A SIMPLE ELECTROSTATIC MODEL CAN EXPLAIN THE EFFECT OF pH UPON THE FORCE-pCa RELATION OF SKINNED FROG SKELETAL MUSCLE FIBERS

R. E. GODT, *Department of Physiology, Medical College of Georgia, Augusta, Georgia 30912 U.S.A.*

ABSTRACT The relative force-pCa relation of skinned frog skeletal muscle fibers is shifted along the pCa axis by changes in pH. This shift has been interpreted as arising from competition between H^+ and Ca^{2+} for a binding site on troponin. Unfortunately, binding studies have been unable to confirm such competition. Alternatively, however, the data fit a model where H^+ influences the degree of dissociation of ionizable groups on the surface of the thin filaments, thus altering the electrostatic potential surrounding the filaments. Alterations in the potential will, in turn, change the concentration of Ca^{2+} near the troponin binding sites in accordance with the Boltzmann relation. A simple model, based upon the Gouy-Chapman relation between surface potential and charge density, provides a quantitative explanation for the shift of the relative force-pCa curve with pH, given a reasonable estimate of the surface charge density on the thin filament. A best fit is obtained when the ionizable groups giving rise to the potential have a log proton ionization constant (pK_a) of 6.1, similar to that for the imidazole group on histidine, and when the density of these groups is near that estimated from amino acid analysis of thin filament proteins and from filament geometry. In preliminary experiments, reaction of skinned frog fibers with diethylpyrocarbonate (DEP) at pH 6 shifted the force-pCa curve toward lower Ca^{2+}. This would be expected in the model since DEP at pH 6 is reported to specifically react with histidine imidazole groups and to irreversibly decrease their pK_a, which would increase the net negative charge of the filaments.

INTRODUCTION

Recently Robertson and Kerrick (1979) reported that pH has a marked effect upon the activation of force by Ca^{2+} in skinned frog skeletal muscle fibers. They found that decreasing the pH from 7.5 to 5.5 progressively shifted the relative force-pCa relation to the right, i.e., toward increasing Ca^{2+} concentrations. They interpreted this shift as the result of a competition between H^+ and Ca^{2+} for the Ca^{2+}-binding sites on troponin which are thought to be responsible for regulation of contraction. In support of this, they refer to experiments on separated troponin C (TnC) from rabbit skeletal and bovine cardiac muscle (Robertson et al., 1978*a*, *b*) which indirectly demonstrated such a competition from observation of the influence of pH upon the Ca^{2+} dependence of fluorescence of labeled TnC. This, however, is in contradiction to an earlier report by Potter et al. (1977) that, in an unbuffered solution, addition of 6 mol of Ca^{2+}/mol of rabbit TnC (enough Ca^{2+} to saturate both classes of calcium binding sites) led to a release of < 0.01 mol of H^+/mol TnC, indicating little or no competition between H^+ and Ca^{2+}. Similarly, studies of the effect of pH on Ca^{2+} binding to either rabbit

troponin (Fuchs, 1974) or to glycerinated rabbit muscle fibers (Fuchs, 1979) do not demonstrate any H^+-Ca^{2+} competition for troponin binding sites.

In this paper, I propose an alternative hypothesis to explain the shift of the relative force-pCa curve with pH. It is known that the myofilaments bear a net negative charge at near neutral pH (Elliott, 1973) and that the resultant electrostatic field surrounding the myofilaments tends to attract cations and to repel anions. Changes in solution pH will alter the surface charge and hence the field, and will thus alter the concentration of ions immediately around each myofilament. Since the Ca^{2+} binding sites on the thin filament are exposed to the Ca^{2+} concentration near the filament surface, changes in the electrostatic field from titration of the ionized surface charges by protons can indirectly change the Ca^{2+} saturation of the sites and can thus influence force production. In this model, H^+ does not compete directly with Ca^{2+} at a binding site; rather, H^+ alters the electrostatic field surrounding the thin filament and thus indirectly affects the effective Ca^{2+} concentration at or near the Ca^{2+} binding sites. It is proposed that, over the pH range 5.5–7.5, the concentration of Ca^{2+} at the filament surface necessary for force activation is the same, but that different Ca^{2+} concentrations in the bath are necessary to achieve constant surface concentrations as pH is altered.

