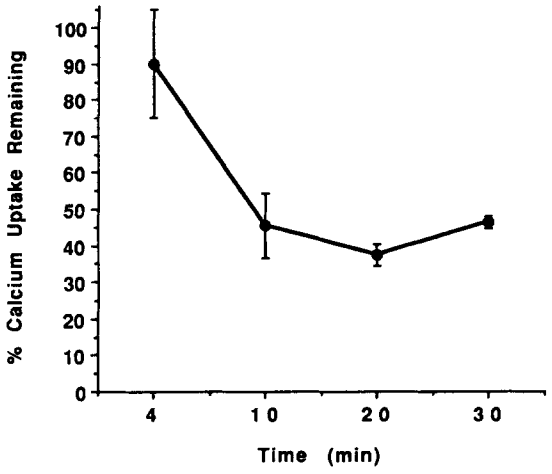

FIGURE 11. Percentage of ^{45}Ca uptake fueled by glycolysis which remains in the presence of the agarose-immobilized hexokinase-based ATP trap. Data at each time point are the average of experiments using two or three different PMV preparations. The data from the individual preparations were at least in triplicate (±SD).

DISCUSSION

PMV as a Model of Intact Smooth Muscle Plasmalemma

To directly test our hypothesis that, in smooth muscle, aerobic glycolysis is linked to membrane ion pump function, we used isolated smooth muscle PMV as a model system of the membrane in the intact muscle. The advantages of this model system are that we were able to examine whether glycolytic enzymes are indeed associated with the plasma membrane and what role these glycolytic enzymes play in the support of ion pump activity in relative isolation from other metabolic enzyme cascades. The isolation of PMV from mitochondrial ATP production allowed us to examine the role of ATP, produced some distance from the plasma membrane, in the support of membrane ion pump activity. Thus the ATP added via an infusion pump in our model system may be considered analogous to ATP produced by the mitochondria in the intact smooth muscle cell. The importance of ATP produced by the membrane-associated glycolytic cascade in the support of ion pump function can then be evaluated in this model system.

Glycolysis Is Associated with PMV

We have previously shown that all the glycolytic enzymes responsible for catabolizing fructose 1,6-diphosphate to lactate are associated with PMV and capable of producing ATP when provided with glycolytic substrate (Paul et al., 1989). It is unlikely that the glycolytic enzymes measured in PMV have simply been trapped during the preparation of the PMV fraction. For example, assuming that the glycolytic enzymes distribute uniformly throughout all volumes during the preparation, the predicted activity of aldolase in PMV would be 0.004 U/mg based on our measurement of aldolase in the tissue of 4 U/g wet weight. The actual aldolase activity in PMV was 0.215 U/mg (Paul et al., 1989), a factor of 50 greater than predicted. Similarly, the activity of pyruvate kinase in intact tissue would be 1,300 U/g wet weight based on our measurement of pyruvate kinase in PMV of 1.4 U/mg (Paul et al., 1989). Pyruvate kinase activity in vascular smooth muscle is ~5 U/g wet weight (Kirk, 1969). Thus, simple distribution of the soluble glycolytic enzymes in the PMV fraction during the preparation cannot account for the relatively high activities of glycolytic enzymes measured in PMV. In addition, these enzymes do not appear to be loosely associated with PMV as significant glycolytic enzyme activity remained after treatment with 600 mM KCl (Paul et al., 1989). The association of glycolytic enzymes with cellular membranes is not a novel finding. There is a long history of studies on the association of glycolytic enzymes with the membranes of the erythrocyte. A complete glycolytic cascade was concluded to be associated with the erythrocyte plasma membrane with some enzymes being completely membrane associated (Green, Murer, Hultin, Richardson, Salmon, Brierley, and Baum, 1965). More recently, using a rapid hemolysis/filtration system, glyceraldehyde-3-phosphate dehydrogenase (Kliman and Steck, 1980), phosphofructokinase, and aldolase (Jenkins, Madden, and Steck, 1984) were determined to be 65, 50, and 40% bound to the plasmalemma, respectively. The enzyme binding was reversible and dependent on the metabolic state of the cell (Jenkins et al., 1984). In cardiac tissue, a glycogenolytic particle was found to be associated with sarcoplasmic reticular membranes (Entman, Kanike, Goldstein, Nelson, Bornet, Futch, and Schwartz, 1976) and glycolytic enzymes were found to specifically bind to both sarcoplasmic reticular and sarcolemmal membranes (Pierce and Philipson, 1985). Similar findings were obtained for skeletal muscle (Entman, Keslensky, Chu, and Van Winkle, 1980; Caswell and Corbett, 1985). Thus the association of glycolytic enzymes with cellular membranes may be a general feature of eukaryotic cells.

