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THE MYOPLASMIC FIXED CHARGES OF THE BARNACLE MUSCLE FIBER AND THE FREE Ca^{2+} CONCENTRATION

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Summary

An experimental model was recently developed to measure the myoplasm density of fixed charges (Caillé, J.P. (1979) Biochim. Biophys. Acta 585, 300—313). The effect of ATP, Mg^{2+} and Ca^{2+} suggests that the myoplasmic thermodynamically effective charge density (ϕX) decreases during muscular contraction. In order to determine if this reduction was due to either ATP, Mg^{2+} or Ca^{2+}, the ϕX dependence on those substances was studied. The results indicated that the addition of $MgCl_2$ (2 mM) reduced ϕX by 10 to 30% depending on the water content of the myoplasm. A reduction of ϕX was also observed with the addition of (10^{-4} M) Ca^{2+}, but the presence of ATP was necessary for this reduction to occur. Finally when EGTA was added to the external solution in order to control the level of free Ca^{2+}, an important reduction of ϕX (40%) was observed for a p*Ca* equal to 5.7 (water content 76.1 ± 0.3 ml/100 g). These results allowed a correlation between the reduction of the myoplasmic fixed charges and the turning on of the contractile machinery.

Introduction

The presence of a charged matrix (polyelectrolytes [1], collodion membranes [2] or parchment-supported inorganic precipitate membrane [3]) in the liquid junction formed by two solutions modifies the transference numbers of small ions in the partition and the electrical potential across the junction [4]. An interesting method has been developed to evaluate the effective charge density from the electrical potential across a partition formed with the charged matrix [5]. Recently we applied this method to calculate the effective charge in a myoplasmic sample of barnacle muscle fibers [6]. We observed that endogene-

ous ATP, Ca^{2+} and Mg^{2+} induced a reduction of the myoplasmic density of fixed charges. These results suggest that a reduction of the fixed charges occurs during muscular contraction. The myoplasmic fixed charges were first evaluated from the Donnan potential on glycerinated fibers of frog muscle [7—9] and of rabbit psoas muscle [10] and recently on chemically-skinned barnacle muscle fibers [11]. Their characteristics were: (a) a relatively high value and (b) a reduction of the fixed charges for muscle fibers in contracted and in rigor states.

During the last ten years different hypotheses were developed which proposed a role for the myoplasmic fixed charges in the ionic distribution, in the equilibrium between the contractile filaments and in muscular contraction [12—19]. The objective of this research was to determine whether any change in the fixed charges of the contractile proteins is associated with muscular contraction. In order to determine if the reduction of the fixed charges observed with the simultaneous addition of ATP, Mg^{2+} or Ca^{2+} [6] was due to either one of these substances, their influence on ϕX was studied. In addition, ϕX was measured as p*Ca* was decreased from 9.0 to 4.0.

The experimental model described previously was modified in order to determine the myoplasmic thermodynamically effective charge density (ϕX) [6]. One important feature of this model is that it permits the control of the hydration of the myoplasmic sample and consequently of the interfilament distance which is an important factor in the measurement of ϕX [3]. Our findings indicate that: (a) a low concentration of Mg^{2+} (2 mM) reduces significantly ϕX; (b) the addition of ATP (over 1 mM) produces an important reduction of ϕX which is absent when Ca is buffered with 0.5 mM EGTA, (c) the addition of Ca^{2+} in the presence of ATP and Mg^{2+} also reduced ϕX, and (d) ATP is essential for ϕX to decrease upon addition of Ca^{2+}.

Methods

Muscle fibers and disks of myoplasm

The isolated muscle fiber of the barnacle (*Balanus nubilus*) was used as a model in these experiments. The barnacles were caught in the region of Vancouver (Canada) and kept in an aquarium for a maximum of three months. The muscle fibers were isolated as described before [20] and rinsed in an isotonic sucrose solution. Each fiber was gently pulled into a cylindrical cavity 5 mm long. The diameter of the cavity was chosen to fit the diameter of the muscle fiber. During this operation the length of the fiber was kept at approximately the same length as the fiber in vivo.

