Acetyl Phosphate as Substrate for Ca$^{2+}$ Uptake in Skeletal Muscle Microsomes

INHIBITION BY ALKALI IONS

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SUMMARY

In skeletal muscle microsomes, Ca$^{2+}$ uptake activated by acetyl phosphate is inhibited by alkali ions. The inhibitory activity of these ions depends on the acetyl-P concentration in the assay medium. For 0.2 mM acetyl-P the pattern of inhibition is Li$^+$ > Na$^+$ > K$^+$ = Rb$^+$ = Cs$^+$ and for 2.0 mM acetyl-P it is Na$^+$ > Li$^+$ > K$^+$. In assay media containing 7.1 mM Ca$^{2+}$ and either 20 mM KCl, 120 mM KCl, or 120 mM NaCl, the plot of the initial rate of Ca$^{2+}$ uptake against acetyl-P concentration yields hyperbolic saturation curves. In an assay medium containing 120 mM LiCl, this saturation curve has a sigmoidal shape. In the presence of 2 mM acetyl-P, the initial rate of Ca$^{2+}$ uptake measured as a function of the Ca$^{2+}$ concentration in the assay medium displays a sigmoidal shape. However, if ATP is used as substrate, a hyperbolic saturation curve is obtained. For 2 mM acetyl-P, the inhibitory activity of the alkali ions depends on the Ca$^{2+}$ concentration in the assay medium. For Ca$^{2+}$ concentrations ranging from 1 to 2 mM, the pattern of inhibition is Li$^+$ > Na$^+$ > K$^+$, and for Ca$^{2+}$ concentration of 7.1 mM, the pattern of inhibition is Na$^+$ > Li$^+$ > K$^+$. Acetyl-P is used as substrate, the kinetics of Ca$^{2+}$ transport by skeletal muscle microsomes resembles that of allosteric enzymes.

METHODS

Preparation of Microsomes. These were prepared by the method of Hasselbach and Makinosel which is the standard procedure of the laboratory. All operations were performed at 4°C. The hind leg and back muscles of a rabbit were ground in a meat grinder. Approximately 500 g of the minced muscle were mixed with 1,500 ml of a cold solution containing 100 mM KCl, 2 mM EDTA, 2.5 KH$_2$PO$_4$, and 2.5 mM K$_2$HPO$_4$. The mixture was homogenized in a Waring Blender for 2 min. The myofibrils were sedimented by centrifugation at 6,500 × g for 15 min. The supernatant was centrifuged at 10,000 × g for 15 min to remove mitochondria. The supernatant was centrifuged in a Spinco model L preparative ultracentrifuge at 44,000 × g for 1 hour. The pellet was then resuspended in 25 ml of KCl, 0.1 M, and stored at 4°C. This preparation proved to be active for at least 1 week. Protein was estimated by the micro-Kjeldahl method assuming the nitrogen content of the protein to be 16%.

Standard Assay—Unless otherwise stated, the incubation medium consisted of 10 mM Tris-maleate buffer, pH 7.0, 5 mM MgCl$_2$, 0.10 mM $^{45}$CaCl$_2$, 0.15 mM EGTA, 4 mM potassium oxalate, and the specified concentrations of acetyl-P and either NaCl, KCl, or LiCl. For these CaCl$_2$ and EGTA concentrations, the free calcium ion concentration calculated was 6.5 × 10$^{-6}$ M. The reaction was started by the addition of microsomes, 0.15 mg of protein per ml. After 2 min of incubation at 37°C, the reaction was stopped by removal of particles with Millipore filters. In all experiments, controls were performed both (a)
without microsomes and (b) with microsomes but without acetyl-P.

Measurement of Ca\(^{2+}\) Uptake—\(^{45}\)CaCl\(_2\) was determined with a liquid scintillation counter. The percentage of Ca\(^{2+}\) bound to microsomes was calculated from the radioactivity of the microsome-free medium and that of the microsome-containing sample. In the range of microsomal protein concentration used, the amount of Ca\(^{2+}\) bound to microsomes in the absence of acetyl-P was less than 0.025 amole per mg of protein, independent of the concentration of monovalent cations in the assay medium.

Apparent Binding Constant of EGTA for Calcium—The solubility method described by Murphy and Hasselbach (7) was used to determine the extent of calcium-EGTA complex formation in the presence of alkali ions. The difference in soluble calcium concentration with and without the addition of EGTA to a suspension of a sparingly soluble calcium salt was measured.

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**RESULTS**

**Ca\(^{2+}\) Uptake as Function of Acetyl-P Concentration**—In these experiments the initial velocity of Ca\(^{2+}\) uptake was measured. When one of the activating divalent cations or the substrate was varied, the other components were added at optimal concentrations. This experimental procedure was used to study the inhibition of Ca\(^{2+}\) uptake by monovalent cations.