It will be shown that this simple model provides a quantitative explanation of the data of Robertson and Kerrick (1979), given reasonable estimates of thin filament properties, if, over the pH range 5.5–7.5, the net surface charge is predominantly due to ionizable groups with an intrinsic pK_a of 6.1. The fit to their data is sensitive to this constant, although the choice of the optimum constant is rather insensitive to the actual density of surface charges. This analysis also provides an estimate of surface charge density, which is close to that derived from amino acid analysis of thin filament proteins (Elliott, 1973).

METHODS AND RESULTS

At the ionic strength used by Robertson and Kerrick (1979) (0.2 M), the Debye length is 0.68 nm. Thus, insofar as the thin filament diameter is some 8 nm (Hanson, 1968), the field geometry around the thin filament is essentially planar. One can then relate the surface charge density on the filament to the electrostatic potential surrounding the filament using Gouy-Chapman theory. In the present treatment, it is assumed that, for all practical purposes, the bathing solution is a 1:1 electrolyte, that the surface charges are uniformly distributed on the filament surface, and that the surface charge density is determined by the total number of ionizable groups and by their proton ionization constant. Once the potential $\psi(r)$ is known, the ionic concentration at any distance r from the filament, $C(r)$, is given by the Boltzmann equation:

$$C(r) = C \exp\left[-ze\,\psi(r)/kT\right] \tag{1}$$

where C is the concentration far from the filament, z is the valence of the ion, $-e$ the electronic charge, k the Boltzmann constant, and T the temperature. For purposes of the model, ions will be treated as point charges.

The troponin binding sites experience the Ca^{2+} concentration at the filament surface. From Gouy-Chapman theory (Barlow, 1970), the electrostatic potential at the surface will be:

$$\psi_0 = \frac{2kT}{e} \sinh^{-1}(q/2A) \tag{2}$$

where q is the charge density, and (for mks units)

$$A = (2\epsilon n_0 kT)^{1/2}$$

where ϵ is the dielectric constant of the medium and n_0 is the number concentration of the bulk electrolyte. If the charge density is predominantly due to a single class of dissociable groups with an intrinsic ionization constant K_a (Tanford, 1961), it is easy to show that, at any pH,

$$q = \frac{q_T K_a}{K_a + [H^+]_0} \tag{3}$$

where q_T is the maximal charge density due to the ionizable groups and $[H^+]_0$ is the hydrogen ion concentration at the filament surface. When ionized, these hypothetical groups are assumed to be negatively charged (but see Discussion).

Most of these quantities are known or can be estimated accurately. For the ionic strength used by Robertson and Kerrick (1979), n_0 is $1.2 \times 10^{26}/m^3$ and at their temperature (22°C), kT is 4.07×10^{-21} J. The electronic charge is 1.6×10^{-19} C and the dielectric constant of the medium is essentially that of water, 6.59×10^{-10} $C^2/N\ m^2$. Two quantities are not known: the maximal charge density and K_a. The net charge density on the thin filament can be estimated, however, from its valence and surface area. From amino acid analysis, Elliott (1973) estimated the valence of the thin filament to be 2.1×10^4 net negative charges/filament at near neutral pH. For a thin filament 2.0 μm long (end-to-end across the Z-line) and 8 nm diam, charge density will thus be -7×10^{-2} C/m^2. The only variable left unknown is the ionization constant, K_a, of the ionizable groups.

With the idea that pH affects the force-pCa relation solely by influencing the field, one can postulate that over the range of pH studied by Robertson and Kerrick (1979), the concentration of Ca^{2+} at the surface of the thin filament, Q, was the same at any given level of activation. From the Boltzmann relation this means that, for instance, at 50% activation, the product $Ca_{50} \exp[-ze\,\psi_0/kT]$ is some constant, Q_{50}, where Ca_{50} is the bath Ca^{2+} concentration necessary for 50% activation. Inserting the Gouy-Chapman expression for ψ_0 and taking the logarithm, with manipulation we see that

$$S = -\sinh^{-1}(q/2A) = (\ln Q_{50}/2z) + (2.3/2z)\ pCa_{50}$$

where pCa_{50} is $-\log Ca_{50}$ and is equal to $-\log K$, where K is the Hill equation parameter used by Robertson and Kerrick to fit their data. For the model to adequately explain the shift of the relative force-pCa curve, a plot of S vs. pCa_{50} should be linear for some value of q_T and K_a. Moreover, since the valence of calcium is 2, the slope of the plot should be 0.575.