Measurement of ΔV

A disk of myoplasm was used as a partition between two compartments containing solutions A_2 and A_1. The major ionic constituent of the solutions was KCl, C_2 and C_1 representing the KCl concentration in compartments 2 and 1 respectively. The ratio C_2/C_1 was fixed to 5.0. The difference of electrical potential (ΔV) between compartments 2 and 1 was measured with a Keithley electrometer (model 604) and a Honeywell recorder (model 196). The measurements were carried out at 10° C. Once the disk of myoplasm was installed, a

TABLE I

COMPOSITION OF THE EXPERIMENTAL SOLUTIONS A_1 AND A_2, IN mM

	$[K^+]$	$[Cl^-]$	[ATP]	$[Mg^{2+}]$	$[Ca^{2+}]$	[THAM]	[Choline]	[EGTA]	*pCa*
A_1	41.2	41.3	4	2	0	0.5	12.8	0.5	8.5
A_2	204.4	204.0	4	2	0	0.5	12.8	0.5	8.5

period of 30 min was required to reach the equilibrium. To consider the measurement valid ΔV had to remain constant for another 30 min. The recorded values of ΔV were corrected for liquid junction potentials [21].

Solutions

The muscle fibers were isolated and kept in a Ringer solution containing 450 mM NaCl/8 mM KCl/20 mM $CaCl_2$/10 mM $MgCl_2$ and 25 mM Tris. The isotonic sucrose solution contained: 650 mM sucrose and 25 mM Tris. The pH of both solutions was adjusted to 7.6 ± 0.05 by adding HCl. The experimental solutions A_1 and A_2 used to measure ΔV were prepared with certified reagents. The addition of ATP, Ca^{2+}, Mg^{2+} and EGTA in solutions A_1 and A_2 was done in such a way that C_1 and C_2 (the concentration of KCl in A_1 and A_2) did not change by more than 2% [6]. The addition of $CaCl_2$ was done as follows: a solution of 0.1 ml $CaCl_2$ was prepared and its concentration was measured with an atomic absorption spectrophotometer. In our previous work [6], the use of EGTA was avoided but in these experiments the control of Ca^{2+} was obtained with the addition of EGTA. To obtain the desired concentration of Cl^-, choline chloride was added to complete the Cl^- content. An example of solutions A_1 and A_2 is presented in Table I. In the solutions with EGTA p*Ca* was reduced by adding $CaCl_2$ from a 0.1 M $CaCl_2$ solution and the p*Ca* was calculated as proposed by Portzehl et al. [22]. In some experiments the disks of the myoplasm were soaked in a relaxing solution which contained 204 mM KCl/4 mM ATP/10 mM EGTA/41 mM choline chloride and 0.5 mM Tris, pH and p*Ca* of this solution were 7.0 and approx. 9.0 respectively.

Water content of the myoplasm

After the ΔV measurement, the sample of myoplasm was immediately weighed and dried to a constant weight at 95°C. The water content was expressed as ml per 100 g.

Determination of ϕX

The method used to obtain ϕX from ΔV has been described [5]. ϕX was calculated according to:

$$\phi X = \bar{c} \; \frac{\dfrac{1 - t^-_{app}}{\alpha} - 1}{\sqrt{t^-_{app}\left(\dfrac{1 - t^-_{app}}{\alpha} - 1 + t^-_{app}\right)}} \tag{1}$$

In this equation, $\bar{c}$ is the mean concentration in the external solution (C_2 +

$C_1)/2$ and α is the transport number of the counter-ion in the external solution. In a recent publication [6] α was evaluated to be 0.52 for this experimental model. t^-_{app} is the apparent transport number of the co-ions in the myoplasm, and t^-_{app} was obtained from the Nernst-Plank equation:

$$\Delta V = (RT/F)\,(1 - 2t^-_{app})\,\ln\,(C_2/C_1) \qquad (2)$$

where R, T and F have their usual significance.

Results

We previously reported [6] that simultaneous addition of ATP, Mg^{2+} and Ca^{2+} reduced ϕX by 30%. The effect of these substances was studied to establish whether this reduction of ϕX may be related to the contracted state of myoplasm.

ϕX was reduced when Mg^{2+} was added to the external solution. The results obtained for low and high water content are summarized in Table II. The measurements of ϕX are grouped for water content within ±2 ml per 100 g. This rule was applied to all the mean values of ϕX here. The difference in the reduction of ϕX caused by Mg^{2+} for low and high water content groups was statistically significant ($P < 0.05$). The measurements of ϕX were not extended to lower values of $\bar{c}$ because the addition of $MgCl_2$ modifies the concentration of Cl^- for $\bar{c} < 0.090$ M.

When Ca^{2+} was added to solutions A_1 and A_2 in presence of $MgCl_2$ (2 mM) and ATP (2 mM) but without EGTA, an important reduction of ϕX was observed. This effect of Ca^{2+} on ϕX is summarized in Fig. 1. The experimental conditions are described in the legend. Without ATP or in the presence of a low ATP concentration (0.1 mM) the addition of Ca^{2+} had no significant effect on ϕX. The small increase of ϕX with 0.1 mM ATP is not statistically significant ($P < 0.20$) (except at $[Ca^{2+}] = 10^{-6}$ M). The reduction in ϕX observed with zero Ca^{2+} when [ATP] increased to 2 mM is significant ($P < 0.01$).