**Fig. 1** shows Ca\(^{2+}\) uptake as a function of acetyl-P concentration. Incubation medium and experimental conditions were as described under "Methods." In each experiment, the percentage of inhibition was calculated relative to a control containing 15 mM KCl. On the other hand, this relationship was observed. The apparent \(K_a\) measured in five different microsomal preparations was found to range from 2.0 \(\times 10^{-4}\) to 2.5 \(\times 10^{-4}\) M. When the monovalent cation concentration of the assay medium was increased to 120 mM with either KCl or NaCl, the uptake of Ca\(^{2+}\) was inhibited (Fig. 1). Na\(^+\) was a more effective inhibitor than K\(^+\) at any of the acetyl-P concentrations tested. In both cases a hyperbolic relationship between Ca\(^{2+}\) uptake and acetyl-P concentration was obtained.

**Fig. 2** shows the inhibition of Ca\(^{2+}\) uptake by alkali ions. Incubation medium and experimental conditions were as described under "Methods." In each experiment, the percentage of inhibition was calculated relative to a control containing 15 mM KCl in the assay medium. The values of each column represent the average \(\pm S.E.\) of 8 experiments.

**Chemicals**—Acetyl-P, ATP, phosphoenolpyruvate, and pyruvate phosphofluorase (EC 2.7.1.40) were supplied by C. P. Boehringer and Soehne (Mannheim) and \(^{45}\)CaCl\(_2\) by Buchler and Company, Frankfurt, Germany. All the other chemicals were analytical grade.

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**Fig. 2** shows that, at an acetyl-P concentration of 0.2 mM, their relative inhibitory activity was Li\(^+\) > Na\(^+\) > K\(^+\). At 2.0 mM acetyl-P it changed to Na\(^+\) > Li\(^+\) > K\(^+\). Fig. 3 shows the progressive inhibition induced by increasing concentrations of alkali ions at acetyl-P concentrations of 0.2 and 2.0 mM. RbCl and CsCl also inhibit microsomal Ca\(^{2+}\) uptake.
FIG. 3. Influence of salt concentration on Ca²⁺ uptake. Incubation medium and experimental conditions were as described under "Methods." Left, acetyl-P, 0.2 mM; right, acetyl-P, 2.0 mM. ○ O, KCl; △ △, NaCl; □ □, LiCl.

It was found in four preparations tested, that the inhibitory activity of Rb⁺ and Cs⁺ was essentially the same as that of K⁺ both at 0.2 and 2.0 mM acetyl-P.

In control experiments, the Ca²⁺ uptake was measured in 0.2 mM acetyl-P both with 10 mM KCl and with 10 mM KCl plus 240 mM sucrose. It was observed that the amount of Ca²⁺ taken up was essentially the same, thus excluding the possibility of an osmotic influence on Ca²⁺ uptake.

Effect of Monovalent Cations on Dissociation Constant of Calcium-EGTA Complex—In order to study the Ca²⁺ uptake as a function of the Ca²⁺ concentration in the assay medium, a CaCl₂ plus EGTA system was used. It has been shown that the apparent dissociation constant of the complex calcium-EGTA varies with the composition of the assay medium (9-12). To determine whether the different concentrations of monovalent cations used modify this dissociation constant, the solubility method described under "Methods" was used. In 15 different determinations with 120 mM of either KCl or NaCl, the value found for $K_{\text{dis}}$ was $3.95 \times 10^{-6}$ M ± S.E. 0.24. This value is in the range reported by Ebashi and Ogawa at pH 6.8 and is about 20 times higher than the value calculated from Schwarzenbach's data for pH 7.0 (9-10). Table I shows that this constant was not significantly altered when the KCl concentration of the medium was raised from 15 to 120 mM or when NaCl and LiCl were substituted for KCl in the same concentration range. In addition, no significant differences were found when: (a) the free calcium concentration of the medium was modified by increasing the oxalate concentration from 4 to 16 mM; (b) the EGTA concentration was raised from 0.1 to 0.2 mM; or (c) 2 mM acetyl-P or 2 mM phosphoenolpyruvate plus 10⁻⁵ M ATP were added to the medium. Thus, in the following experiments, the value of $K_{\text{dis}} = 3.95 \times 10^{-6}$ M was used to calculate the free calcium concentrations for given CaCl₂ and EGTA concentrations.

Ca²⁺ Uptake as Function of Ca²⁺ Concentration—Fig. 4 shows Ca²⁺ uptake in media containing 2 mM acetyl-P, a low concentration of KCl, and different concentrations of ionic calcium. Sigmoidal saturation curves were observed in the several mi-

TABLE I

| Addition to incubation medium | No. of experiments | $K_{\text{dis}}$ |
|------------------------------|-------------------|----------------|
| KCl, 15 mM                   | 5                 | 3.7 ± 0.35     |
| KCl, 120 mM                  | 5                 | 4.1 ± 0.35     |
| NaCl, 120 mM                 | 6                 | 3.9 ± 0.45     |
| LiCl, 120 mM                 | 4                 | 3.9 ± 0.56     |

