A RAPID AND ULTRASENSITIVE METHOD TO MEASURE Ca^{++} MOVEMENTS ACROSS BIOLOGICAL MEMBRANES

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SUMMARY

An electrometric system was used to measure Ca^{++} uptake by sarcoplasmic reticulum vesicles (SR). The method permits continuous recording of Ca^{++} uptake and thus the valuation of kinetic parameters. Furthermore, the ultrasensitivity of the method permits to follow changes in Ca^{++} concentration below 10^{-6} M.

INTRODUCTION

Various methods have been described to measure Ca^{++} uptake by biological membrane systems. Some of them involve centrifugation procedures and analyses of Ca^{++} either by radioisotope techniques or by atomic absorption spectrometry (1, 2). Other methods take advantage of filtration techniques followed by Ca^{++} analysis either in the filtrates or in the material trapped on the filters (3).Double beam spectrophotometry has been applied to follow the trace of murexide absorbance in specialized and rather expensive equipment (4, 5). Fluorescent probes such as chlorotetracicline have been also used to distinguish between Ca^{++} bound or free (6, 7). However, all these methods have limited application mainly when one wants to follow the kinetics of Ca^{++} uptake at low Ca^{++} concentrations in a rapid and simple way.

I describe here a simple and ultrasensitive method by using a potentiometric technique and standard equipment normally available in most laboratories. The method permits following changes in the Ca^{++} in the medium of the order of 1-10 nmoles

even in the presence of a relative large background of Ca^{++} and other cations. The method also works at concentrations of Ca^{++} as low as 10^{-6} M.

MATERIALS AND METHODS

Sarcoplasmic reticulum membranes (SR) were isolated from rabbit skeletal muscles as described elsewhere (8). The membranes were kept in 0.1 M KCl and 10 mM Tris-maleate (pH 7.0) at about 20-25 mg of protein per ml of suspension. The protein was determined by the biuret method (9).

The potentiometric set up for measuring Ca^{++} uptake is composed of a Radiometer Ca^{++} electrode, a Radiometer pH meter, Model PHM 64, a Perkin Elmer recorder, Model 56, and an intermediate bucking voltage device (Fig. 1) which was inserted in series between the pH meter output and the recorder input. The pH mode of the pH meter was used without the A 3701 adapter plug.

The reaction media (pH 7.0) contained 50 mM KCl, 5 mM $MgCl_2$, 5 mM Tris, 0.2 mM ATP, $CaCl_2$ and other reagents as desired. The reactions were conducted at 18°C in a thermostated vessel containing the SR membranes (0.1 to 1.0 mg protein) in a total volume of 2.5 ml.

The calibration of the system by additions of small amounts

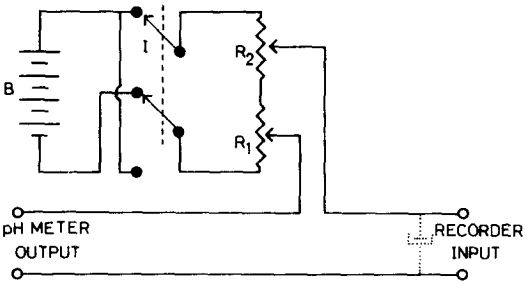

Fig. 1 - Circuit of the bucking voltage device inserted in the experimental set up. When very high sensitivity is desired (10 mV full scale in the recorder), a capacitor (10-30 μF) inserted as shown by the dotted trace reduces the noise. Symbols: R_1, 10 KΩ; R_2, 1 KΩ; B, 6 V battery; I, reverser switch.

of Ca^{++} was made at the end of the experiments, since we verified that the presence of SR protein and ATP do not change the sensitivity of the electrode.

Movements of Mg^{++} and H^{+} during the Ca^{++} uptake by SR do not influence the electrode response, since additions of Mg^{++} and H^{+} in relative large amounts do not have measurable effects.