In order to study the direct effect of ATP on ϕX, measurements were taken

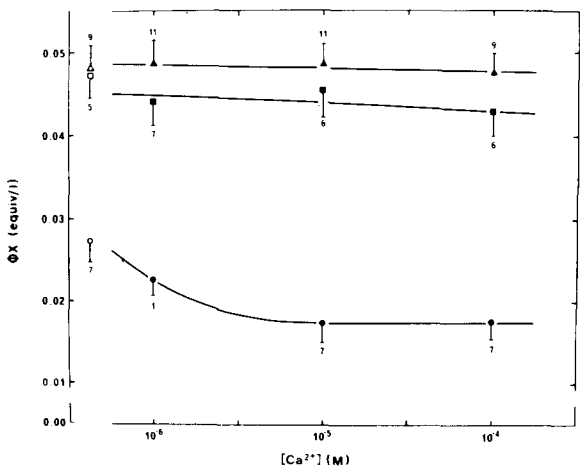

Fig. 1. Effect of $[Ca^{2+}]$ on ϕX as a function of [ATP]. EGTA was not added in solutions A_1 and A_2. From top to bottom, solutions A_1 and A_2 contained; ATP, 0.1 mM (closed triangles, water content 75.3 ± 0.2 ml per 100 g)/ATP, 0.0 mM (closed squares, water content 73.4 ± 0.2 ml per 100 g)/ATP, 2.0 mM (closed circles, water content 75.2 ± 0.2 ml per 100 g). The open symbols represent the values obtained with $[Ca^{2+}] = 0$. The $\bar{c}$ was equal to 0.150 M. The values are means and vertical bars represent the standard error of the mean. Curves were drawn by hand. The numbers indicate the number of measurements.

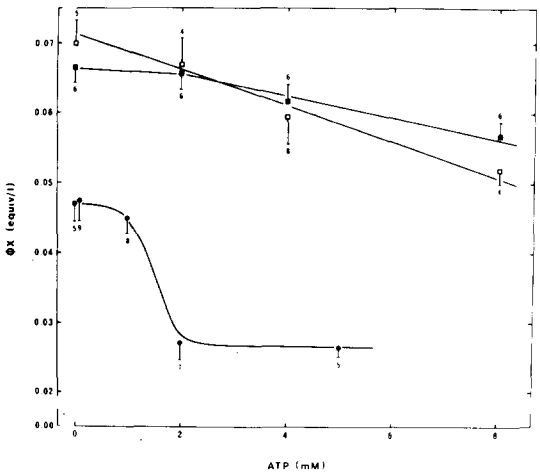

Fig. 2. Dependence of ϕX on [ATP]. Open squares, the myoplasmic disks were treated for 2 h in a relaxing solution (EGTA, 10 mM; p*Ca* 9.0) and then incubated for at least 15 h in a solution A_2 containing: EGTA, 0.5 mM, p*Ca* = 8.5, before the measurements which were performed with solutions A_1 and A_2 ($\overline{c}$ = 0.123 M), the mean water content of those myoplasmic disks was 72.0 ± 0.1 ml per 100 g. Closed squares, same conditions as above but $MgCl_2$ (2 mM) was added to solutions A_1 and A_2, the mean water content of those myoplasmic disks was 71.0 ± 0.1 ml per 100 g. Closed circles, the myoplasmic disks were not treated in the relaxing solution, but were incubated for at least 15 h in a solution A_2 without EGTA before the measurements which were performed with solutions A_1 and A_2 ($\overline{c}$ = 0.150 M) without EGTA, the mean water content of those myoplasmic disks was 73.0 ± 0.3 ml per 100 g. Values are means and vertical bars represent the standard error of the mean. The curves were drawn by hand. The numbers indicate the numbers of measurements.

