Ca^{2+} Activation of Force and Actomyosin ATPase Activity in Skinned Muscle Fibers: Effects of [MgATP] and Temperature

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Abstract

We examined the effects of varied [MgATP] and temperature on the Ca^{2+} activation of force and actomyosin ATPase activity (ATPase) using skinned frog (*Rana pipiens*) skeletal muscle fibres. At 21°C and 2.0mM MgATP, the $[Ca^{2+}]_{50}$ of force (2.64∀0.14μM) was significantly greater than that of ATPase (1.58∀0.08μM, $p<.05$). Reducing [MgATP] to #0.5mM decreased the $[Ca^{2+}]_{50}$ of force and the slope of the force-free $[Ca^{2+}]$ relationship (N) but did not alter those of ATPase. At 0.25mM MgATP, the $[Ca^{2+}]_{50}$ values of the force and ATPase were not significantly different. Reducing temperature from 21°C to 15 or 10°C also significantly reduced the $[Ca^{2+}]_{50}$ and N of force but not those of ATPase such that at 10°C, the values were not significantly different. Using the model of Huxley, the ratio of ATPase to force is proportional to g_{app}, the rate constant for dissociation of force generating crossbridges. At 21°C and 5.0mM MgATP, this measure of g_{app} shows considerable Ca^{2+} sensitivity, decreasing by approximately 80% with increasing free $[Ca^{2+}]$. On the other hand, at reduced [MgATP] (21°C, 0.25mM MgATP), the magnitude of g_{app} is reduced and its variance with free $[Ca^{2+}]$ is attenuated. Similar effects were found at lower temperature (10°C) and high MgATP (5.0mM). These results show that both temperature and [MgATP] affect the magnitude and Ca^{2+} sensitivity of g_{app}. In addition, decreasing the overall magnitude of g_{app} shifts the force-free $[Ca^{2+}]$ relationship to lower free $[Ca^{2+}]$ and decreases the Ca^{2+} sensitivity of g_{app}.

Key words: contractile apparatus, crossbridge cycling kinetics, energetics, skeletal muscle.

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Force output by the contractile apparatus results from the interaction of actin-myosin crossbridges. In simplified form, force regulation at the level of the crossbridge can be described as a two state model which includes groups of weak binding (WB) or non-force generating states and strong binding (SB) or force producing states [12]. When free $[Ca^{2+}]$ is increased above a threshold level, crossbridges continually cycle between these states. Transitions between SB and WB involve a number of biochemical steps and are characterized by the apparent rate constant for transition from WB to SB (f_{app}) and the apparent rate constant for the transition from SB to WB state (g_{app}).

Brenner [3, 4] suggests that Ca^{2+} regulation of crossbridge force output by this system is due to the kinetics of crossbridge cycling between the SB and WB states. The fraction of crossbridges in the SB state (F_S) can be defined as:

$$F_S = f_{app} / (f_{app} + g_{app}) \quad [1]$$

and isometric force production (F) as:

$$F = F_{av} \$ [M] \$ A \$ L_2 \$ f_{app} / (f_{app} + g_{app}) \quad [2]$$

where [M] is the concentration of myosin per liter, A is the fiber cross-sectional area, L_2 is the length of one-half sarcomere and F_{av} is the average force of a single myosin head [4, 13]. Assuming that one ATP molecule is hydrolyzed per cross-bridge cycle, actomyosin ATPase activity (ATPase) can be defined as:

$$ATPase = [M] \$ A \$ a \$ L_2 \$ g_{app} \$ (f_{app} / (f_{app} + g_{app})) \quad [3]$$

where a is the number of half-sarcomeres within the fiber. Using equations [2] and [3], the ratio of ATPase and force is proportional to g_{app}:

$$ATPase / F = g_{app} \$ (a / F_{av}) \quad [4]$$

Brenner [3, 4] contends that f_{app} increases with increasing free [Ca^{2+}] in a sigmoidal manner whereas g_{app} remains relatively constant across levels of free [Ca^{2+}]. This conclusion is based, in part, on his finding that the relationship between force and ATPase is linear. That is, ATPase/F does not vary substantially with increasing free [Ca^{2+}] and hence, g_{app} must remain constant. On the other hand, Kerrick et al. [13] argue that g_{app} is Ca^{2+} sensitive, decreasing by fivefold with increasing free [Ca^{2+}]. The basis for this conclusion is that there is a separation of the force- and ATPase-free [Ca^{2+}] curves with the Ca^{2+} sensitivity of ATPase being greater than that of force. This leads to larger relative ATPase/F values at intermediate free [Ca^{2+}] than at high free [Ca^{2+}]. This observation is also supported by results of Williams et al. [20, 21].

