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Continuous recording of pH and pCa during calcium binding by muscle microsomes

The methods which have been routinely used for studying calcium binding by muscle microsomes involve separating the particles from their suspension medium, either by centrifugation (*e.g.* ref. 1) or filtration (*e.g.* ref. 2). These procedures impose limits on the time resolution obtainable. In addition, the properties and contents of the microsomal membranes are subject to alteration by forces acting on the particles during filtration or centrifugation. The uptake of calcium by isolated sarcotubular vesicles has been detected in measurements of turbidity changes, which can be monitored continuously[3]. OHNISHI AND EBASHI[4] have utilized an indicator technique to obtain continuous measurements during calcium binding. HERNIO AND SARIS[5] have described experiments in which pH was recorded continuously. The changes in pH were found to be correlated both with H^+ production resulting from ATP hydrolysis and with ammonia evolution accompanying adenylate deamination[5]. The approach described in the present paper permits continuous and sensitive measurements of net calcium movements and associated changes in energy utilization.

In our experiments, pH and pCa were simultaneously recorded. The A. H. Thomas No. 4858-L15 combination pH electrode and the Orion[6] calcium electrode were used. The electrodes were connected, with the pH electrode reference in common, to Radiometer model 22 pH meters. The outputs of the meters were further amplified and then recorded by means of a dual channel recorder.

In the experiment shown in Fig. 1A, the endogenous creatine kinase of the microsomal preparation, together with added creatine phosphate, served as an ATP-regenerating system. The use of this regenerating system simplifies the interpretation of H^+ changes. When the added adenine nucleotide is maintained primarily in the form of ATP, ammonia production due to adenylate deamination is minimized and

does not contribute significantly to pH changes. The level of creatine kinase in the microsomal fraction appears to be sufficient so that rephosphorylation of ADP is not a rate-limiting step in the overall hydrolysis. To check this, ATP was determined enzymatically[9] in aliquots of the reaction mixture, in experiments similar to that of Fig. 1A. Within the experimental error (maximal variation about the mean approx. $\pm$ 10 %), the ATP level in the presence or absence of calcium was identical to the total concentration of adenine nucleotide added. The addition of 0.12 mg/ml of creatine kinase (Boehringer) did not alter the measured ATP concentration.

As shown in Fig. 1A, there is a fairly linear change in pH, which is accelerated by the addition of calcium. Phosphate analyses in similar experiments, carried out by the method of WAHLER AND WOLLENBERGER[10], have shown that the percentage increase[11] in the rate of release of P_i in the presence of calcium is approximately equal to the corresponding increase in the rate of OH^- production. In two experiments, the ratios of the rates of stimulated/control (a) ATPase activity and (b) OH^- production were (a) 2.1 and (b) 1.9 (Expt. 1), and (a) 1.8 and (b) 1.7 (Expt. 2). Under the conditions of the experiment of Fig. 1A, about 0.4 OH^- is released per creatine phosphate split in the overall reaction. Since approximately the same OH^-/P_i ratio of about 0.4 is observed either in the presence or absence of calcium, the pH changes appear to be predominantly associated with the creatine phosphate hydrolysis which accompanies ATP splitting. The changes in slope of the pH traces, which are quite sharp, can be used to estimate the length of time required for a given addition of calcium to be bound.

The application of the calcium electrode to somewhat similar mitochondrial experiments has been described by CHANCE AND YOSHIOKA[12]. Certain reaction conditions required for the microsomal experiments complicate the interpretation of calcium electrode responses. The microsomal calcium binding is dependent on ATP and magnesium[1,11]. The calcium electrode shows some sensitivity to magnesium, at the level of this cation required to support microsomal calcium binding. Responses