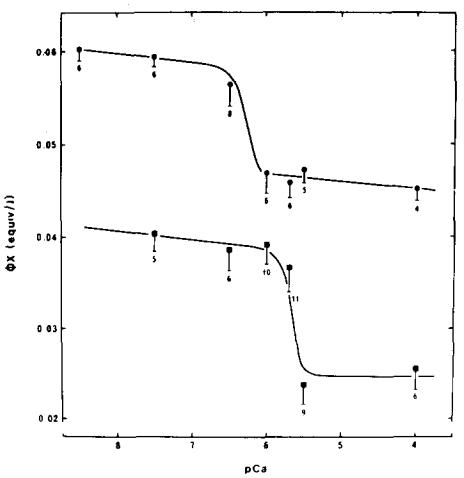

Fig. 3. Dependence of ϕX on p*Ca*. The myoplasmic disks were not treated in a relaxing solution but were incubated for at least 15 h in solution A_2 described in Table I. The measurements were done with solutions A_1 and A_2 ($\overline{c}$ = 0.123 M). p*Ca* was reduced by adding $CaCl_2$. The results were grouped according to two water contents: 71.2 ± 0.2 ml per 100 g (closed circles) and 76.1 ± 0.3 ml per 100 g (closed squares). The vertical bars represent the standard error of the mean and the curves were drawn by hand. The numbers indicate the number of measurements.

in Ca^{2+}-free solutions. The dependence of ϕX on ATP is summarized in Fig. 2. The experimental conditions are described in the legend. In the presence of $MgCl_2$ (2 mM) and without EGTA an important decrease of ϕX occurred when [ATP] reached 1 mM. When the disks of myoplasm were previously treated with a relaxing solution, the measurements of ϕX in the presence of EGTA (0.5 mM) did not show the important reduction observed for [ATP] = 2 mM with the untreated disks. However, even in the treated disks of myoplasm the addition of larger quantities of ATP (4 and 8 mM) reduced ϕX significantly ($P < 0.05$).

ϕX was measured with ATP (4 mM)/$MgCl_2$ (2 mM) and EGTA (0.5 mM) as a function of p*Ca*. The free Ca^{2+} concentration in solutions A_1 and A_2 was calculated as mentioned in Methods. To prevent the entry of large amounts of EGTA into the disks of myoplasm which could introduce an error in the estimations of free Ca^{2+}, the disks of myoplasm were not treated in the relaxing solution. When EGTA was added, previous treatment with the relaxing solution did not increase ϕX significantly. The values of ϕX in treated and untreated myoplasm, measured under the conditions described above were 0.061 equiv./l and 0.060 equiv./l respectively. Fig. 3 shows a slow decrease in ϕX as p*Ca* was reduced from 8.5 to 7.5 with [ATP], [$MgCl_2$] and [EGTA] equal to 4, 2 and 0.5 mM respectively. An important reduction of ϕX (40%) was observed between p*Ca* 6.5 and 5. This reduction (25%) was smaller when the water content was lower. Moreover, there was a shift (Fig. 3) toward higher values of p*Ca* causing this major decrease of ϕX when the water content was reduced. With a water content of 71.2 ml per 100 g 50% of the reduction appeared at p*Ca* = 6.3 instead of p*Ca* = 5.7 for a water content of 76.1 ml per 100 g.

Discussion

Conductivity measurements on barnacle muscle fibers [23] recently revealed that Mg^{2+} reduced the volume fraction and the specific conductivity of the contractile filaments. On frog skeletal muscle fibers mechanically skinned, an increase of free Mg^{2+} causes a shrinkage of the fiber [24]. This decrease of the fiber volume may be associated to a reduction of the net negative charge of the myofilaments. This was interpreted in terms of a screening of fixed charges by Mg^{2+}. The accumulation of monovalent cations by the membrane-damaged muscle fibers [25] corroborates this interpretation since the excess of cations is reduced when Mg^{2+} is added to the external electrolyte solution. Moreover, the addition of Mg^{2+} (0.1 M) reverses the exclusion of the anion (Cl^-) into an accumulation. The effect of Mg^{2+} on the thermodynamically effective charge density may be explained in terms of a reduction of ϕX due to the screening of Mg^{2+} on the fixed charges. The effective fixed charge density is dependent on the hydration of the myoplasm [6] or on the number of myofilaments contained in a unit volume. Elliott and his co-workers [10] demonstrated the importance of the distance between thick filaments in the interpretation of Donnan potential in glycerinated rabbit psoas muscle. In the measurements described above the number of myofilaments contained in a unit volume and the length of the fiber were kept constant. These conditions were chosen to prevent changes of the interfilament distance in the network. When Mg^{2+} is added even if the hydration of myoplasm is kept constant, the volume fraction occupied by the filaments is reduced [23]. This could also explain partially the lower value of ϕX with Mg^{2+}. These results indicate that Mg^{2+} must be present when the effect of ATP and Ca^{2+} is studied.