Glycolysis Fuels the Calcium Pump Independently of Solution [ATP]

To determine whether ATP produced by glycolytic enzymes localized on unsealed membrane fragments significantly contributes to calcium uptake in PMV, PMV was serially diluted 10-fold and glycolytically fueled calcium uptake was measured. At a 10-fold dilution of PMV, the bath [ATP] decreases ~10-fold. If the endogenous glycolytic cascade were functioning analogously to a soluble ATP regenerating system, such as the exogenously added PCr and creatine phosphokinase, then as the [ATP] in the bath decreased, calcium uptake per milligram protein would decrease. Since calcium uptake per milligram protein did not change with a 10-fold dilution of

PMV (using three different preparations), we conclude that the glycolytically produced ATP that fuels calcium uptake is produced almost entirely by localized enzymes.

The independence of glycolytically supported calcium uptake from bath [ATP] may indicate some form of channeling of ATP from the kinases of glycolysis to the calcium pump. Evidence for channeling of ATP between phosphoglycerate kinase and the sodium pump, via a membrane-associated pool of ATP, has been reported for the erythrocyte (Parker and Hoffman, 1967; Proverbio and Hoffman, 1977). The Ca^{2+}-ATPase of the erythrocyte was also found to partially utilize membrane-associated ATP rather than ATP in the bath (Proverbio, Shoemaker, and Hoffman, 1988). Channeling of glycolytic substrates between the sequential enzymes of glycolysis has been studied in a variety of tissues (see Srere, 1987 for a review). The data from the serial dilution experiment agree well with the hypothesis that glycolytically produced ATP may be channeled to the calcium pump, perhaps via a membrane-associated pool, although the nature of this apparent channeling remains unclear.

Glycolytically Produced ATP Is Preferred by the Calcium Pump

To directly compare the ability of glycolytically produced ATP to fuel the calcium pump with that of ATP produced exogenously, we matched the ATP production rates of the two systems. Calcium uptake fueled by exogenously added ATP was 62 and 56% of that fueled by glycolysis at 10 and 20 min of incubation, respectively. Thus, glycolytically produced ATP appears to be preferentially utilized by the calcium pump, by almost a factor of two, compared with exogenously added ATP. This technique of matching the ATP production rates is based on the assumption that flux through the sequential enzymes glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase is equal during the linear phase of glycolytic NADH production. By this technique, the initial rate of glycolytic NADH production (via glyceraldehyde-3-phosphate dehydrogenase) is taken as being equal to the initial rate of glycolytic ATP production (via phosphoglycerate kinase). If any mismatch occurs, it would be that the flux through GAPDH is greater than the flux through PGK, resulting in an overestimate of the initial rate of glycolytic ATP production. Thus, when ATP is produced by other systems at a rate matched to the rate of glycolytic NADH production, ATP may have been added at a rate greater than the actual ATP production rate of glycolysis. Additionally, infused ATP was added at a rate matched to the estimated initial rate of glycolytic ATP production, which is an overestimate of the actual rate of glycolytic ATP production over 30 min of incubation (see Fig. 5). Thus glycolytically produced ATP may be utilized preferentially by the calcium pump to a greater extent than was observed. The slightly greater calcium uptake fueled by other endogenous sources of ATP may have resulted from the same overestimation of the rate of glycolytic ATP production.

The membrane calcium pump also preferentially utilizes ATP produced by either the endogenous creatine kinase or endogenous pyruvate kinase as compared with exogenously added ATP (see Fig. 7). The preferential use, by cardiac plasmalemmal Na/K-ATPase, of ATP produced by the endogenous creatine kinase (Grosse, Spitzer, Kupriyanov, Saks, and Repke, 1980), and more recently, calcium uptake by sarcoplasmic reticulum (Rossi, Eppenberger, Volpe, Cotrufo, and Wallimann, 1990) has

been reported. Thus, in another vesicular system different ion pumps appear to preferentially utilize endogenously produced ATP. Since three different endogenous sources of ATP appear to fuel different membrane ion pumps preferentially, it is likely that the endogenously produced ATP forms a membrane-associated common pool which can then be utilized by membrane ATPases. Such a mechanism has been previously proposed for the erythrocyte where phosphoglycerate kinase contributes to a membrane pool of ATP which can support both the Mg-ATPase and the Na-ATPase (Proverbio and Hoffman, 1977). The preferential utilization, by membrane ATP-requiring processes, of a membrane associated pool of ATP produced by membrane-associated kinases may represent a common feature of cellular energy utilization.

