THE PARTICIPATION OF PARVALBUMINS IN THE ACTIVATION–RELAXATION CYCLE OF VERTEBRATE FAST SKELETAL-MUSCLE

J.-F. PECHÈRE, J. DERANCOURT and J. HAIECH
Centre de Recherches de Biochimie Macromoléculaire du CNRS, B.P. 5051, 34033 Montpellier, France

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1. Introduction

Although the physiological role of muscular parvalbumins [1–3] remains an open question, several studies [4,5] have restricted the range of possibilities and suggested that these proteins constitute a non-interacting regulatory system of the sarcoplasmic Ca^{2+}-level which operates essentially in fast contracting muscles so as to facilitate their cyclic relaxation. In addition, a scheme has been proposed [5], in which the affinity of parvalbumins for Ca^{2+} (thus their possible competition with troponin C for this ion) could be modulated by changes in hydrogen ion-concentration associated with the contraction process. This allows undisturbed activation of the myofibrils at higher pH, while ensuring their complete relaxation at lower pH. Experiments reported recently in the literature [6,7] have substantiated the validity of several steps of the above scheme. Thus, they have shown that calcium-free parvalbumins indeed have the potential to block contraction or ATPase activity of Ca^{2+}-activated myofibrils. Sarcoplasmic reticulum, in turn, is able to remove efficiently all the Ca^{2+}-ions which can be bound by parvalbumins.

The first experiments reported in the present communication were designed independently in order to evaluate more specifically the capability of hydrogen ions to modulate the competition of parvalbumins and myofibrils for calcium. Their negative outcome led to the idea that kinetic effects, rather than changes in affinities at equilibrium, come into play in order to preserve the potential for activation of myofibrils in presence of parvalbumins, as is the case in vivo where these proteins are present often at a millimolar concentration [4]. The additional experiments described below show that such is indeed the case. This allows a description of calcium regulation of fast skeletal-muscle activity which is now compatible with all the experimental data.

Abbreviations: EGTA ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetra-acetate, DTT dithiothreitol, HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, Tris (tris hydroxy methyl)aminomethane, P_i inorganic phosphate, TCA trichloroacetic acid

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2. Materials and methods

Myofibrils were prepared from fresh hind-leg frog-muscles according to the procedure of Etlinger and Fischmann [8] and were stocked under nitrogen in relaxing buffer (0.1 M KCl, 0.01 M imidazole, 2 mM Mg^{2+}, 2 mM EGTA, 1 mM DTT, pH 7.0). Before use, they were equilibrated by repeated washings in the cold with Ca^{2+}-free buffer (0.15 M KCl, 0.05 M HEPES, 0.5 mM DTT, pH 7.3, passed over excess Chelex resin). Their concentration was estimated by the Biuret reaction [9].

Frog parvalbumin p*I* 4.50 [10] was used throughout the present work. The Ca^{2+}-free form of the protein was obtained by extensive dialysis first versus the relaxing buffer above and then versus the Ca^{2+}-free buffer. Its concentration was determined by measuring the absorbance of the solutions at 259 nm [11].

In the study of the competition of myofibrils and

parvalbumin for Ca^{2+}, a small amount (approx. 0.16 nmol) of $^{45}CaCl_2$ (spec. act. 3.61 μCi/nmol) was added to 30 mg Ca^{2+}-free myofibrils suspended in 10 ml Ca^{2+}-free 60 mM KCl, 30 mM imidazole, 30 mM Tris at pH 6.8 and pH 8.2, and the suspension was titrated at 4°C with 1 mM cold $CaCl_2$ or 0.5 mM parvalbumin containing 2 Ca^{2+}/mol, dissolved in one of the two buffers used to suspend the myofibrils. The titration was followed by measuring the appearance of the radioactivity in the supernatant after centrifugation of aliquots of the suspension at 800 X *g* for 8 min in the cold.

The influence of parvalbumins on the ATPase activity of myofibrils (2.5 mg/ml) was examined under conditions similar to those described by Bendall [12]. The reactions were followed under nitrogen either in a pH-stat or by measuring the appearance of P_i by the Fiske and SubbaRow method [13] in the TCA supernatants of aliquots of the reaction mixture removed at appropriate times. The experiments were performed at 15°C in 0.15 M KCl, 0.001 M (pH-stat) or 0.05 M (P_i analysis) HEPES, 0.5 mM DTT, 5 mM ATP, 5 mM $MgCl_2$, at pH 7.3. The activation of the initially Ca^{2+}-free myofibrils was achieved, in the absence or in the presence of 0.60×10^{-4} M Ca^{2+}-free parvalbumin, by the addition of 0.1 μmol $CaCl_2$/ml suspension, resulting in a final concentration of approx. 0.25×10^{-4} M in free Ca^{2+}-ions.