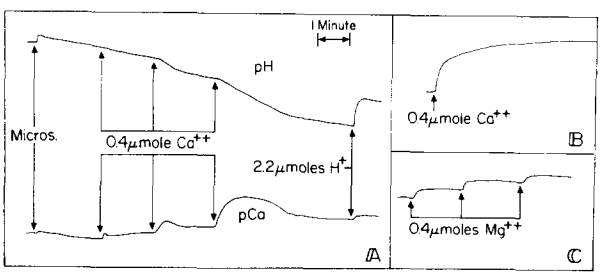

Fig. 1. Microsomes were isolated from rabbit skeletal muscle in a medium containing 0.1 M KCl and 5 mM histidine, essentially by the method of MARTONOSI AND FERETOS[7]. After the two initial centrifugations in which myofibrils and mitochondria were removed, the microsomes were sedimented by centrifugation at 78000 $\times$ *g* for 30 min. The possibility that the Ca^{2+} binding properties of the preparation might be attributable to mitochondrial contamination was ruled out by experiments similar to this in which mitochondrial inhibitors[8], such as oligomycin (1.3 μg/mg protein) and azide (5 mM) were found to have little effect. In (A) the reaction mixture contained the following substances in a total volume of 8 ml: 0.1 M KCl, 20 mM histidine (pH 6.8), 0.5 mM disodium ATP (Sigma), 0.5 mM $MgCl_2$, 3.7 mM disodium creatine phosphate (Boehringer), 10 mg microsomal protein. In addition, ionic solutes added with the microsomes included 44 μmoles Mg^{2+} and 12 μmoles Ca^{2+} per g of protein. The temperature was 20°. In (B) and (C) conditions were the same, except that no microsomes were added. The calcium electrode traces only are shown, at the same gain and time scale as in (A).

of the electrode to equivalent additions of calcium and magnesium, under the experimental conditions of this paper, in the absence of microsomes, are shown in Fig. 1, B and C. While correlations between increments in electrode potential and changes in calcium concentration are readily obtained, meaningful calibration of the electrode responses in terms of calcium activity is difficult, when a complexing agent such as ATP is present, along with magnesium. The practical range of sensitivity of the electrode also excludes some concentrations relevant to the microsomal experiments. Muscle microsomes, supplemented with magnesium and ATP, have been reported to be capable of lowering the calcium activity of a suspension medium to 10^{-7} M or lower[13]. The calcium electrode does not show a response to calcium, under the conditions of interest, below about 10^{-6} M.

As is evident from Fig. 1B, the response of the calcium electrode is slow compared to the calcium uptake rates. When an uptake cycle is short enough, the calcium electrode does not have time to respond fully before the calcium activity has returned to a low level (see Fig. 1A). Although slower in its response than the pH electrode, the calcium electrode does serve to confirm the time course and extent of the uptake of a calcium addition. The inclusion of an ATP-regenerating system is critical. If a regenerating system is not used, magnesium and calcium are released from chelation by ATP as the ATP is hydrolyzed. The result is an apparent increase in bivalent ion activity on the calcium electrode trace, which can obscure changes in calcium activity due to calcium uptake by the microsomes. When an ATP-regenerating system is used, as in Fig. 1A, the calcium electrode potential returns to almost the initial level when an uptake cycle is completed. There is, however, a slight shift of the baseline as each addition of calcium is bound. This may arise in part from the release of magnesium from the microsomes in exchange for calcium[14]. Additions of magnesium in amounts equivalent to the quantities of calcium bound cause comparable changes (see Fig. 1C).

In the absence of a precipitant, sequential additions of calcium are taken up at increasingly slower rates, as expected from earlier studies (*e.g.* ref. 2). The progressive slowing of the uptake rate is not seen if oxalate or phosphate is added, or if sufficient P_i is released as a consequence of creatine phosphate hydrolysis. Even in the presence of a precipitant such as oxalate, however, a small amount of calcium is initially bound at a more rapid rate than the rate of binding of subsequent calcium additions. For example, in an experiment in which 1 mM oxalate was included, other conditions being comparable to Fig. 1, four consecutive additions of 35 μmoles calcium per g of protein, made over a period of 6 min, were taken up in 17, 67, 50, and 53 sec (uptake times estimated from the pH recordings).

The Ca/ATP ratio for inward calcium flux has been reported to be about 2, when measured by isotope techniques[2,15,16]. Ca/ATP ratios of close to 2 have also been reported for net calcium uptake, in the absence of a precipitant, prior to saturation[2]. From our data, Ca/ATP ratios for net calcium transport can be calculated, by correlating the calcium uptake time, estimated from the pH trace, with the rate of "extra"[15] ATP splitting, determined by phosphate analysis. In an experiment similar to that of Fig. 1, the net Ca/ATP ratios for three successive additions of 27 μmoles of calcium per g of protein were, respectively, 1.3, 0.31 and 0.14 moles of calcium bound per "extra" ATP split. If the reported Ca/ATP ratio of 2 for unidirectional transport[2,15,16] is applicable to these experiments, it is evident that there is considerable recycling of calcium through the microsomal membranes.

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Johnson Research Foundation,
University of Pennsylvania,
Philadelphia, Pa. (U.S.A.)

JOYCE H. JOHNSON
BERTON C. PRESSMAN

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