Discrepancies in the Ca^{2+} sensitivity of g_{app} between these studies may due to the different experimental conditions employed. In the studies by Brenner [3, 4], experiments were conducted at 5°C and 1.0mM MgATP. Kerrick et al. [13] and Williams et al. [20, 21] used 21°C and 0.5 and 2mM MgATP. As lowered temperature likely slows crossbridge cycling kinetics and lowers g_{app}, it may mask the Ca^{2+} sensitivity of g_{app}. Also, Kerrick et al. [13] show that lowered [MgATP] reduces much of the change in g_{app} with Ca^{2+} activation. In an attempt to reconcile the results of these studies, we examined the effects of varied [MgATP] and temperature on the Ca^{2+} activation of force and ATPase. We found that both of these conditions result in marked changes in the Ca^{2+} sensitivity and overall magnitude of g_{app}.

Methods

Incubation solutions contained 85 mM K^+ plus Na^+, 1mM Mg^{2+}, 7mM EGTA, 10^{-9} (relaxing solution) or 10^{-4} M Ca^{2+}_{free} (activating solution) and propionate as the major anion. $MgATP^{2-}$ was varied from 0.25 to 5.0mM. Ionic strength was adjusted to 180mM and pH maintained at 7.0 with imidazole propionate. For measurements of ATPase activity, the above solutions were supplemented with 0.4mM NADH, 0.2mM P^1, P^5-di(adenosine-5)pentaphosphate (AP_5A, to inhibit myokinase), 5mM phophoenol pyruvate (PEP), 100 U\$$ml^{-1}$ pyruvate kinase (PK) and 140 U\$$ml^{-1}$ lactate dehydrogenase (LDH) [7, 10, 15]. The concentrations of various ionic species were determined by solving ionic equilibrium equations using published binding constants [5].

Semiteninosus muscles of male grass frogs (*Rana Pipiens*) were removed and placed in relaxing solution (10^{-9} M Ca^{2+}) supplemented with 50% glycerol (v/v). Small bundles of fibers (~50 fibers per bundle) were dissected free and chemically skinned in glycerol-supplemented relaxing solution containing 1% Triton X-100 for 20 min at room temperature. Single fibers were then dissected from fiber bundles and mounted in the Güth Muscle Research System [7]. The fiber was secured between a pair of micro tweezers, one of which is attached to a photo diode force transducer and the other to a length controller, then placed in a quartz cuvette ($1mm^2$ x 1cm). Sarcomere length was adjusted to 2.5µm with the aid of a He/Ne laser. Using slack tests, end compliance of this system was found to be 3-4% of fiber length. Following fiber mounting, the solution in the cuvette was illuminated by a high pressure xenon lamp with light filtered at 340 nm. A microscope photometer, housing a 470 nm interference filter, was used for detection of NADH fluorescence in the solution.

The force- and ATPase-free Ca^{2+} relationships were determined by exposing the skinned fibers to solutions which contained incremental levels of free [Ca^{2+}] while monitoring isometric force and NADH fluorescence ([20, 21]; Figure 1). Step changes in free [Ca^{2+}] were created with a gradient device in which solutions were periodically perfused through the cuvette in 28µl increments. This procedure resulted in exposure of the fiber to between 60-80 increments of free [Ca^{2+}] (pCa 9.0 - 4.5 (where pCa = -log free [Ca^{2+}])). The solution in the cuvette was exchanged every 15s which allowed sufficient time for increases in force to reach a steady plateau and ATPase to reach a steady rate. ATPase values were not affected by inclusion of cyclopiazonic acid, a specific