Note that while pH affects the potential, the potential influences $[H^+]_0$ in turn, via the Boltzmann relation (Eq. 1). Thus, to be self-consistent, we must have

$$[H^+]_0 = [H^+]_B \exp(-e\,\psi_0/kT) \tag{4}$$

where $[H^+]_B$ is the hydrogen ion concentration in the bulk medium (cf. Ninham and Parsegian, 1971) calculated from bath pH:

$$[H^+]_B = 10^{-pH}/\alpha_{H^+}$$

where α_{H^+} is the hydrogen ion activity coefficient (taken to be 0.75; Harned and Owen, 1958, Table 14-2-1A, for HCl in 0.2m KCl solution at room temperature). Self consistency was achieved by inserting Eqs. 2 and 4 into Eq. 3 and solving by iteration for the value of q appropriate to each choice of q_T, K_a and bath pH, using a microcomputer.

The relation between S and pCa_{50} for values of pK_a between 4 and 7.5, using the initial (Elliott, 1973) estimate of maximal charge density, is shown in Fig. 1. Note that the plots change curvature as pK_a increases and that for pK_a of 6 it appears reasonably linear. Linear regression analysis of the data indicates that at a pK_a of 6.1 the data fall on an optimal straight line ($r = 0.9994$) and that the

correlation coefficient decreases for pK_a larger and smaller than 6.1. The slope of the plot is 0.514, not far from that predicted by the model.

Although there is some uncertainty in the estimate of thin filament valence, the choice of optimum pK_a in this analysis is not very sensitive to the absolute value used for q_T. Plots of S vs. pCa_{50} (not shown) for charge densities half and twice the value used in Fig. 1 change curvature at similar values of pK_a. Linear regression analysis indicates that the plots are best fit by a straight line at a pK_a of 6.2 (r = 0.9994) when charge density is halved and a pK_a of 6.0 (r = 0.9979) when charge density is doubled. The slopes are quite different, however, being 0.325 and 0.694 for one-half and twice the charge density, respectively.

By trial-and-error, it was determined that if the maximal charge density is 26% higher than the initial estimate (i.e., $q_T = -8.8 \times 10^{-2}$ C/m^2), the plot of S vs. pCa_{50} is optimally linear (r = 0.9984) for pK_a of 6.1 and has a slope of 0.576. The y-intercept was -2.272. Thus Q_{50} was 1.1×10^{-4} M so that, at any pH, the pCa at the filament surface at 50% activation was 3.96. Table I shows the close relation between the pCa_{50} predicted from the model, using these values of pK_a and charge density, and those observed by Robertson and Kerrick (1979).

DISCUSSION

This analysis shows that a simple model involving the electrostatic field around the thin filament can quantitatively explain the shift of the relative force-pCa curve with pH as seen by Robertson and Kerrick (1979). The model is simple in that it assumes that, over the pH range 5.5–7.5, the thin filament can be represented as a negatively-charged polyion whose net charge is dominated by ionizable groups that are uniformly distributed on its surface and have a single intrinsic pK_a. The field set up by these charges accumulates Ca^{2+} near the filament and it is the Ca^{2+} concentration at the filament surface (and not that in the bulk medium) that is important for activation of contraction.