RESULTS AND DISCUSSION

Fig. 2 shows the electrode response to additions of Ca^{++}, ATP and Mg^{++}. The additions of Ca^{++} produce a rather linear response in the range of Ca^{++} concentration utilized throughout. Addition of ATP up to 0.2 mM does not interfer apreciably with the signal and additions of Mg^{++} do not have any measurable effect even when the concentration added is ten times higher than that of Ca^{++}.

When the SR protein (1.0 mg) is added to the medium (Fig. 2), there is a small increase in the signal probably due

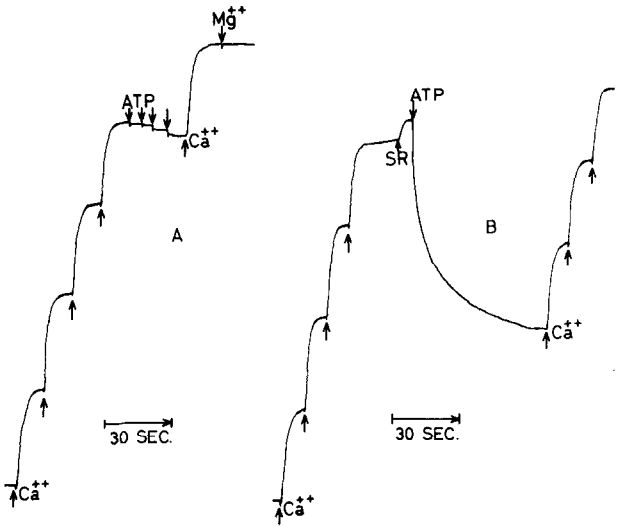

Fig. 2 - Signals produced by Ca^{++}, ATP, Mg^{++} and SR. (A) Each Ca^{++} addition was 50 nmoles and the concentration of Ca^{++} was 10^{-5} M prior to the first addition. The final concentration of ATP was 50 µM after each addition. The amount of Mg^{++} added was 500 nmoles. (B) The amount of Ca^{++} taken up by SR in 1 min. was about 130 nmoles. Additions were: Ca^{++}, 50 nmoles; ATP, 0.1 mM; SR, 1.0 mg.

to the contaminating Ca^{++} of the suspension added. Further addition of ATP causes a rapid deflection on the Ca^{++} trace due to Ca^{++} uptake by the SR vesicles. At the end of 1 min., about 130 nmoles of Ca^{++} are taken up per mg of SR protein. Note that the Ca^{++} additions at the end of the reaction (Fig. 2) produce signals similar to those before the additions of protein and ATP. Thus, the calibration of the trace may be done at the end of the experiments, when Ca^{++} uptake is essentially complete.

Under the conditions utilized above, care should be taken to avoid a drop of Ca^{++} concentration in the medium below about 10^{-5} M, since the response of the electrode below this concentration is slow and not linear. Thus the amounts of added protein and Ca^{++} must be controled in such a way that the Ca^{++} concentration does not drop below 10^{-5} M. However, the electrode can work satisfactorily below 10^{-5} M if EGTA is added to the medium. In this circunstances it is possible to work at about 10^{-6} M or even below (Fig. 3). Other methods using double bleam spectrophotometry of murexide absorbance have failed to work at these low Ca^{++} levels (4, 5).

We show in Fig. 4 that it is possible to estimate the initial Ca^{++} uptake rates since the electrode response is fast enough. Note that additions of Ca^{++} at the end of the experiment

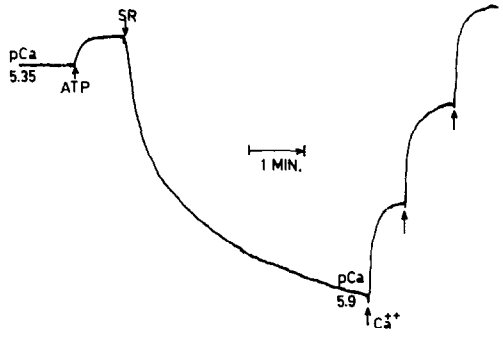

Fig. 3 - Ca^{++} uptake at low Ca^{++} concentration. The reaction was conducted in the standard reaction medium containing 0.1 mM Ca^{++} and 0.1 mM EGTA. The estimated free Ca^{++} concentration before SR addition (0.2 mg) was about 4.4×10^{-6} M (pCa 5.35). In the lowest point of the Ca^{++} trace the Ca^{++} concentration was 1.2×10^{-6} M (pCa 5.9). The trace was calibrated at the end of the experiment by adding 10 nmoles of Ca^{++} where indicated.