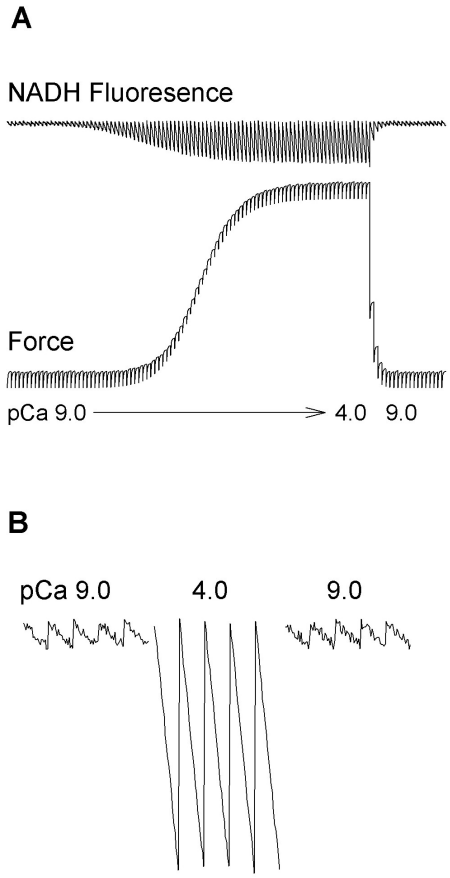

Figure 1. A, Raw records of NADH fluorescence and force assayed simultaneously in a single skinned frog fiber. B., Expanded records of representative segments of NADH fluorescence recoreds from Panel A.

inhibitor of the sarcoplasmic reticulum ATPase [9]. Further, inducing sinusoidal length changes (2.5%, 300Hz) resulted in a two-fold increase in ATPase indicating that maximal ATPase values recorded during isometric contraction are not limited by the coupled enzyme system used. It is also important to emphasize that the time course of ATPase activity in skinned fibers follows three phases, an initial Aphosphate burst@ which is followed by fast linear and slow steady state phases [1, 11, 14]. In our protocol and system, ATPase activity during the third, steady state phase is computed.

The gradient device (Scientific Instruments, GmbH) consisted of an upper chamber which contained activating solution and a lower, mixing chamber which initially contained relaxing solution. With each pump step, 28µl of solution was withdrawn from the mixing chamber and replaced with 28µl of activating solution from the upper chamber resulting in gradual incremental increases in free [Ca^{2+}] within the lower mixing chamber. The volume of the tubing and the volume of solution delivered by the peristaltic pump were periodically calibrated. Free [Ca^{2+}] delivered to the cuvette with each pump step was computed via an algorithm provided by Dr. Konrad Güth. In addition, free [Ca^{2+}] determined by the algorithm was routinely verified using the fluorescent indicator calcium green-2 (480nm excitation, 515 emission). Free [Ca^{2+}] determined by the two methods typically differed by less than 0.02 pCa units.

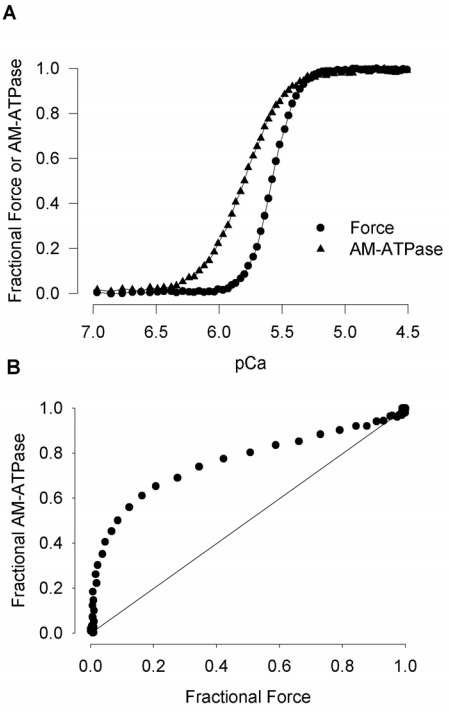

*Figure 2. Force- and ATPase-free [Ca^{2+}] relationships in an individual skinned frog fiber. **A**: force and ATPase plotted as a function of free [Ca^{2+}]. **B**: Relationship between fractional force and ATPase. Each symbol represents an individual force and ATPase measurement at a given level of free [Ca^{2+}]. Data were obtained at 21°C and 5.0mM MgATP. □, force and □, ATPase. Maximal Ca^{2+} force and ATPase activities in this fiber were 221.5 mNAmm^{-2} and 413.7 µMAs^{-1}, respectively.*

In most cases, experiments were conducted at room temperature (21°C). Lower temperatures were obtained by blowing chilled air on the cuvette and tubing. By varying the air temperature and flow, we could reliably lower and maintain temperature within the cuvette at 10 and 15°C. It should also be noted that for experiments conducted at 10 and 15°C, solutions changes were made every 30 and 20 sec respectively. This allowed for greater resolution of the ATPase measurement at the lower temperatures.

