Ion-specific and General Ionic Effects on Contraction of Skinned Fast-Twitch Skeletal Muscle from the Rabbit

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ABSTRACT We used single fibers from rabbit psoas muscle, chemically skinned with Triton X-100 nonionic detergent, to determine the salts best suited for adjusting ionic strength of bathing solutions for skinned fibers. As criteria we measured maximal calcium-activated force (F_{max}), fiber swelling estimated optically, and protein extraction from single fibers determined by polyacrylamide gel electrophoresis with ultrasensitive silver staining. All things considered, the best uni-univalent salt was potassium methanesulfonate, while a number of uni-divalent potassium salts of phosphocreatine, hexamethylenediamine *N,N,N',N'*-tetraacetic acid, sulfate, and succinate were equally acceptable. Using these salts, we determined that changes in F_{max} correlated best with variations of ionic strength ($1/2\ \Sigma\ c_i\ z_i^2$, where c_i is the concentration of ion i, and z_i is its valence) rather than ionic equivalents ($1/2\ \Sigma\ c_i|z_i|$). Our data indicate that increased ionic strength per se decreases F_{max}, probably by destabilizing the cross-bridge structure in addition to increasing electrostatic shielding of actomyosin interactions.

INTRODUCTION

In the "skinned" muscle fiber preparation, the sarcolemma is removed (Natori, 1954) or made permeable (e.g., Szent-Györgyi, 1949) so that the solution bathing the contractile apparatus is under experimental control. One is then faced with the problem of composing the experimental solution. One option is to mimic the intracellular milieu as closely as possible. This dictates not only the choice and concentration of constituents, but also other measures of the ionic environment such as ionic strength (which for vertebrate skeletal muscle is ~0.18 M; see Godt and Maughan, 1988). In vivo, the major constituents of skeletal muscle sarcoplasm are potassium and phosphocreatine (Beis and Newsholme, 1975; Godt and Maughan, 1988). Phosphocreatine, however, is labile, and costly in the dipotassium form. Therefore, investigators commonly use experimental solutions that contain some

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phosphocreatine (to buffer ATP levels), but in which ionic strength is adjusted by adding the potassium salt of a more stable, less costly anion such as chloride, acetate, propionate, or methanesulfonate.

Normally, investigators maintain skinned fiber bathing solutions at a constant ionic strength ($\mu_s = 1/2\ \Sigma\ c_i\ z_i^2$, where c_i is the concentration of ion i, and z_i is its valence). However, recently Johansson (1975) and Smith and Miller (1985) found that stability constants in polyvalent salt solutions depend on ionic equivalents ($\mu_e = 1/2\ \Sigma\ c_i|z_i|$) rather than ionic strength. In addition, comparing maximal force produced by skinned rat and toad muscles in solutions with increasing concentrations of potassium chloride or K_2EGTA, Fink et al. (1986) concluded that it is more appropriate to control ionic equivalents than ionic strength when studying the contraction of skinned muscle fibers. In solutions that contain only univalent ions, μ_e and μ_s are identical. But in solutions containing a mixture of uni- and polyvalent ions, such as those used in skinned fiber solutions, μ_e and μ_s differ. Moreover, different concentrations of univalent ions are required to substitute for polyvalent ions if μ_e rather than μ_s is kept constant.

The purpose of this study was to determine the salt best suited to adjust the ionic strength of skinned skeletal fiber bathing solutions by monitoring maximal calcium-activated force (F_{max}) of chemically skinned rabbit psoas fibers. The mechanism of salt-specific effects was examined by observing swelling of the fibers and extraction of muscle proteins. The best salt should show the least decline of F_{max} and the least swelling as ionic strength is increased, and should not extract proteins from the fibers. Finally, we used the best uni- and polyvalent salts to determine whether ionic strength or ionic equivalents is the measure of the ionic environment to which skinned fibers are more sensitive.

METHODS

Skinned Fiber Preparations

Fibers of the fast-twitch psoas major muscle of domestic rabbits were used in all experiments. The rabbits were killed by sodium pentobarbital overdose and small bundles of muscle were excised and chemically skinned in a skinning solution containing (in mM): 1 Mg^{2+}, 2 MgATP, 5 EGTA, 20 imidazole, and 60 KCl or potassium methanesulfonate ($MeSO_3^-$) to make ionic strength 150 mM, pCa > 8.5, pH 7.0, and 0.5% vol/vol purified Triton X-100, a nonionic detergent (Boehringer Mannheim Corp., Indianapolis, IN). After skinning, the fibers were stored at −20°C in a similar solution which contained 0.1 mM leupeptin, cytidine-5′-triphosphate (CTP) instead of ATP, and 50% vol/vol glycerol. Storage in CTP and leupeptin was undertaken to prevent phosphorylation of the myosin light chains because leupeptin inhibits the proteolysis of myosin light chain kinase (MLCK) to a calcium-insensitive form and CTP is not a substrate for MLCK (Pires and Perry, 1977; Srivastava and Hartshorne, 1983). It is our experience that fibers can be stored in this manner up to 1 mo without evidence of mechanical deterioration (e.g., breaking during calcium activation, or a decrement in maximal velocity of shortening) or of significant extraction of contractile proteins as evidenced by changes in maximal calcium-activated force or calcium sensitivity.

Immediately before experimentation, a fiber bundle was removed from the freezer and bathed in skinning solution (see above) at room temperature (~22°C). Single fibers were dissected from the fiber bundle and attached between the arm of an optoelectronic force

transducer and a stationary arm by wrapping the fiber around sand-blasted hooks. The fiber was stretched to a sarcomere length of 2.6 μm as determined using He-Ne laser diffraction. For further experimental details see Godt and Nosek (1989).

Composition of Bathing Solutions

The solutions used in these experiments were formulated according to microcomputer programs written in Turbo Pascal (Borland International, Scotts Valley, CA). These programs

TABLE I

Stability Constants Used (M^{-1})