FIG. 4. Ca²⁺ uptake as a function of the free Ca concentration in medium containing ATP or acetyl-P. **CaCl₂, 0.2 mM; EGTA** was as stated in the figure; KCl, 15 mM. Other additions and experimental conditions were as described under "Methods." ○——○, acetyl-P, 2.0 mM; ■——■, ATP, 10 μM; phosphoenolpyruvate, 2 mM; pyruvic phosphoferase, 25 μg per ml. Ca²⁺ was calculated as described under "Methods"; with the use of $K_{\text{dis}}$ calculated from Schwarzenbach's data, the free calcium concentration would be about 20 times lower than that reported in the figure.
crosomal preparations tested. This implies that the apparent \( K_m \) for \( Ca^{2+} \) was progressively modified by increasing concentrations of this cation (14). Kinetics such as this have been deemed characteristic of allosteric enzymes. In a separate paper (15), it has been shown that a hyperbolic saturation curve is obtained when ATP was used as substrate in concentrations of \( 10^{-5} \) M or higher. Fig. 4 shows this experiment, repeated as a control, with the same microsomal preparation used for the test in the presence of acetyl-P. Marked differences between the saturation curves obtained with ATP and acetyl-P can be observed. Fig. 5 shows the inhibitory effect of alkali ions. In the presence of 120 mM KCl or 120 mM NaCl, sigmoidal curves were obtained with a shape similar to that observed with a control medium containing a low concentration of KCl. However, when 120 mM LiCl was added to the control assay medium, a sigmoidal curve of different shape was observed. At lower \( Ca^{2+} \) concentrations, Li\(^+\) was a strong inhibitor, at least as strong as Na\(^+\). Its activity decreased when the \( Ca^{2+} \) concentration was raised, and at 3.8 \( \mu M \) \( Ca^{2+} \) the activity of Li\(^+\) was similar to that of K\(^+\).

**Fig. 5.** Effect of alkali ions on microsomal \( Ca^{2+} \) uptake. Acetyl-P, 2.0 mM; \( ^4CaCl_2, 0.2 \) mM; EGTA was as stated in the figure. Other additions and experimental conditions were as described under “Methods.” \( \bullet \) control, KCl, 15 mM; \( \bigtriangleup \) control plus 120 mM KCl; \( \Delta \) control plus NaCl, 210 mM; \( \square \) control plus 120 mM LiCl.

\( Ca^{2+} \) Uptake as Function of \( Mg^{2+} \) Concentration—Fig. 6 shows that the pattern of activation induced by \( Mg^{2+} \) was essentially the same in media containing either 0.2 or 20 mM acetyl-P. Maximum activity was obtained in the range of 4 to 10 mM MgCl\(_2\). Addition of excess \( Mg^{2+} \) inhibited \( Ca^{2+} \) uptake. The pattern of this inhibition was similar for the two concentrations of acetyl-P used.

**DISCUSSION**

The data presented show that when acetyl-P was used as a substrate the \( Ca^{2+} \) transport system of skeletal muscle microsomes displayed kinetics similar to that described for allosteric enzymes (14). Different kinetics data toward \( Ca^{2+} \) were observed depending on the choice of substrate. When acetyl-P was used, the saturation curve toward \( Ca^{2+} \) showed a sigmoidal shape indicating a cooperative effect, where at least two calcium ions interact with the carrier system. The binding of one ion in some manner facilitates the binding of the next, i.e. \( Ca^{2+} \) was acting both as substrate and as activator (14, 16). On the other hand, an ATP concentration of \( 10^{-5} \) M elicited first order kinetics toward \( Ca^{2+} \). These data suggest that the binding of ATP to the microsomal membrane promotes a conformational change in the \( Ca^{2+} \) carrier system which results in a modification of its affinity for \( Ca^{2+} \).

In a separate paper (15), data have been presented showing that the alkali ions do not damage the microsomal membrane nor do they interfere with the precipitation of calcium oxalate in the interior of the vesicles. Thus, the described inhibition of \( Ca^{2+} \) transport is related to a specific effect of these alkali ions at the site of the \( Ca^{2+} \) pump. The data of Figs. 1 and 2 show that the inhibitory effect of the alkali ions decreases when the acetyl-P concentration of the assay medium was raised. Yamamoto and Tonomura (17), measuring the \( Ca^{2+}\)-dependent ATPase activity of sarcoplasmic reticulum, have shown that the \( Ca^{2+} \) affinity of the vesicles was enhanced by raising the ATP concentrations in the assay medium. It is possible that in the case with ATP, increasing concentrations of acetyl-P also increased the affinity of the \( Ca^{2+} \) transport system for calcium ions and that the observed inhibition was the result of different degrees of competition between \( Ca^{2+} \) and the alkali ions. In a...
separate paper (15), it has been shown that the alkali ions inhibit the Ca\textsuperscript{2+} transport supported by ATP concentrations of 1 to 10 μM in a manner similar to that described in this paper for acetyl-P preparations.

Finally, it should be emphasized that there is additional evidence that the Ca\textsuperscript{2+} transport system exhibits characteristics similar to those described for allosteric enzymes (17, 18). Weber, by means of the heavy fraction of the sarcoplasmic reticulum, has shown that the effect of caffeine on Ca\textsuperscript{2+} transport varies with the ATP concentration in the assay medium (18).

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