Fig. 1 and Table I demonstrate that the model fits the data well if the maximal charge density is -8.8×10^{-2} C/m^2 and pK_a is 6.1. These are reasonable values, given what is known about muscle proteins. Given the dimensions of the thin filament, the maximal charge density corresponds to a thin filament valence of 2.6×10^4 net negative charges. At pH 7 the valence will be 1.4×10^4 net negative charges which is near that estimated by Elliott (1973) from amino acid analysis. In the model the net negative charge on the filament is assumed for simplicity to arise from the ionization of a single class of negatively charged groups, whose pK_a is 6.1. Thus below pH 5.5 the net charge on the filament will be small. This is mathematically similar to the actual physiological situation, over the pH range 5.5–7.5, where the net charge on the filament is low at pH 5.5 (since the isoelectric points of the major myofilament proteins are between 5 and 5.5; Collins and Edwards, 1971), and becomes increasing negative as pH rises to 7.5, due mainly to a decrease in the positive charge contributed by protonated imidazolium groups on histidines. In view of the good fit between model and experiment, it is reasonable to speculate that, between pH 5.5 and 7.5, the net negative charge on the thin filament is modulated predominantly by deprotonation of these imidazolium groups, whose pK_a is 6 for the free amino acid (Edsall and Wyman, 1958). This seems especially likely since histidine is ubiquitous in muscle protein, with an estimated intracellular concentration of 36 mM in skeletal muscle (Curtin and Woledge, 1978). Thus values and assumptions used in the analysis are consistent with muscle biochemistry.

It is widely agreed that the myofilaments possess a net negative charge when bathed in media at near neutral pH (Elliott, 1973). The field due to this charge will influence the

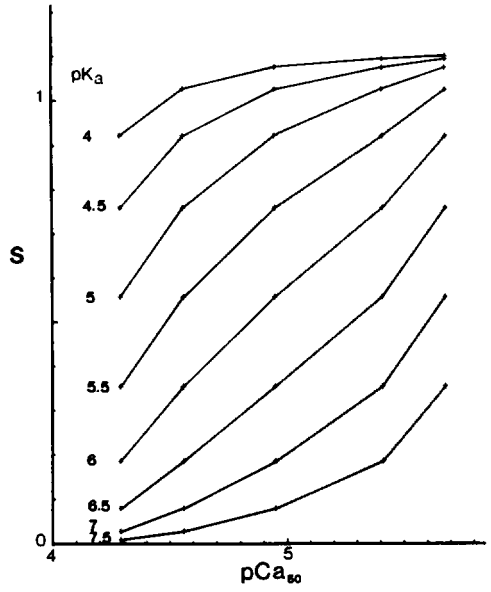

FIGURE 1 Relation between $S = -\sinh^{-1}(q/2A)$ and pCa_{50} for different values of log proton ionization constant (pK_a) of ionizable groups. Maximal charge density on thin filament calculated using Elliott (1973) estimate of thin filament valence. Points for each pK_a connected for clarity.

concentration of ions in the immediate vicinity of the filaments and, given the radial dependence of the potential, one can utilize the Boltzmann equation to calculate the ionic concentrations at any distance from the filament. Since the Ca^{2+}-binding sites on troponin are in equilibrium with the Ca^{2+} concentration immediately surrounding the thin filament, it seems reasonable to assume that factors that influence the electrostatic field, such as pH, would have effects upon the Ca^{2+} saturation of these binding sites.

Although bath Ca^{2+} concentrations in skinned fiber experiments are controlled with EGTA, the presence of the chelator does not influence the local accumulation of Ca^{2+} by the

TABLE I
SHIFT OF ψ_0 and pCa_{50} WITH pH

pH	ψ_0	pCa_{50}	
		Predicted*	Observed‡
	(mV)		
7.5	−52	5.71	5.68
7	−42	5.36	5.41
6.5	−30	4.96	4.95
6	−19	4.58	4.56
5.5	−10	4.28	4.29

*Using $pK_a = 6.1$ and $q_T = -8.8 \times 10^{-2}$ C/m^2.
‡Robertson and Kerrick (1979), calculated as –log K where K is the Hill equation constant.

thin filaments. In the binding reaction, $CaEGTA^{2-}$ is in equilibrium with Ca^{2+} and $EGTA^{4-}$, and the stability constant of this reaction:

$$K = \frac{[\text{Ca EGTA}^{2-}]}{[\text{Ca}^{2+}]\,[\text{EGTA}^{4-}]} \tag{5}$$

is unaffected by the presence of an electrostatic field. (This is the case, insofar as the change in chemical potential of an ion of valence z, due to the field at any point r, is given by $ze\psi(r)$ (Aveyard and Haydon, 1973) thus, for the equilibrium state, these terms cancel since the reaction is electroneutral). In the model, ionic concentration at any distance from the filament is assumed to obey the Boltzmann relation. Thus, near the negatively-charged filament, $[Ca^{2+}]$ is high and $[Ca\ EGTA^{2-}]$ and $[EGTA^{4-}]$ are low. Furthermore, the Boltzmann relation is consistent with, and independent of the binding equilibrium, as can be seen by inserting the Boltzmann equation (Eq. 1) for each ion into Eq. 5, noting that the potential-dependent terms cancel because of electroneutrality.