Ionic species	Reference	(22°C)
HImid/H-Imid	Murphy and Koss (1968)	1.23×10^7
CaAcetate/Ca-Acet	Martell and Smith (1977)	3.39
MgAcetate/Mg-Acet	Martell and Smith (1977)	3.24
HAcetate/H-Acet	Martell and Smith (1977)	3.63×10^4
CaPropionate/Ca-Prop	Martell and Smith (1977)	3.16
MgPropionate/Mg-Prop	Martell and Smith (1977)	3.47
HPropionate/H-Prop	Martell and Smith (1977)	4.68×10^4
$CaMeSO_3$/Ca-$MeSO_3$	Andrews (1989)	1.0
$MgMeSO_3$/Mg-$MeSO_3$	Iino (1981)	1.0
CaLactate/Ca-Lac	Dawson et al. (1986)	15.85
MgLactate/Mg-Lac	Dawson et al. (1986)	19.95
HLactate/H-Lac	Dawson et al. (1986)	5.37×10^3
CaIsethionate/Ca-Iseth	Assumed similar to $MeSO_3$	1.0
MgIsethionate/Mg-Iseth	Assumed similar to $MeSO_3$	1.0
HIsethionate/H-Iseth	Assumed similar to ethanesulfonate of Martell and Smith (1977)	47.86
CaNitrate/Ca-Nit	Smith and Martell (1976)	1.14
MgNitrate/Mg-Nit	Smith and Martell (1976)	0.42
HNitrate/H-Nit	Martell and Smith (1982)	870.0
CaH2HDTA/Ca-H2HDTA	Martell and Smith (1974)	1.41×10^4
MgH2HDTA/Mg-H2HDTA	Martell and Smith (1974)	1.62×10^4
H3HDTA/H-H2HDTA	Martell and Smith (1974)	5.01×10^2
CaSuccinate/Ca-Suc	Dawson et al. (1986)	15.85
MgSuccinate/Mg-Suc	Dawson et al. (1986)	15.85
HSuccinate-H-Suc	Dawson et al. (1986)	1.55×10^4
CaSulfate/Ca-Sulf	Dawson et al. (1986)	2.69×10^2
MgSulfate/Mg-Sulf	Dawson et al. (1986)	2.00×10^2
HSulfate/H-Sulf	Dawson et al. (1986)	1.78×10^4
MgPEP/Mg-PEP	Dawson et al. (1986)	199.5
HPEP/H-PEP	Unpublished titration data	1.66×10^6
NaATP/Na-ATP	Cation binding to ATP is assumed similar to K-ATP of Godt and Lindley (1982)	8.00
TMAATP/TMA-ATP		8.00
CholineATP/Chol-ATP		8.00

solve the set of simultaneous equations describing the multiple equilibria of ions in the solutions, using previously reported association constants (Godt and Lindley, 1982) and others reported here (Table I).

The basic relaxing solution contained (in mM): 1 Mg^{2+}, 1 MgATP, 15 Na_2 phosphocreatine, 5 EGTA, 20 imidazole, 10 KCl, and ~100 U/ml creatine kinase, pH 7.00 (±0.01). The calculated ionic strength of this solution was 90 mM, while the ionic equivalent was 69 mM. The

basic activating solution was similar but contained $CaCl_2$ to adjust the concentration of free calcium. KCl was omitted from the basic activating solution to keep ionic strength at 90 mM. All pH adjustments were made using the acid and/or base of the salt being tested.

Procedures for Force Measurements

After being mounted on the force transducer, the fibers were transferred through a series of experimental solutions contained in rows of Plexiglas troughs. Triton X-100 (0.5% vol/vol) was added to each trough to reduce the surface tension of the solutions.

Experiments conducted to determine the least deleterious anion (to F_{max}) used the potassium salts of $MeSO_3^-$, acetate, lactate, isethionate, propionate, chloride, nitrate, and the sodium salt of perchlorate (used since potassium perchlorate is insoluble in water). Each salt was added to the basic relaxing and activating solutions in amounts appropriate to bring the total ionic strength of the bathing solutions to 165, 240, and 390 mM.

Three activation solutions (pCa = 5, 4.5, and 4) were prepared for each salt at each ionic strength. These were used to determine the level of calcium that yielded maximal activation of the fiber (pCa_{max}). This procedure was used with the first two fibers of each experimental group to ensure that the fibers were maximally activated. In all solutions, force levels at these calcium concentrations were quite similar (differing by no more than a few percent), however, except for solutions with K lactate, force at pCa 4 was slightly higher (i.e., pCa_{max} was taken to be 4). In K lactate, pCa_{max} was 4.5 since force was not at all higher in pCa 4.

After determination of pCa_{max}, fibers were relaxed in a low-calcium solution (pCa > 8.5) of the salt being tested at an ionic strength of 165 mM. In most cases the fiber was then randomly activated, at pCa_{max}, in solutions of 90, 165, 240, and 390 mM total ionic strength. For salts whose effects were irreversible at high ionic strength, e.g., KNO_3 and $NaClO_4$, contractions at high ionic strength were done last so as not to confound data at lower ionic strengths. Also, in the case of $NaClO_4$, fibers could not be activated at both 240 and 390 mM ionic strength, so additional fibers had to be used for these treatments. The activation procedure involved a plateau method, which involved continuous activation of fibers through all four ionic strengths before return to the low-calcium control solution. Activation time was kept brief (less than ~10 s) in each salt. Each fiber acted as its own control. F_{max} is reported as a percentage of F_{max} in the basic 90 mM μ_s solution.

To control for a decrease in F_{max} with repeated contractions, each group of contractions was bracketed by contractions in a control solution. Force in any experimental solution was expressed relative to the average of the control force before and after. Except for the deleterious salts given above, fibers tolerated this activation procedure quite well in that F_{max} in control solution after the procedure was >80% of that observed originally. We found that exposure to the 90 mM μ_s relaxing solution caused an increase in basal tension. To limit any deleterious effects of such low ionic strength solutions, each fiber was subjected to the 90 mM activation solution only once. Therefore, the control solutions contained $KMeSO_3$ and had a total ionic strength of 165 mM.

We found that $MeSO_3^-$ was the least deleterious anion. Therefore, a similar set of experiments were run to determine the least deleterious cation using the $MeSO_3^-$ salt of tetramethylammonium (TMA), choline, sodium, and potassium. Again, each salt was added to the basic solution to attain total ionic strengths of 165, 240, and 390 mM.

Measurement of the Calcium Sensitivity of Skinned Fibers

In experiments comparing the effects of various potassium salts of $MeSO_3^-$, acetate, propionate, and chloride on calcium sensitivity, fibers were activated in solutions of 200 mM ionic strength over a range of calcium concentrations from pCa 8.5 to 4. The two parameters of calcium

sensitivity, K (the calcium concentration at which half-maximal activation occurred), and N, the Hill coefficient (a measure of the slope of the force–pCa relationship), were determined by least-squares fit to a Hill equation of the form (Godt and Lindley, 1982):

$$\%F_{max} = 100\,[Ca^{2+}]^N/(K^N + [Ca^{2+}]^N)$$

Determination of Skinned Fiber Width

The possibility of differential fiber swelling was determined in a subset of the solutions noted above. A representative number of anions were used: $MeSO_3^-$, acetate, propionate, chloride, and nitrate (as potassium salts). Measurements of fiber width were made using a LaSico micrometer mounted in the optical system of a Zeiss inverted microscope at magnifications of 400× and 100×. This system allowed fiber width to be measured to the nearest micrometer. The fibers were transferred among solutions contained in 1-ml troughs mounted on the microscope stage. All measurements were made on activated fibers since fiber diameter is known to decrease upon activation (Maughan and Godt, 1981). Fiber widths stabilized within 10–20 s after bathing solutions were changed. Simultaneous force recordings assured that the fibers reacted to the salt solutions as they had previously, while photographs were taken to document the alterations in fiber width. Widths are reported as a percentage of the fiber width in basic 90 mM μ_s (pCa = 4) solution.