produce 90% of the maximum signal in about 2 sec. The estimated initial rate of Ca^{++} uptake at 18°C (660 nmoles/min./mg protein) is comparable with the rates previously reported (4, 5, 10). A control with "leaky" (sonicated) membranes showed that the Ca^{++} trace remains stable since there is no Ca^{++} acummulation. The slightly increase in Ca^{++} concentration upon the addition of sonicated vesicles is probably due to some endogenous Ca^{++} which leaks out from the disrupted membranes.

Fig. 4 shows also that most of the Ca^{++} is taken up by SR in the first 10 sec. However, the uptake proceeds at a slow rate for several minutes probably due to precipitation of calcium phosphates inside the vesicles (3, 11). The sonicated vesicles do not induce measurable alteration on the Ca^{2+} activity of the medium. Therefore, the slow rate of Ca^{++} activity decrease is due to transport of Ca^{++} into the SR vesicles.

Fig. 4 shows that the amount of Ca^{++} taken up by SR is a function of the amount of protein added and that it is possible to work with minute amounts of material with measurable signals. The stability of the electrode system permits to increase this sensitivity by a factor of 5 to 10 without apreciable noise

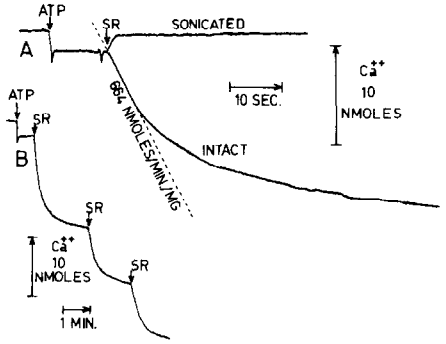

Fig. 4 - Ca^{++} uptake by intact and sonicated SR vesicles. (A) The estimated initial rate of uptake was about 664 nmoles/min./mg protein. The concentration of Ca^{++} was 60 µM (150 nmoles) and that of ATP was 0.2 mM. The SR added was 0.1 mg. (B) The amount of Ca^{++} removed from the medium is a function of the amount of SR added. The first addition was 0.2 mg followed by two additions of 0.1 mg. Total Ca^{++} concentration prior to addition of SR was 60 µM (150 nmoles) and ATP 0.2 mM.

which can be diminished by the insertion of a capacitor between the input terminals of the recorder (Fig. 1).

We tried also to use other comercially available electrodes such us the Orion system. Although this electrode have similar performances to those of the Radiometer in pure Ca^{++} solutions, it does not respond to small additions of Ca^{++} in the reaction medium referred to above. Probably the high Mg^{++} background of the medium (5 mM) lowers the sensitivity of the Orion electrode so that it does not respond anymore to Ca^{++} additions in the range of interest in our studies. Furthermore, as it has been pointed out previously (12), the response of the Orion electrode is rather slow so that it is useless in following the rapid kinetics of Ca^{++} uptake. In contrast, the response of the Radiometer electrode is very fast under the conditions utilized permiting estimation of initial rates.

We believe that the method here described may be very useful in studies of Ca^{++} movements across biological membranes, such us sarcoplasmic reticulum, mitochondria, plasma membranes and other biological materials, since it permits estimating very accurately the rates of transport, total uptake capacity and other valuable parameters. Furthermore, the present method is of easy application, is rapid and ultrasensitive and requires standard equipment available in most laboratories.

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