This electrostatic model is in contrast to the model proposed by Robertson to explain his data (Robertson et al., 1978*a*, *b*; Robertson and Kerrick, 1979). He proposes that there is a direct competition between H^+ and Ca^{2+} at the binding site on troponin responsible for contractile activation. This model is not in accord, however, with recent binding studies on separated troponin as well as glycerinated fibers (Fuchs, 1974 and 1979; Potter et al., 1977) where no such H^+-Ca^{2+} competition was observed.

The electrostatic model can be tested in several ways. One would expect that an increase in ionic strength, by increasing the shielding of surface charges, should decrease the surface potential. If, as proposed, myofilament surface charges play a role in activation of muscle fibers, this decrease would tend to shift the force-pCa curve to the right, since higher bath concentrations of Ca^{2+} would be required to achieve the same level of Ca^{2+} at the filament surface. This prediction accords well with the observations of Ashley and Moisescu (1977) in skinned barnacle muscle fibers. Similarly, increasing ionic strength shifts the relation between myofibrillar ATPase activity and pCa to the right (Portzehl et al., 1971; Solaro and Briggs, 1974). Another test of the model would be to observe the influence of chemical reagents that modify imidazole groups (e.g. diethylpyrocarbonate, DEP; Means and Feeny, 1971) upon the force-pCa relation of skinned fibers. If, between pH 5.5 and 7.5, thin filament net charge is affected predominantly by titration of histidine imidazole groups, one would expect their carbethoxylation by DEP to shift the force-pCa curve to the left (i.e., toward lower Ca^{2+} concentrations) because DEP markedly decreases their pK_a (Mühlrád et al., 1967). This would tend to increase the net negativity of the thin filament which, in turn, would lead to an increased concentration of Ca^{2+} at the filament surface. These experiments provide a direct test of the model since Ca^{2+} binding to troponin is reportedly unaffected by changes in ionic strength over the range 0.05–0.6 M or by reaction with DEP (Fuchs, 1974).

Preliminary experiments with DEP on mechanically skinned fibers from the frog *Rana pipiens* are encouraging. In DEP-free solutions at 15°C containing (in mM): 1 Mg^{2+}; 3.13 MgATP; 5 EGTA; 15 phosphocreatine; 20 PIPES buffer; 16–30 KCl (so that ionic strength was 0.15 M), pH 7.0; 0.5 mg/ml creatine kinase, and variable $CaCl_2$, the pCa_{50} was 5.87 (see Godt, 1974, for general experimental details). Fibers were reacted for 5 min with 1 mM DEP in a pCa 8.5 solution at pH 6, and tested again, after a rinse, in the same DEP-free solutions.

The pCa_{50} of DEP-reacted fibers was 6.08, a shift of 0.21 log units to the left. Maximal Ca^{2+}-activated force at pCa 4.65 was unaffected. Longer exposure to DEP (30 min) did not appear to further change pCa_{50} although maximal force was decreased to 40% of control. On the other hand, immersion of fibers up to 15 min in the pH 6 solution without DEP did not affect either pCa_{50} or maximal force.

It is the intent of this paper to put forth a simple electrostatic model which can explain the otherwise puzzling pH data and, further, to indicate that apparently competitive effects need not necessarily be due solely to direct competition at a binding site. One must not ignore the role that might be played by myofilament surface charges in the regulation of contractile activation. Moreover, if myofilament surface charge has an influence on ionic equilibria in the manner proposed, the apparent binding constants derived for troponin from experiments on separated protein might not be the same as the constants for troponin attached to the thin filaments.

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