Similar experiments were also run with the dipotassium salt of creatine phosphate and the $MeSO_3^-$ salts of sodium, potassium, choline, and TMA.

Compression of Skinned Fibers

After skinning, the otherwise swollen fibers were compressed to widths near that seen when fibers were bathed in the basic 90 mM μ_s solutions (Godt and Maughan, 1977; Maughan and Godt, 1979) by increasing solution osmolarity with the addition of Dextran T 500 (a long-chain polymer of number average mol wt 500,000; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as in Godt and Maughan (1981).

Dextran T 500 concentrations of 4 and 10% wt/vol (g/100 ml) were used in each experimental solution. A Dextran concentration of 4% was usually sufficient to compress fibers to within ±1–3% of the widths seen in the 90 mM μ_s solution. With a 10% Dextran concentration, fibers were compressed well below widths seen when fibers were bathed in the basic 90 mM μ_s solutions (<95%) and F_{max} in a given solution was significantly reduced.

Determination of Protein Extraction from Skinned Fibers

To determine the effects of various anions on protein extraction, protein concentrations were measured by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970; Giulian et al., 1983) with 15% acrylamide. Because of the small amounts of protein present in single fibers (total volume in the 10–50-nl range) and the smaller amounts extracted, silver stain was used to quantify protein concentration (Switzer et al., 1979; Oakley et al., 1980). Fibers of known volume (determined optically) were immersed, under mineral oil, in a series of three 5-μl aliquots of relaxing solution for 1-, 9-, and 20-min intervals (30 min total). 4 μl of each aliquot containing any extracted protein was then recovered with a micropipette and placed in 8 μl of sample buffer containing 62.5 mM Tris (pH 6.8), 1% wt/vol SDS, 0.01% wt/vol Bromphenol blue, 15% vol/vol glycerol, and 5% vol/vol β-mercaptoethanol. The remaining fiber (i.e., the fiber matrix) was placed in 5 μl of sample buffer and sonicated for 2 min. This solution was diluted 10- and 100-fold and 4 μl of each diluted solution was loaded onto the gel in lanes adjacent to the sample of extracted protein for comparison. Thus, if, for example, the density of any protein band in the lane containing extracted protein were the same as that in

the lane containing 100-fold diluted fiber, the concentration of extracted protein would be 1% of that in the original fiber. Bands in the lane containing extracted protein could be quantified at one-tenth of this level, i.e., 0.1% of the protein concentration in the original fiber. To identify each protein extracted, purified samples (Sigma Chemical Co., St. Louis, MO) of known muscle proteins were run alongside the experimental solutions and fiber matrices. The solutions assessed were of 165 and 390 mM ionic strength (75 and 300 mM added salt, respectively), and included one of the salts found to be least deleterious to F_{max}, namely, $KMeSO_3$ (n = 5), and one of the salts found to be most deleterious to F_{max}, KCl (n = 5). Two fibers and their aliquots were analyzed in the case of potassium salts of acetate and propionate.

Evaluation of Measures of the Ionic Environment

The strategy used in the experiments to determine whether fibers were sensitive to ionic strength or ionic equivalents involved altering the basic bathing solution by adding an increasing amount of the neutral salt of the univalent cation and the polyvalent anion found to be the least deleterious to F_{max}. A complementary set of solutions was mixed in which the added neutral salt was of the monovalent cation and anion found to be least deleterious to F_{max}. Concentrations were such that ionic strength (μ_s) was equal in correspondingly numbered solutions, while ionic equivalents (μ_e) were allowed to vary (Table II).

TABLE II

Solutions

	Solutions with:	
	K_2PCr μ_e/μ_s	$KMeSO_3$ μ_e/μ_s
Solution set 1	119/165	144/165
Solution set 2	169/240	219/240
Solution set 3	269/390	369/390

Experimental Design and Statistics

All experiments involving force and width measurement used a modified Latin-square design, with each fiber acting as its own control and with six fibers per experimental group (unless otherwise noted). All force and width data were normalized and reported as a percentage of maximal calcium-activated force, or width, in the basic (90 mM μ_s) solution. Means, standard errors, and analyses of variance were calculated using Statgraphics (STSC Inc., Rockville, MD). Post hoc testing (alpha level = 0.05) of means were conducted using the Student-Newman-Keuls method (Sokal and Rohlf, 1969) in F_{max} and swelling experiments. Paired one-tailed t tests (alpha level = 0.05) were used during analysis of data determined from optical scanning of the SDS-PAGE gels of KCl- and $KMeSO_3$-treated fibers. Regression analysis with the data of each group of salt solutions fitted by a logarithmic or linear function (95% confidence intervals) was also used.

RESULTS

Ion-specific Effects on F_{max} and Fiber Width: Univalent Salts

As ionic strength was raised by addition of uni-univalent salts, the F_{max} of chemically skinned rabbit psoas fibers decreased monotonically. The slope of the relationship, however, is dependent on the choice of anion and cation. Fig. 1 shows this

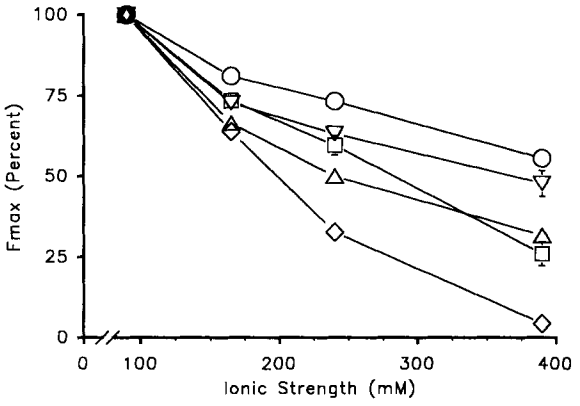

FIGURE 1. The effect of increased ionic strength and anion substitution on F_{max} of skinned single rabbit psoas fibers as ionic strength was increased by addition of the potassium salt of $MeSO_3$ (○), acetate (▽), propionate (△), chloride (□), or nitrate (◇). For simplicity, all results are expressed as a percentage of F_{max} in the 90 mM ionic strength solution (including standard error bars, visible when larger than the symbols).

relationship with five salts, some of which are commonly used to adjust ionic strength of skinned fiber bathing solutions. Fig. 1 and Table III illustrate that for potassium salts, F_{max} decreases least if methanesulfonate is the anion. When cations were varied (as salts of $MeSO_3^-$), there was little difference in the depression of F_{max} with increases of ionic strength, although, on the basis of maintaining F_{max}, choline and TMA were slightly better than potassium and sodium (Table IV). Except for solutions containing KNO_3 at 390 mM and $NaClO_4$ at 240 and 390 mM total ionic strength, which cause an irreversible reduction of F_{max} within a few seconds, the effects of increased ionic strength on F_{max} are reversible.