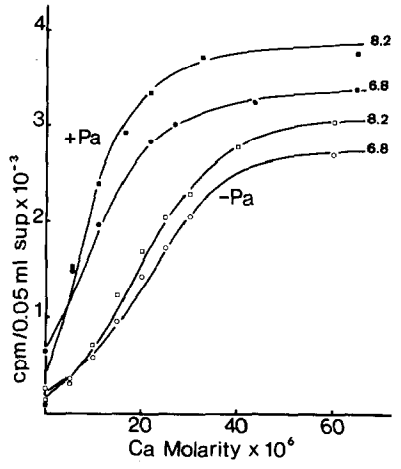

Fig.1. Competition for Ca^{2+} between myofibrils and parvalbumins, at pH 6.8 and pH 8.2. The displacement of $^{45}C^{2+}$ from the myofibrils into the supernatant is plotted in function of the quantity of cold Ca^{2+} added either under the form of $CaCl_2$ (○,□) or as Ca_2-parvalbumin (●,■). When the reasoning of Colowick and Womack [14] is applied to the first pair of curves (○,□), dissociation constants of 0.66×10^{-6} M and 0.52×10^{-6} M can be calculated for 4.6 groups and 3.9 groups/mol myofibril, at pH 6.8 and pH 8.2, respectively. This conforms with the values found, in absence of Mg^{2+}, for the two Ca^{2+}-specific sites of troponin that are involved in the regulation of the myofibrillar ATPase activity [16]. The two sites with higher affinity were titrated too rapidly to permit the determination of their dissociation constant.

3. Results and discussion

The results of the competition experiments are reported in fig.1. They clearly show that, within experimental error, parvalbumins are able to remove Ca^{2+}-ions from myofibrils equally well, at pH 8.2 or at pH 6.5. This result is in agreement with the observations of Fischer et al. [6] and of Gerday and Gillis [7] who showed that Ca^{2+}-free parvalbumins are able to block the contraction or the ATPase activity of Ca^{2+}-activated myofibrils in the same way as EGTA does. The validity of their conclusions can now be extended over the entire pH-range which might perhaps be considered, under extreme local conditions, to prevail during contraction. On the other hand, the possibility considered earlier [5] that the Ca^{2+}-binding capacity of parvalbumins could be modulated by hydrogen ions, has thus to be abandoned. As, in fast contracting muscle, these proteins are present at a concentration sufficient to prevent Ca^{2+} released from the sarcoplasmic reticulum reaching the myofibrils. The mechanism by which this ion initiates their contraction remains an intriguing problem.

It must be observed, however, that the above arguments refer to a situation at equilibrium. In the considered physiological process, this situation may not prevail. It therefore seemed appropriate to examine if an initial phase of myofibrillar activity upon Ca^{2+}-activation might not be preserved, before the subsequent inhibition of the steady-state activity. That this is actually the case is suggested by the experiments reported in fig.2. After addition of Ca^{2+}, the curves obtained in presence of Ca^{2+}-free parvalbumin indeed run parallel to those corresponding to the preceding weak activity of the relaxed-