Isometric force and ATPase activities during each step were collected via computer and analyzed off-line. The position and the shape for the force- and ATPase - free [Ca^{2+}] relationship were determined by fitting the developed force and ATPase data obtained from each fiber to the modified Hill equation using a nonlinear curve fitting routine (SigmaPlot, Jandel Scientific):

$$F/F_{max} = [Ca^{2+}]^N \cong ([Ca^{2+}]_{50}{}^N + [Ca^{2+}]^N)^{-1}$$

where N is the slope (Hill coefficient) of the relationship and $[Ca^{2+}]_{50}$ represents the Ca^{2+} concentration required to evoke 50% of maximal isometric force (F_{max}) or maximal ATPase activity (A_{max}). This non-linear approach yielded r^2 values above 0.98 in all cases. Due to the large number of data points comprising each curve, statistical comparisons were made using F_{max}, A_{max}, $[Ca^{2+}]_{50}$ and N values. We found that in six fibers repeatedly subjected to the gradient procedure, variation in the above variables was 3-7%. Also, the derived values were the same whether using the gradient procedure or using randomly administered free [Ca^{2+}] (J.H. Williams & C.W. Ward, unpublished).

Results

Figure 2A shows the relationships between force, ATPase and free [Ca^{2+}] in a single skinned fiber. In this example, both fractional force and fractional ATPase are plotted. As can be seen, there is a clear separation of the two curves with the ATPase curve showing increased Ca^{2+} sensitivity. In this example, force and ATPase activities were determined at 21°C and 5.0mM MgATP. In 10 fibers, the $[Ca^{2+}]_{50}$ of force (2.64∀0.14µM) was significantly greater than that of ATPase (1.58∀0.08µM, p<.05). In addition, N of force was greater than that of ATPase (4.74∀0.45, 2.85∀0.23, p<.05). It is clear from Figure 2B that at 21°C and 2mM MgATP the relationship between force and ATPase is non-linear. ATPase increases at much lower free [Ca^{2+}] than does force. In fact, ATPase reaches nearly 30% of A_{max} before detectable force is apparent.

Kerrick et al. [13] demonstrate that reducing [MgATP] from 2.0 to 0.5mM decreases the difference in $[Ca^{2+}]_{50}$

between force and ATPase. However, at 0.5mM, they show that the Ca^{2+} sensitivity of ATPase remained somewhat greater than that of force. Here, we used a wider range of [MgATP] levels. Decreasing [MgATP] to 1.0mM had little effect on the Ca^{2+} activation of either force or ATPase (Figure 3). At these MgATP concentrations, the $[Ca^{2+}]_{50}$ and N values of force and ATPase were not different from those recorded at 5.0mM. In contrast, reducing [MgATP] to 0.5 and 0.25mM significantly reduced the $[Ca^{2+}]_{50}$ and N of force but did not affect those of ATPase. At 0.25mM MgATP, the $[Ca^{2+}]_{50}$ values for force and ATPase were not significantly different. At this concentration of MgATP, the force- and ATPase-free $[Ca^{2+}]$ curves overlap indicating a near-linear relationship between force and ATPase. At 5.0mM MgATP, the correlation coefficient (r^2) between fractional force and ATPase was 0.905 whereas at 0.25mM it was 0.996.

Likewise, reducing temperature to 15 and 10°C shifted the force curve towards the ATPase curve. At the lowered temperatures, $[Ca^{2+}]_{50}$ and N of force were significantly reduced but those of ATPase were unaffected (Figure 4). At 15°C, $[Ca^{2+}]_{50}$ of force and ATPase remained significantly different. This difference, however, was eliminated at 10°C. As occurred at 0.25mM MgATP and 20°C, a near-linear relationship between the two variables was found ($r^2 = 0.991$).

Values for g_{app} at pCa 4.0 were derived as described by Kerrick et al. [13] using equation 3. This calculation required three assumptions. First, we assumed that under conditions of maximal Ca^{2+} activation, the fraction of XB's in the force generating state $F_S = 0.95$ We found that exposing skinned fibers to elevated MgADP (10mM) increased F_{max} by 5.07∀0.09% and that additional MgATP had little added effect. Elevated [MgADP] is thought to slow the detachment of myosin crossbridges such that nearly all remain in the SB states [13]. Second, we assumed a concentration of 154 μmol of myosin heads per fiber liter [6]. Third, with g_{app} at maximal Ca^{2+} activation computed, values at intermediate free $[Ca^{2+}]$ were calculated from the ratio of fractional force and fractional ATPase (equation 4). It should be pointed out that g_{app} was computed only at pCa values where significant force could be measured, that is, where F∃10% of F_{max}).