It is well known that as ionic strength of bathing solutions is increased, fibers tend to swell (e.g., Godt and Maughan, 1977; Maughan and Godt, 1980). We found that the degree of swelling depended on the salt used to increase ionic strength (Fig. 2 for potassium salts; Table V for $MeSO_3^-$ salts). Of the potassium salts tested, fibers swelled

TABLE III

Percent of Basic (90 mM μ_i) F_{max} attained as Ionic Strength and Anion Composition Are Altered (Arranged in Descending Order at 240 mM Ionic Strength, n = 6)

Salt	Ionic strength		
	165 mM	240 mM	390 mM
	%	%	%
K $MeSO_3$	81.2 ± 1.2*	73.3 ± 1.8*	55.6 ± 1.8‡
K lactate	72.5 ± 1.5	64.0 ± 0.9	55.2 ± 1.8‡
K acetate	72.8 ± 1.6	63.3 ± 1.3	47.8 ± 4.0
K chloride	73.5 ± 2.1	59.6 ± 3.0	26.0 ± 3.7
K isethionate	69.6 ± 2.5	59.4 ± 2.8	46.9 ± 2.3
K propionate	66.5 ± 2.1	50.0 ± 1.8	31.7 ± 2.0
K nitrate	63.9 ± 1.4	32.7 ± 1.6	4.3 ± 1.9§
Na perchlorate	73.7 ± 3.5	12.2 ± 4.8§	0.0 ± 0.0§

*Significantly greater force was generated in this salt solution than in any other at 165 and 240 mM ionic strength ($P < 0.05$).

‡Significantly greater force was generated in these salt solutions than in any other at 390 mM ionic strength, with no significant difference noted between the two conditions ($P < 0.05$).

§F_{max} was irreversibly decreased after activation in these solutions.

TABLE IV

Percent of Basic (90 mM μ_s) F_{max} Attained as Ionic Strength and Cation Composition Are Altered (Arranged in Descending Order at 240 mM Ionic Strength, n = 6)

Salt	Ionic strength		
	165 mM	240 mM	390 mM
	%	%	%
Choline $MeSO_3$	91.9 ± 0.9*	79.3 ± 1.0‡	42.9 ± 1.6
TMA $MeSO_3$	91.1 ± 1.8*	75.8 ± 1.6	57.9 ± 2.2
K $MeSO_3$	81.2 ± 1.2	73.3 ± 1.8	55.6 ± 1.8
Na $MeSO_3$	83.1 ± 1.2	69.3 ± 1.4	50.3 ± 2.7

*Significantly greater force was generated in these salt solutions than in any other at 165 mM ionic strength, with no significant difference noted between the two conditions ($P < 0.05$).

‡Significantly greater force was generated in this salt solution than in any other at 240 mM ionic strength ($P < 0.05$).

least with $MeSO_3^-$. Likewise, swelling was least in the potassium or sodium salt of $MeSO_3^-$ (Table V). Such swelling was fully reversible in all salt solutions tested.

Could salt-specific effects on F_{max} be related simply to differences in fiber swelling? As can be noted, Figs. 1 and 2 argue against this; while swelling was minimal between 165 and 390 mM ionic strength, there was a substantial decrease in F_{max} over the same range. Furthermore, the effect of swelling on F_{max} was specifically tested by osmotically restoring fiber widths to near those seen in the basic 90 mM μ_s solution by using 4% Dextran. In the potassium salts tested, Table VI shows that compression had little or no effect (< 10%) on F_{max}, except in solutions containing KCl at 390 mM ionic strength. The salt-specific effects on F_{max} remain after compression, indicating that differences in swelling do not explain the differences in the salt-specific effects on F_{max}. KNO_3 solutions were not tested due to their irreversible effects on force generation.

Anion-specific Protein Extraction from Skinned Fibers

Protein extraction from skinned fibers was assessed by using polyacrylamide gels of the fibers themselves and of the solutions in which they had been bathed. Typical gels

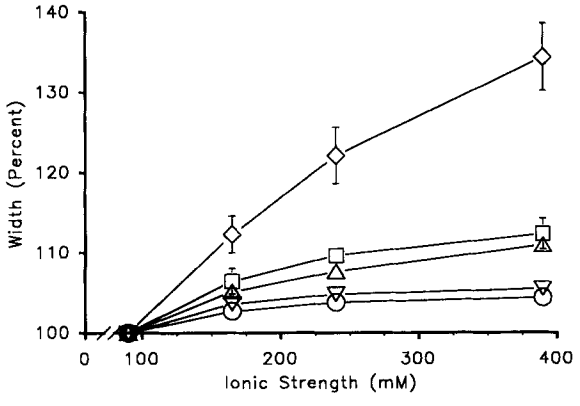

FIGURE 2. The effect of increased ionic strength and anion substitution on width of single skinned rabbit psoas fibers as ionic strength was increased by addition of the potassium salt of $MeSO_3$ (○), acetate (▽), propionate (△), chloride (□), or nitrate (◇). For simplicity, all results are expressed as a percentage of width in the 90 mM ionic strength solution (including standard error bars, visible when larger than the symbols).

TABLE V

Percent of Basic (90 mM μ_s) Fiber Width Attained as Ionic Strengh Is Increased with the Given Salt ($MeSO_3$ Salts Are Arranged in Descending Order at 240 mM Ionic Strength, n = 6)

Salt	Ionic strength		
	165 mM	240 mM	390 mM
	%	%	%
Na $MeSO_3$	102.7 ± 0.3*	103.5 ± 0.6*	104.1 ± 0.7*
K $MeSO_3$	102.7 ± 0.9*	103.8 ± 1.0*	104.4 ± 0.9*
TMA $MeSO_3$	104.4 ± 0.9	107.2 ± 0.9	108.7 ± 0.7
Choline $MeSO_3$	105.0 ± 0.8	108.0 ± 0.9	108.8 ± 2.2
K_2PCr	103.2 ± 1.7	105.3 ± 2.2	109.8 ± 2.2

*Swelling of fibers in solutions containing either Na^+ or K^+ $MeSO_3$ does not significantly differ, but is statistically different from width in the basic solution and from all other $MeSO_3$ salts at each ionic strength ($P < 0.05$).

are shown for fibers bathed in solutions containing KCl and $KMeSO_3$ (Fig. 3). Table VII lists the protein extractions that resulted at 300 mM (390 mM μ_s) added salt. At 165 mM μ_s, there was minimal extraction of myofilament proteins. In contrast, in solutions adjusted to 390 mM μ_s with addition of KCl there was substantial time-dependent extraction of myosin heavy and light chains, and a 60-kD protein (perhaps α-actinin). No other salt tested caused substantial protein extraction.