Restricted Diffusion of Endogenously Produced ATP

The ATP produced by the endogenous ATP-producing systems continues to fuel the calcium pump despite the presence of a hexokinase-based ATP trap (5 U/ml) capable of eliminating calcium uptake fueled by ATP added exogenously to the bath. At 2 U/ml of hexokinase, calcium uptake fueled by infused ATP was still distinguishable from calcium binding (data not shown). Therefore, 5 U/ml of hexokinase was chosen as a concentration that could consistently eliminate the calcium uptake fueled by infused ATP. As shown in Fig. 11, by 4 min of incubation glycolytically fueled calcium uptake in the presence of the ATP trap is ~90% of that in the absence of the ATP trap. This finding is consistent with the ATP produced by enzymes endogenous to PMV having restricted diffusion away from the vesicle. Similar conclusions were reached for the sodium pump in the erythrocyte ghosts (Mercer and Dunham, 1981) using a hexokinase-based ATP trap. In addition, glycolytically produced ATP has been shown to prevent ATP-dependent potassium channels from opening in cardiac myocytes despite the presence of an ATP trap (Weiss and Lamp, 1987). These results indicate that the ATP produced at the level of the membrane readily diffuses to membrane ATP-requiring processes but not to the bulk solution.

Compartmentalization of Energy Transduction

The heterogeneous localization of glycolytic enzymes within the cytosol may represent a localization of glycolytic function. In this study we have shown that a glycolytic cascade or creatine kinase localized to the plasmalemma preferentially supports the calcium pump. We have previously observed a functional compartmentalization of glycolysis and glycogenolysis in smooth muscle with glycolysis correlated with membrane ion pump function, and glycogenolysis associated with oxidative phosphorylation which supports maintenance of tension (Lynch and Paul, 1985). Moreover, creatine kinase localized at the myofibrils of cardiac tissue has been reported to specifically utilize ADP produced by myofibrillar Mg^{2+}-ATPase, while creatine kinase localized at the plasmalemma specifically utilizes ADP produced by plasmalemmal Mg^{2+}-ATPase (Saks, Ventura-Clapier, Huchua, Preobrazhensky, and Emelin, 1984). Therefore, energy-producing systems, composed of the same enzymes but localized differently within the cell, may support different cell functions. The localization of energy transduction within the cytosol may therefore determine which energy-

producing enzymes will support a given energy-requiring process. The functional colocalization of energy-producing and -transducing enzymes may be a common characteristic of cellular energy transduction.

The nature of this functional coupling of energy-producing to energy-consuming enzymes remains unclear. Mercer and Dunham (1981) showed that a membrane-associated glycolytic cascade supported the sodium pump in erythrocyte inside-out vesicles despite the inclusion of soluble hexokinase and glucose. Weiss and Lamp (1987) used intact cardiac myocytes to show that glycolytically produced ATP inhibited an ATP-dependent potassium channel, also despite the inclusion of the soluble ATP trap. However, in our experiments a soluble hexokinase-based ATP trap (7.5 U/ml), in contrast to the agarose-bound, hexokinase-based ATP trap reported above, eliminated calcium uptake in PMV fueled by either endogenous or exogenous sources of ATP (Fig. 10). This difference may be a reflection that the degree of the preservation of the native state of the membranes or the degree of incorporation of hexokinase within the membranes differs among experimental preparations. We believe this may be an important distinction, but at this stage further speculation is not warranted.

For smooth muscle, this is the first demonstration that ATP produced by membrane-associated glycolytic enzymes preferentially supports a membrane ATP-requiring process. This may be of particular interest, for in contrast to the erythrocyte, smooth muscle has both oxidative and glycolytic metabolism. In addition, we have compared multiple endogenous ATP sources with an exogenous ATP source and found that all three endogenous sources of ATP supported the calcium pump equally well, and all endogenous ATP sources were utilized preferentially by the calcium pump. Although other studies, primarily using cardiac and erythrocyte membranes, have provided some evidence for preferential use of ATP produced at the membrane for membrane ATP-requiring processes, our finding that ATP produced by various endogenous enzymes are approximately equally well utilized indicates that the mechanism of the preferential use of such ATP may be by creating a unified membrane-associated pool of ATP which is supplied by various enzymes and used by various ATPases.

The localized functional coupling of glycolysis to specific ATP-requiring processes has a number of important physiological implications. Such localized energy transduction allows for independent regulation of the separate pools of glycolysis and glycogenolysis as proposed by Lynch and Paul (1985). In addition, the close juxtaposition of energy-producing and -consuming systems may result in a kinetic advantage of the transfer of ATP and ADP between enzymes, thereby enabling ATP production and consumption of the coupled system to be closely and rapidly matched. More detailed knowledge of the structure of the "cytosol" will be required to elucidate its role in energy transduction.

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