At 5.0mM MgATP (20°C), g_{app} shows considerable Ca^{2+} sensitivity, decreasing by more than 80% as free $[Ca^{2+}]$ increases (Figure 5A). However, as [MgATP] is lowered, the Ca^{2+} sensitivity of g_{app} is progressively reduced as well as the overall magnitude. At 0.50 and 0.25mM MgATP, the decrease in the magnitude of g_{app} at maximal Ca^{2+} activation is 72 and 46% respectively along with a decrease in the Ca^{2+} sensitivity of g_{app}. Similar results were found when temperature was reduced (Figure 5B). As temperature was lowered to 15 and 10°C, the decrease in magnitude of g_{app} at maximal Ca^{2+} activation was

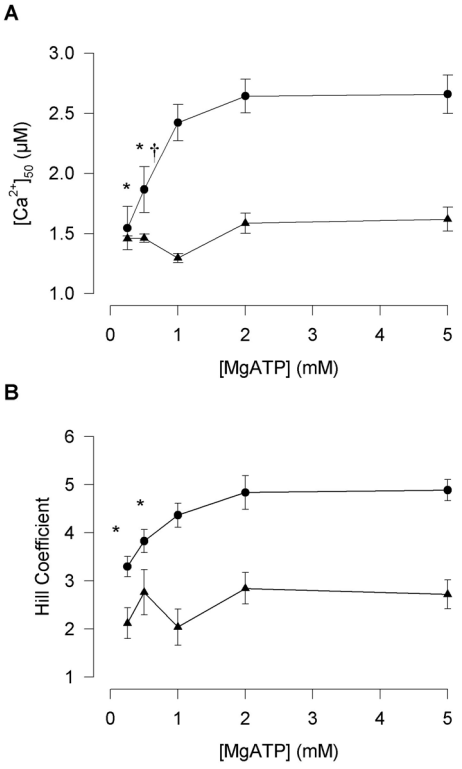

*Figure 3. Changes in the $[Ca^{2+}]_{50}$ (**A**) and N (**B**) of force and ATPase with variations in [MgATP]. *p<.05 versus 2.0 and 5.0mM, Hp<.05 versus ATPase. □, force and □, ATPase.*

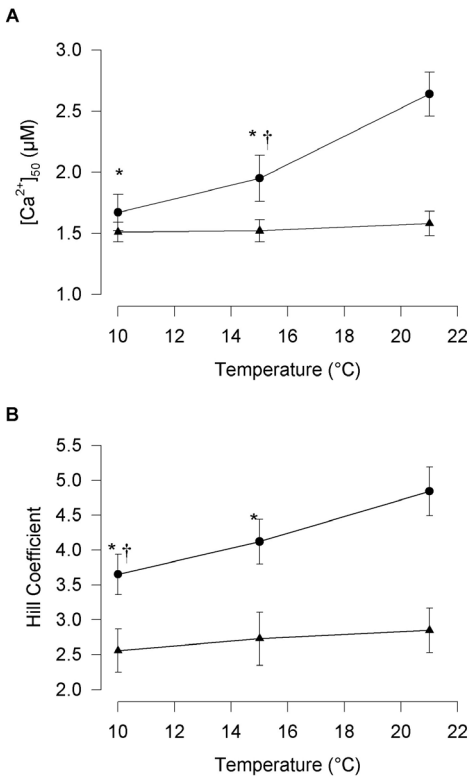

*Figure 4. Changes in the $[Ca^{2+}]_{50}$ (**A**) and N (**B**) of force and ATPase with variations in temperature. *p<.05 versus 21°C, Hp<.05 versus ATPase. □, force and □, ATPase.*

consistently smaller, 70 and 59% respectively, and the Ca^{2+} sensitivity of g_{app} was almost completely eliminated at 10°C.