Anion Effects on the Force–pCa Relationship

The force–pCa relation was very similar among the four potassium salts tested. Table VIII shows that at 200 mM μ_s, calcium sensitivity (K) did not depend on the salt used to adjust ionic strength. The only significant difference is that the slope of the force–pCa relation (N) with acetate was greater than that with chloride ($P > 0.05$).

Ion-specific Effects of Polyvalent Salts

The potassium salts of the divalent anions phosphocreatine (PCr), sulfate, succinate, and hexamethylenediamine *N,N,N',N'*-tetraacetic acid (HDTA) had similar effects on F_{max} as ionic strength is increased (Table IX). Note that the sodium salt of phosphoenol pyruvate (PEP), predominantly trivalent at neutral pH, causes force to decline more precipitously than any divalent anion.

TABLE VI

Alteration of F_{max} during Compression of Fibers with 4% Dextran T 500 to Near Width Seen in the Basic (90 mM μ_s) Solution, n = 6

Salt	Ionic strength		
	165 mM	240 mM	390 mM
	%	%	%
K $MeSO_3$	−3.87 ± 1.10	+0.50 ± 0.76	+6.23 ± 3.39
K acetate	−6.42 ± 4.53	+0.79 ± 0.86	+7.11 ± 1.57*
K propionate	−3.72 ± 0.49	+3.02 ± 2.58	+8.01 ± 1.22*
K chloride	−3.18 ± 0.64	+6.72 ± 1.10*	+26.37 ± 4.62*

*F_{max} was significantly increased by compression under these conditions ($P < 0.05$).

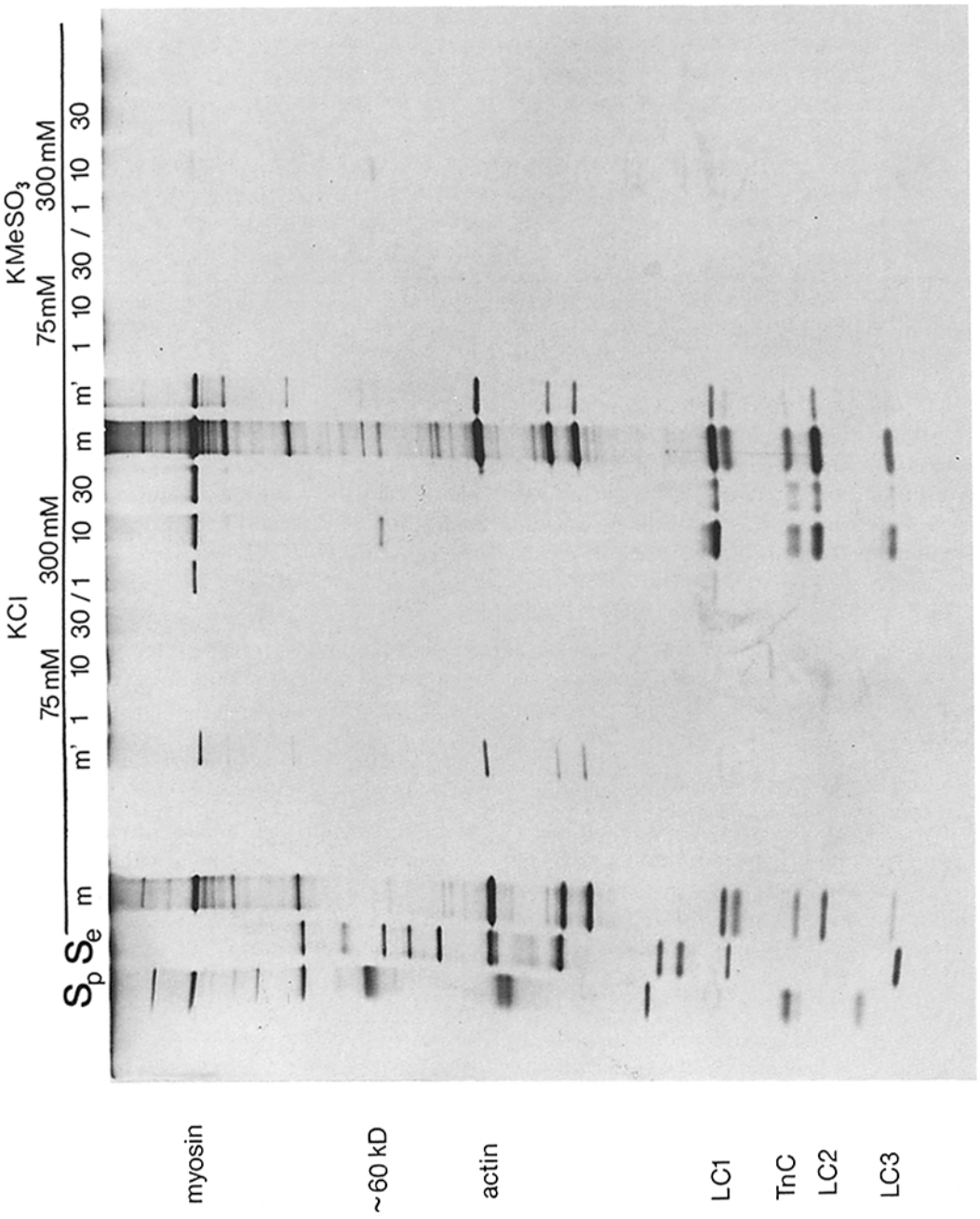
KCl
75 mM
300 mM
KMeSO3
75 mM
300 mM
m' 1 10 30 / 1 10 30 m m' 1 10 30 / 1 10 30
Sp Se m
myosin
~60 kD
actin
LC1
TnC
LC2
LC3

FIGURE 3

TABLE VII

Average Cumulative Protein Extraction at 390 mM Total Ionic Strength (n = 5) for Chloride and $MeSO_3$, Mean and [Range] shown; n = 2 for Acetate and Propionate, Both Values Shown)