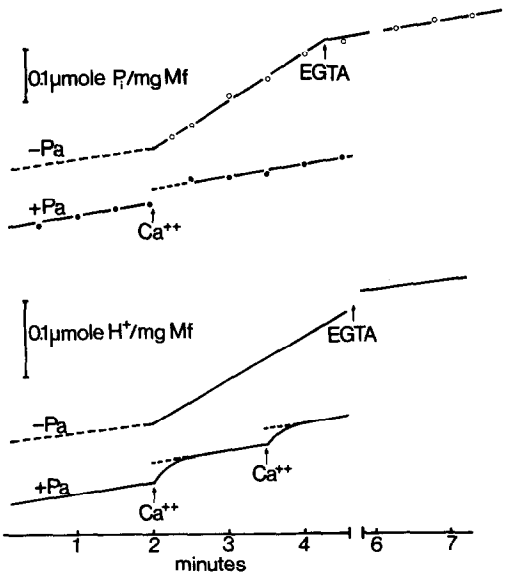

Fig.2. Splitting of ATP by myofibrils initially in the Ca^{2+}-free state and subsequently activated by the addition of $CaCl_2$. The production of hydrogen ions (——) or of P_i (○,●) appearing in a 0.79/1.00 ratio, is reported in function of time, either in absence (– Pa) or in presence (+ Pa) of Ca^{2+}-free parvalbumin. In absence of Ca^{2+}-free parvalbumin, the levels of ATPase activity corresponding to the relaxed state were measured at the end of the experiments by addition of EGTA (final concentration 2 mM) and are reported as dotted lines at the start of the experiments.

state. They repeatedly do so at a higher level, indicating that a transient hydrolysis of ATP must have taken place immediately after each activation, at a rate seemingly comparable to that found in the control samples, devoid of parvalbumin. A biochemical basis is thus provided for the tension-transients, alluded to [6] under supposedly similar conditions.

In view of the known values of the relative affinities for Ca^{2+} of parvalbumins ($K_d \simeq 10^{-7}$ M [15]) and troponin ($K_d \simeq 10^{-6}$ M [16]) the simplest explanation of the above situation is kinetic. If the capture of Ca^{2+} by parvalbumins is sufficiently slow in comparison to that by troponin, it is understandable that the myofibrils will be affected first and that their activation will be preserved. In contrast, after this has happened, the parvalbumins with stronger affinity for Ca^{2+}, will effectively contribute to the rapid relaxation of the myofibrils. This sequence of

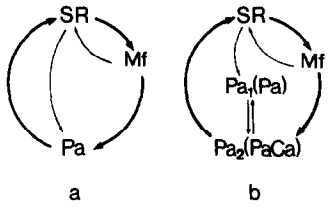

Fig.3. Scheme depicting the possible involvement of parvalbumins in the movement of Ca^{2+}-ions during a contraction cycle in fast skeletal vertebrate-muscle. The width of the arrows symbolically reflects the magnitude of the Ca^{2+}-fluxes resulting from the overall kinetics of the different steps considered. (a) Scheme involving one parvalbumin state. (b) Scheme involving two parvalbumin states whose interconversion could be Ca^{2+}-dependent.

events is exactly what is expected in rapidly contracting muscles which contain parvalbumins almost exclusively [4].

The situation can be adequately represented by a scheme (fig.3) analogous to that presented earlier [5] but where the width of the arrows refers to relative Ca^{2+}-fluxes associated with magnitudes of kinetic constants, rather than of affinities at equilibrium. The simplest version of this scheme (fig.3a) considers only one Ca^{2+}-capturing state for parvalbumins. A somewhat more elaborate variant (fig.3b) calls for two Ca^{2+}-capturing states in these proteins, a slow-capture form and a rapid-capture form. It is attractive to think that they could correspond to their Ca^{2+}-free form and to the one Ca^{2+}/mol-form, respectively. The transformation between the two states could be effected by the Ca^{2+}-ions themselves, without excluding, perhaps, the participation of hydrogen-ions.

Experiments are presently under way which, it is hoped, will distinguish between these possibilities as well as extend the above observations to the millisecond range where the preservation of the transient ATPase activity, in presence of parvalbumins, might be directly recorded and the actual kinetic constants of the system evaluated. The results of the present and of the cited [5–7] experiments already imply, however, that the performance of fast skeletal vertebrate-muscle is not determined by a simple back-and-forth movement of Ca^{2+}-ions between the sarcoplasmic reticulum and the myofibrils, but by their transport through a one-way cycle, in which

parvalbumins play an essential role. In this context, it also seems possible that the relaxing action of parvalbumins might compensate for the kinetic insufficiencies of the Ca^{2+}-recapture by the sarcoplasmic reticulum, noted repeatedly [17]. Finally, it is conceivable that a cycle such as reported in fig.3 might operate in situations other than the contraction of fast skeletal-muscle. For instance, parvalbumins have been detected in brain tissue [4] where a troponin C has also been found [18]. The association of these two elements with a membranous Ca^{2+}-releasing and -pumping device could also lead to a rapid transient-activation process, similar to the one envisaged in the present report.

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