Discussion

In this investigation, we found that the Ca^{2+} activation of force varies considerably with changes in [MgATP] and temperature, where as that of ATPase does not. This results in marked alteration in the Ca^{2+} activation of g_{app}, the rate constant for crossbridge transition from SB to WB states. At 20°C and 5.0 mM MgATP, g_{app} shows a considerable decrease with increasing free [Ca^{2+}]. However, at reduced [MgATP] or lowered temperature, the change in g_{app} with increasing free [Ca^{2+}] is attenuated. Brenner [3, 4] reports that g_{app} shows little Ca^{2+} sensitivity whereas Kerrick et al. [13] and Williams et al. [20, 21] show that it decreases by nearly five-fold with increasing free [Ca^{2+}]. Our results suggest that this discrepancy may be due to differences in experimental temperature and different [MgATP].

Unfortunately there are few investigations which report values for g_{app}. Under conditions of maximal Ca^{2+} activation, Brenner [3] reports that in rabbit fast fibers g_{app} in frog fibers is 1-1.5 s^{-1} at 5°C and ~2 at 15°C. This latter value compares favorable with our estimate of g_{app} at 15°C (1.7 s^{-1}). In addition, using Q_{10} values of 1.3-2.0, Brenner=s data predict that g_{app} at 20°C would be between 2.3 and 2.8 s^{-1}, a range consistent with our estimate (2.6 s^{-1}). Other groups report values for A_{max} in rabbit and rat fast fibers at 15°C [2, 18]. Using their data as well as the approach described earlier, estimations of g_{app} range from 1.4 to 2.7 s^{-1}. Thus, our measure of g_{app} in frog fibers seems to lie within the range reported for mammalian fibers.

At 5.0mM MgATP and 20°C, g_{app} decreases by approximately 80% with increasing free [Ca^{2+}]. At free Ca^{2+} levels where force is ~10% of F_{max}, g_{app} is quite large, 10-12 s^{-1}. At similar free [Ca^{2+}], others have reported that the rate constant for tension re-development ($k_{tr} = f_{app} + g_{app}$) in mammalian fibers is much smaller (Metzger et al., [17]). We have found similar results in frog fibers [20]. This raises an inconsistency within the model. How can g_{app} be larger than the sum of f_{app} and g_{app}? There are at least two possible explanations. First , Brenner's model [3, 4] assumes that the backward apparent rate constants (f_{app}^- and g_{app}^-) are small and may be neglected. However, if f_{app}^- is significantly large at low free [Ca^{2+}], then k_{tr} could remain low while g_{app} is large. Second, Brenner [3,4] reports that F_{av} is insensitive to changes in free [Ca^{2+}]. However, his data show that there is considerable scatter of data points at low free [Ca^{2+}]. Equation 3 predicts that if F_{av} was smaller at low free [Ca^{2+}] than at high, then our estimates of g_{app} would be somewhat smaller than is shown in Figure 5. It should be pointed out that according to Brenner's data [3,4], F_{av} would, at most, double with increasing free [Ca^{2+}]. While this change could account for some of the Ca^{2+} sensitivity in g_{app} reported here, it cannot fully explained the 80% decrease reported at 5.0mM MgATP and 20°C.

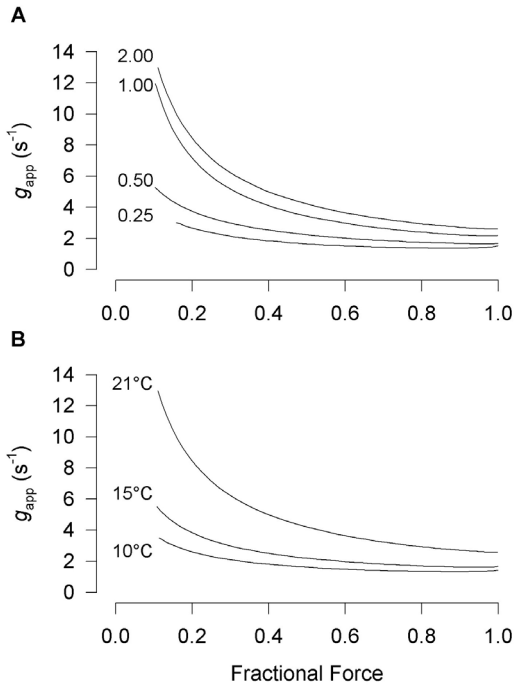

Figure 5. Relationships between g_{app} *and force with variations in [MgATP] (**A**) and variations in temperature (**B**). Curves were calculated using mean* $[Ca^{2+}]_{50}$ *and N values determined at each condition.* g_{app} *values are reported for free* $[Ca^{2+}]$ *where force was ∃10% of* F_{max}.