	Myosin	60 kD	Actin	MLC1	MLC2	MLC3
After 1 min	%	%	%	%	%	%
K chloride	0.9	7.1	0.1	1.2	0.8	0.6
	[0–1.7]	[0–19.8]	[0–0.3]	[0–3.0]	[0–1.8]	[0–1.5]
K $MeSO_3$	*	1.9	*	0	0	0
	[0–0.1]	[0–5.2]	[0–0.2]			
K propionate	*	1.8	0	0	0	0
	0	0	0	0	0	0
K acetate	0	0	0	0	0	0
	0	0	0	0	0	0
After 10 min						
K chloride	4.5	29.1	0.2	13.8	12.8	19.5
	[0–9.8]	[0–85.3]	[0–0.5]	[0–31]	[0–29.5]	[0–51.5]
K $MeSO_3$	*	4.2	*	0	0	0
	[0–0.2]	[0–10.6]	[0–0.2]			
K propionate	*	0.6	0	0	0	0
	*	0.1	*	*	0	0
K acetate	0	5.6	0	0	0	0
	0	0	0	0	0	0
After 30 min						
K chloride	10.0	31.9	0.4	26.3	26.5	37.5
	[0.6–21.4]	[0–85.3]	[0–1.0]	[0–43]	[0–44]	[0–60]
K $MeSO_3$	0.1	5.6	0.1	0	0	0
	[0–0.3]	[0–14.1]	[0–0.2]			
K propionate	*	0.8	0.1	0	0	0
	*	0.2	0	0.2	0	0
K acetate	0	10.1	0	0	0	0
	0	0	0	0	0	0

60 kD, protein of 60 kD; MLC1, myosin light chain 1; MLC2, myosin light chain 2; MLC3, myosin light chain 3.

*Trace amounts (<0.1%) observed.

Ionic Strength vs. Ionic Equivalents

Having examined the effects of a variety of salts on skinned fiber properties, we were then able to answer the question of whether ionic strength or ionic equivalents is the parameter of the ionic environment to which skinned psoas fibers are more sensitive.

FIGURE 3 (*opposite*). Silver-stained polyacrylamide gel of rabbit psoas muscle fiber matrix and muscle proteins extracted by treatments in low-calcium (pCa <8.5) basic 90 mM μ_s solution adjusted to 165 and 390 mM total ionic strength by addition of either 75 or 300 mM KCl, or $KMeSO_3$. S_p, protein standards; S_c, muscle cytosolic protein standards; *m*, 1:10 dilution of fiber matrix after experiment; *m'*, 1:100 dilution of fiber matrix after experiment; *1*, *10*, or *30*, total elapsed time in experimental solution; *60 kD*, protein of ~60 kD; *LC*, myosin light chain (1, 2, or 3); *TnC*, troponin C.

TABLE VIII

The Influence of Anion Substitution on the Force–pCa Relationship of Single Skinned Rabbit Psoas Muscle Fibers at 200 mM Total Ionic Strength (n = 6 for All Conditions)

Salt	Hill coefficient (N)	[Ca^{2+}] at 50% F_{max} (K)
		M
K $MeSO_3$	3.70 ± 0.35	$1.0 \pm 0.08 \times 10^{-6}$
K acetate	4.72 ± 0.34*	$8.56 \pm 0.49 \times 10^{-7}$
K propionate	3.42 ± 0.53	$9.36 \pm 0.46 \times 10^{-7}$
K chloride	3.17 ± 0.12*	$8.59 \pm 0.51 \times 10^{-7}$

*N varied significantly between these two conditions ($P > 0.05$).

We determined F_{max} in two series of solutions: one set contained $KMeSO_3$ to adjust ionic strength, while the other set contained K_2PCr (two of the least deleterious uni- and divalent anions, respectively) to adjust ionic strength. If ionic strength is the appropriate parameter, the relationship between F_{max} and ionic strength should be the same in both salts. In contrast, if ionic equivalents is the appropriate parameter then the plots of F_{max} vs. ionic equivalents for both sets of solutions should be superimposable. To control for fiber to fiber variability, each fiber acted as its own control and was exposed to all solutions. As seen in Fig. 4 *A*, the relation between F_{max} and ionic strength was the same in both salts. Full regression analysis revealed no differences between these plots. However, when expressed with respect to ionic equivalents (Fig. 4 *B*), the decreases in F_{max} differ significantly between $KMeSO_3$ and K_2PCr as ionic concentrations are increased. In addition, experiments (data not shown) comparing $TMAMeSO_3$ and TMA_2HDTA yielded similar conclusions. There-

TABLE IX

Percent of Basic (90 mM μ_i) F_{max} Attained as Dipotassium Salts of Polyvalent Anions Were Used to Increase Ionic Strength (n = 6)

Salt	Ionic strength		
	165 mM	240 mM	390 mM
	%	%	%
K_2HDTA	89.5 ± 1.4*	75.2 ± 1.9	57.0 ± 3.9
TMA_2HDTA	90.2 ± 2.1*	78.1 ± 2.5‡	52.0 ± 4.5
K_2PCr	86.8 ± 1.5	76.8 ± 2.4‡	60.4 ± 3.2
K_2 succinate	84.3 ± 1.7	74.6 ± 2.0	67.2 ± 3.6§
K_2 sulfate	80.2 ± 1.5	71.6 ± 1.3	60.1 ± 2.2
Na_3 PEP	76.6 ± 1.7	53.9 ± 1.6	36.2 ± 1.8

*Significantly greater force was generated in these salt solutions than in any other at 165 mM ionic strength, with no significant difference noted between these two conditions ($P < 0.05$).

‡Significantly greater force was generated in these salt solutions than in any other at 240 mM ionic strength, with no significant difference noted between the two conditions ($P < 0.05$).

§Significantly greater force was generated in this salt solution than in any other at 390 mM ionic strength ($P < 0.05$).