The screening effect of Ca^{2+} on the fixed charge was explored. The concentration range of endogenous Ca^{2+} was maintained between 0 and 10^{-4} M. Higher concentrations in the range of 10^{-3} M denatured the myoplasm as illustrated by the appearance of large myoplasmic vacuoles. With low concentration of endogenous ATP (0.1 mM), [Ca^{2+}] up to 10^{-4} M did not significantly

TABLE II

THERMODYNAMICALLY EFFECTIVE CHARGE DENSITY (ϕX) OF THE MYOPLASM AS A FUNCTION OF THE MEAN ($\bar{c}$) EXTERNAL ELECTROLYTE SOLUTIONS WITH THE ADDITION OF $MgCl_2$ (2 mM)

Values are means ± S.E. Number of measurements in parentheses

	θX (equiv. per l)				Water content (ml/100 g)	$[Mg^{2+}]$ (M)
$0.5(c_1 + c_2)$(M):	0.09	0.150	0.21	0.30		
	0.101	0.134	0.158	0.181	59.0	—
	(6)	(6)	(6)	(6)	(6)	
	±0.011	±0.013	±0.010	±0.011	±0.6	
	0.081	0.115	0.146	0.165	59.9	0.002
	(7)	(7)	(7)	(7)	(7)	
	±0.003	±0.003	±0.004	±0.007	±0.8	
	0.042	0.059	0.062	0.066	71.8	—
	(5)	(5)	(5)	(5)	(5)	
	±0.004	±0.004	±0.008	±0.009	±0.7	
	0.036	0.045	0.049	0.048	71.7	0.002
	(6)	(6)	(6)	(6)	(6)	
	±0.002	±0.003	±0.003	±0.004	±0.8	

change ϕX. This indicates that Ca^{2+} at low concentrations has no screening effect. (The small increase in ϕX observed with the addition of 0.1 mM ATP is not very significant ($P < 0.20$).)

When [ATP] reached 2.0 mM, the addition of Ca^{2+} caused a significant reduction of ϕX. Since Ca^{2+} by itself did not have a significant effect on ϕX, one can speculate that this reduction is associated with a contracted state of myoplasm. As Ca^{2+} has virtually no screening effect the reduction of ϕX may be associated to a modification in the contractile proteins (structural modifications and formation of complexes between the proteins).

The results presented in Fig. 1 indicate that in the presence of Mg^{2+} and without endogenous Ca^{2+}, the addition of 2 mM ATP reduces ϕX. This may appear contradictory to our previous conclusion that 1 mM of endogenous ATP did not modify ϕX but the measurements of ϕX as a function of ATP on disks of myoplasm with and without the addition of EGTA (0.5 mM) resolved this contradiction. One has to remember that disks of myoplasm used to measure ϕX with EGTA were treated for 2 h in a relaxing solution. Three major points derive from results included in Fig. 2. First, the presence of EGTA increased slightly but significantly ($P < 0.05$) ϕX. The value used for the comparison is in Table II (similar water content, $[MgCl_2]$ = 2.0 mM and [ATP] = 0), the values are 0.059 ± 0.004, [5] and 0.066 ± 0.002, [6] equiv./l. A logical explanation is that EGTA binds Ca^{2+} and slightly increases ϕX through a dissociation of Ca^{2+} from its binding sites. In the absence of Ca^{2+} and Mg^{2+} the increase of endogenous ATP up to 4 and 8 mM slightly but significantly reduced ϕX; this phenomenon cannot be explained by the binding of ATP by myosin [26,27], or by a reduction in the fraction of the total volume occupied by the contractile filaments [23]. This may indicate that ATP causes structural changes in the contractile filaments.

On the untreated disks of myoplasm, the addition of ATP in presence of $MgCl_2$ significantly reduced ϕX for concentrations of ATP higher than 1 mM. As this important reduction is not seen on disks treated with EGTA, it is logical to interpret this reduction as a combined effect of ATP, Mg^{2+} and Ca^{2+} because of a weak control over free Ca^{2+} concentration.

The results in Fig. 3 summarize the dependence of ϕX on p*Ca* and indicate an important reduction of ϕX (40% for a water content of 76.1 ml per 100 g and 25% for 71.2 ml/100 g as a function of p*Ca*). The p*Ca* value setting on this reduction is shifted to a higher value when the water content is lowered. The interfilament distance is certainly less when the water content is reduced. How can this influence the Ca^{2+} level necessary to this change in ϕX? No satisfactory explanation has been found for this shift. The value of p*Ca* necessary to observe this reduction is similar to that which induces a contraction in glycerinated barnacle muscle fibers [28]. These results certainly indicate that the modification of the fixed charges obtained in our laboratory [6] and by others [29] under experimental conditions reproducing relaxed and contracted states, can be correlated to the turning of the contractile machinery. The muscular contraction in barnacle muscle fibers may be accompanied by a shortening of thick filaments [30], but since it would cause an increase of ϕX, it cannot be used to explain the results obtained.

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