We found that at each [MgATP] and all three temperatures used, g_{app} showed some Ca^{2+} sensitivity. However, smaller absolute and relative changes in g_{app} were observed at lower [MgATP] and lower temperatures. While Brenner [3] suggests that g_{app} is relatively insensitive to increases in free [Ca^{2+}], he does acknowledge that even at low temperatures (5°C), there might be a slight effect of Ca^{2+} on g_{app}. A close examination of his data reveals that at 5°C there is a small increase in ATPase at low levels of Ca^{2+} compared to force. This results in a reduction in g_{app} of about 33% as free [Ca^{2+}] increases. Brenner [3] also states that this could result from suppression in A_{max} caused by some fiber deterioration. We argue that such a case is not likely. While depressed A_{max} might cause some non-linearity in the force-ATPase relationship, it could not account for the large increase in ATPase at free Ca^{2+} levels where no force is detected and where fiber deterioration is minimal. When taken together, our data and those of Brenner [3, 4], Kerrick et al. [13] and Williams et al. [20, 21] indicate that g_{app} is Ca^{2+} sensitive, the extent of which varies with temperature.

The finding that g_{app} is Ca^{2+} sensitive and affected by [MgATP] and temperature suggest that one or more steps in the transition from SB to WB are also sensitive to these

conditions. Wang and Kawai [19] suggest that this transition involves 1) the release of ADP from the actomyosin complex, 2) binding of ATP, 3) isomerization of ATP and 4) the detachment of actin and myosin. They also show that ATP isomerization and cross-bridge detachment are possible rate-limiting steps. However, Wang and Kawai [19] argue that ADP dissociation is accelerated rather than decreased by increasing free $[Ca^{2+}]$ and Goldman et al. [8] show that the detachment of the rigor crossbridge is independent of $[Ca^{2+}]$. As a result, the ATP isomerization step remains a likely candidate to be slowed as free $[Ca^{2+}]$ is increased. Also it is likely that reduced [MgATP] affects step 2, the binding of ATP or the rigor crossbridge. It remains to be seen which step is sensitive to changes in temperature.

One of the consequences of g_{app} changing during Ca^{2+} activation of the fibers is to make the force-free $[Ca^{2+}]$ relationship steeper (i.e. Hill coefficient larger) than that of ATPase. This is because force is proportional to $f_{app}/(f_{app}+g_{app})$ and ATPase proportional to $g_{app}Af_{app}/(f_{app}+g_{app})$. The results is that decreasing g_{app} during Ca^{2+} activation would cause force to rise faster with increasing free $[Ca^{2+}]$ than it would if only f_{app} was Ca^{2+} sensitive. This is clearly the case as shown in Figure 2. Further, since the Ca^{2+} sensitivity of g_{app} decreases with decreasing [MgATP] and temperature it is predicted that the slope of the force-free $[Ca^{2+}]$ relationship would be less under these conditions. This notion is supported by the data shown in Figures 3 and 4.

We also found that only the $[Ca^{2+}]_{50}$ of force is shifted to lower free $[Ca^{2+}]$ when MgATP and temperature are lowered and not the $[Ca^{2+}]_{50}$ of ATPase activity. These results, in combination with those of Figure 5 showing that the overall value of g_{app} decreases with decreasing MgATP and temperature, suggest that the shift of the force-free $[Ca^{2+}]$ relationship to lower free $[Ca^{2+}]$ could be due to the decrease in g_{app} and rather than to a decrease in the affinity of troponin C for Ca^{2+}. Our results also suggest that changes in the $[Ca^{2+}]_{50}$ of force could be the result of changes in the rate constant for the dissociation of force generating myosin crossbridges and that the steepness of the force-free $[Ca^{2+}]$ relationship is due to changes in the Ca^{2+} sensitivity of g_{app}. Therefore, pharmacological agents, environmental factors, or chronic adaptations which affect contractile apparatus function in skinned fibers should be investigated for their effects upon g_{app} in addition to their effects on the affinity of troponin C for Ca^{2+}.

In summary, we find that both MgATP and temperature affect the rate constant for the dissociation of force generating myosin crossbridges (g_{app}) and this effect is to decrease both the overall value and Ca^{2+} sensitivity of g_{app}. In addition, the results show that these changes in g_{app} are responsible for shifting the force-free $[Ca^{2+}]$ relationship to lower $[Ca^{2+}]$ and decreasing the steepness of the relationship.

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