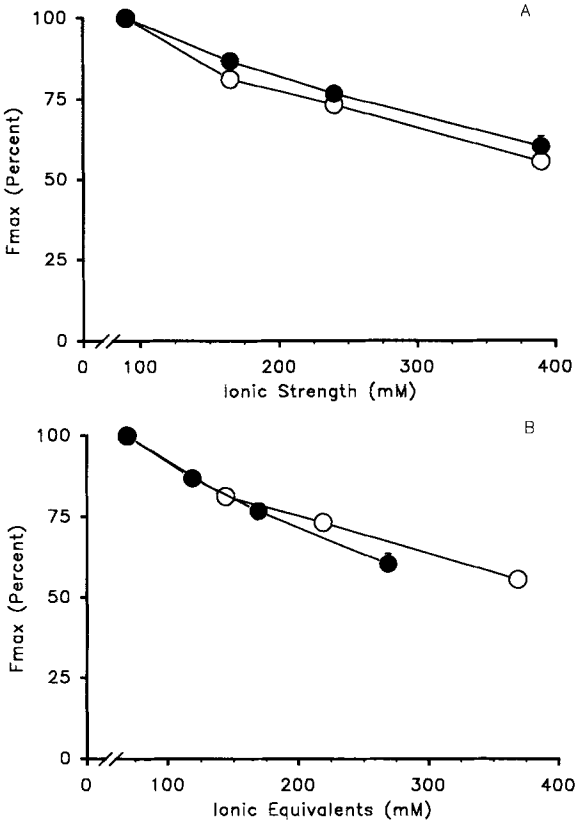

FIGURE 4. The effect on F_{max} of single skinned rabbit psoas fibers as the ionic concentration of the basic activating solution was increased by the addition of either methanesulfonate ($KMeSO_3$; ○) or phosphocreatine (K_2PCr; ●). (*A*) F_{max} plotted vs. ionic strength. Although F_{max}'s in solutions containing $KMeSO_3$ and K_2PCr were statistically different at 165 mM ($P > 0.05$), ANOVA and regression analysis (95% confidence interval) confirmed that the lines describing the data for $KMeSO_3$ and K_2PCr do not differ to a significant degree. (*B*) The same F_{max} data plotted vs. ionic equivalents. ANOVA and regression analysis (95% confidence interval) confirmed that the lines describing the data for $KMeSO_3$ and K_2PCr are significantly different. Results are expressed as a percentage of F_{max} attained in the basic activating solution (including standard error bars, visible when larger than the symbols).

fore, judged by effects on F_{max} (the criterion used by Fink et al., 1986), our data indicate that ionic strength is the most appropriate general ionic parameter to control in skinned muscle fiber bathing solutions.

DISCUSSION

Salt Preference for Adjusting Ionic Strength

On the basis of its minimal effects on F_{max}, swelling, and protein extraction from skinned skeletal muscle fibers, these results present a strong case for the use of $KMeSO_3$ to adjust ionic strength of skinned fiber bathing solutions. Potassium, while being slightly more deleterious to F_{max} than choline or TMA, is preferred because it is the major intracellular cation, and because it causes minimal swelling of skinned skeletal muscle fibers as bathing solution ionic strength is increased. Likewise, $MeSO_3^-$ is to be preferred because it minimizes swelling of the myofilament lattice and is least deleterious to F_{max} as concentrations are increased. In addition, solutions containing $KMeSO_3$ cause negligible extraction of proteins from skinned fibers, even at high (300 mM) concentrations and long (30 min) times. Moreover, the binding of

calcium and magnesium to $MeSO_3^-$ is weak, not differing from the capacity of these ions to bind chloride (Andrews, 1989; Iino, 1981). Increasing ionic strength by the use of K_2PCr, the major intracellular salt, resulted in similar effects on F_{max} (Fig. 4 *A*) and fiber width (Table V) as when solutions containing $KMeSO_3$ were used. There were no significant differences between $KMeSO_3$ and K_2PCr effects on F_{max}, while the two anions cause similar, but not equal swelling of skinned fibers (Fig. 2 and Table V). Finally, $KMeSO_3$ is preferred to K_2PCr because it is considerably cheaper and less labile.

Ionic Strength or Ionic Equivalents?

From plots of F_{max} vs. ionic strength (Fig. 4 *A*) or ionic equivalents (Fig. 4 *B*), it appears that formal ionic strength is the measure of total ionic content of bathing solutions to which skinned muscle fibers respond. As outlined above, $KMeSO_3$ and K_2PCr (or $TMAMeSO_3$ and TMA_2HDTA) appeared to have minimal salt-specific effects. Therefore, when the composition of skinned fiber bathing solutions is altered, ionic strength, rather than ionic equivalents, should be held constant.

This conclusion is opposite to that of Fink et al. (1986), who infer from their data from skinned rat and toad muscle that F_{max} responds to ionic equivalents, not ionic strength. We believe that this discrepancy is not due to species differences, but rather to the choice of salts, since Fink et al. (1986) adjusted the ionic composition of the bathing solution with either KCl or K_2EGTA. The use of KCl probably confounded their results because of an ion-specific effect of chloride on F_{max}. If we compare our F_{max} data of fibers activated in solutions containing KCl, rather than $KMeSO_3$, with the F_{max} of fibers in K_2PCr or K_2HDTA solutions, we also would have concluded that ionic equivalents, and not ionic strength, was the more appropriate parameter (Fig. 5, *A* and *B*).

Effects of Anions on Calcium Sensitivity of Skinned Fibers

It has been determined that bathing solutions containing potassium, sodium, or choline differentially affect Ca^{2+} sensitivity of the contractile apparatus (Fink et al., 1986). However, in the present investigation, various monovalent anions widely used in skinned muscle fiber experiments did not differentially affect the calcium sensitivity of the contractile apparatus at near physiological ionic strength (Table VIII). This contrasts with the markedly different effects exhibited by these anions on F_{max}.

How Does Ionic Strength Affect F_{max}?

Increasing ionic strength decreases F_{max}, even in the best of salts. The mechanism is unclear, but we can think of a number of possibilities: alteration of myofilament spacing, ionic shielding of actomyosin interactions, or destabilization of protein structure/function.

The first mechanism is unlikely to play a major role because alterations of fiber width (and presumably interfilament spacing) over the range of ionic strengths tested are small (Table V). Moreover, osmotic compression with Dextran, to near widths seen when fibers were bathed in the basic 90 mM μ_s solutions, does not reverse the effect of elevated ionic strength on F_{max} (Table VI). Moreover, recent evidence from

Kawai et al. (1990) suggests that changes in myofilament lattice spacing are unable to account for the effects of ionic strength on isometric force.

Increasing ionic strength should decrease the electrostatic interactions between proteins by simple ionic screening. If electrostatic interactions between sites on actin and myosin are important to cross-bridge function, this might explain why F_{max} is affected by ionic strength (Moos, 1973; Highsmith, 1977). In fact, Geeves and Goldmann (1990) showed that the overall affinity of actin for myosin subfragment-1,

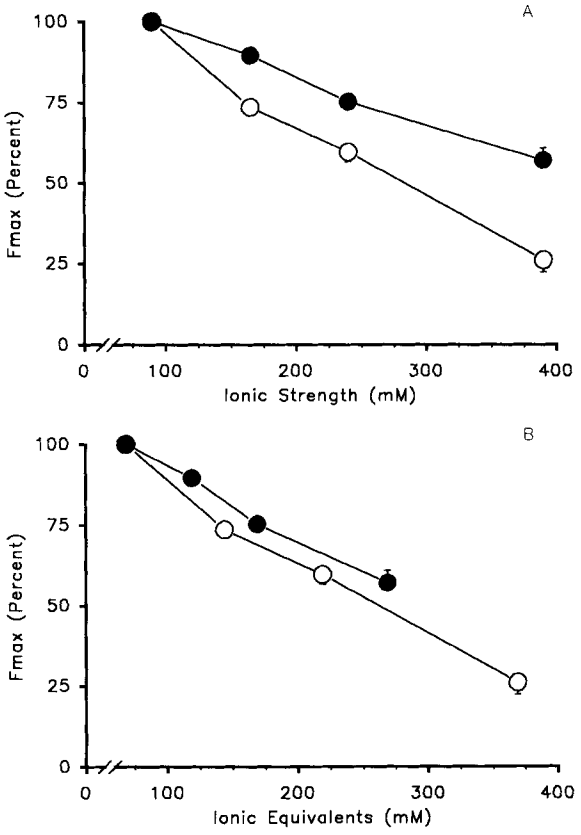

FIGURE 5. The effect on F_{max} of single skinned rabbit psoas fibers as the ionic concentration of the basic activating solution was increased by the addition of K salts of either chloride (○) or hexamethylenediamine-*N,N,N',N'*-tetraacetic acid (K_2HDTA; ●).(*A*) F_{max} plotted vs. ionic strength. F_{max} in solutions containing KCl and K_2HDTA were statistically different at each ionic strength ($P > 0.05$), ANOVA and regression analysis (95% confidence interval) confirmed that the lines describing the data for KCl and K_2HDTA are significantly different. (*B*) The same F_{max} data plotted vs. ionic equivalents. ANOVA and regression analysis (95% confidence interval) confirmed that the lines describing the data for KCl and K_2HDTA are also significantly different, yet are more similar than those presented in *A*. Results are expressed as a percentage of F_{max} attained in the basic activating solution (including standard error bars, visible when larger than the symbols).

measured in vitro by fluorescence titration, is decreased when ionic strength is increased from 0.1 to 0.5 M with potassium acetate (their Table 1). It is well known that changes in solution tonicity affect tetanic force of intact muscle fibers (Gordon and Godt, 1970). Changes in tonicity also alter intracellular ionic strength (Gordon et al., 1973; Homsher et al., 1974). Using bundles of 10–16 intact fibers, Vaughan et al. (1983) have shown that as tonicity of the bathing solution is increased, stiffness of activated fibers decreases; however, force decreases more precipitously than stiffness.

More recent evidence from Månsson (1989) shows no significant alteration of instantaneous stiffness of tetanically stimulated single frog muscle fibers as a function of tonicity. Given that instantaneous stiffness is a measure of the number of strongly attached cross-bridges, this argues against the possibility that changes in ionic screening influence this phase of the cross-bridge cycle, but does not rule out screening effects on other steps of the cycle. With intact fibers however, altering solution tonicity may have influences other than simply changing intracellular ionic strength (i.e., altering interfilament spacing, or affecting signal transduction between transverse tubules and sacroplasmic reticulum). These influences are largely outside of experimental control. Thus, experiments with skinned fibers will be required to fully assess the relative role of ionic screening on cross-bridge function. In point of fact, Kawai et al. (1990) suggest that changes in ionic strength modify the rapid equilibrium between the detached cross-bridge state and the "weakly-attached" state, and that this causes the effect on isometric force. They also conclude that other steps in the cross-bridge cycle are less sensitive to ionic strength.

It is recognized that elevation of ionic strength leads to destabilization of protein structure and function, either directly by ion binding to the protein or indirectly by ionic disruption of the highly ordered lattice of water associated with the proteins (Robinson, 1989; Zaks and Klibanov, 1988). Von Hippel and Schleich (1969) have attempted to explain the ubiquitous alteration of macromolecules (DNA, collagen, and RNA) by ions in aqueous solution in terms of alteration of solvent structure by the added ions. It is noteworthy that their ion series was similar to those observed in the present experiments. On the other hand, Tanford (1970) has argued strongly that the destabilization of the native conformation of protein is not necessarily related to the effects of ions on water structure per se, but may be related to the alteration of the thermodynamics of interactions of hydrophobic side-chains of the protein with the solvent.

Recent evidence from our laboratory argues in favor of a major role for protein destabilization in the effects of elevated ionic strength. We found that a number of zwitterions naturally occurring in high concentration in muscles of euryhaline animals can reverse the effects of elevated ionic strength on the F_{max} of skinned rabbit psoas muscles (Fogaca et al., 1990). These compounds, especially trimethylamine *N*-oxide (TMAO), are known to protect proteins from destabilization of structure and function under a variety of conditions, e.g., elevated salt concentrations (Yancey et al., 1982). The mechanism of this protecting effect is thought to involve a preferential interaction of solute with proteins in that the solute is excluded from the surface of the protein, thus leading to preferential hydration of the protein, resulting in a situation where the native structure is thermodynamically favored (Arakawa and Timasheff, 1985; Carpenter and Crowe, 1988; Timasheff and Arakawa, 1989). Protein destabilization occurs under conditions that favor transition to the denatured state; i.e., as solutes that preferentially bind to the protein are introduced or their concentrations are increased (Arakawa and Timasheff, 1982; Timasheff and Arakawa, 1989). In this view, elevated concentrations of ions such as chloride or nitrate may decrease F_{max} by binding to the muscle proteins, denaturing cross-bridge structure and thereby altering cross-bridge function. These observations do not rule out a role for electrostatic screening on cross-bridge function since, at concentrations of TMAO

found to be most effective (300 mM), F_{max} is still depressed at high ionic strength ($KMeSO_3$, KCl, or KNO_3) although not to the extent that it was in the absence of TMAO (Fogaca et al., 1990; Fogaca, R. T. H., M. A. W. Andrews, and R. E. Godt, unpublished observations).

Salt-specific Effects on F_{max}

The preferential interaction model of protein destabilization could explain the salt-specific effects of increased ionic strength on F_{max} of skinned skeletal muscle fibers (Jacobs and Guthe, 1970; Gordon et al., 1973; Homsher et al., 1974; Fink et al., 1986). In our hands (Fig. 1, Tables III and IV), the anions can be arranged in order of increased potency (capacity to inhibit F_{max}), over the physiological range of ionic strength (165–240 mM), as:

$$\begin{gathered}\text{methanesulfonate} \\ < \text{lactate} \simeq \text{acetate} \\ < \text{chloride} \simeq \text{isethionate} \\ < \text{propionate} \\ \ll \text{nitrate} \\ \ll \text{perchlorate}\end{gathered}$$

Similarly, the cations, in order of increased potency, are:

$$\begin{gathered}\text{choline} \simeq \text{tetramethylammonium} \\ < \text{potassium} \simeq \text{sodium.}\end{gathered}$$

Similar sequences of ions and salts have been shown to affect physico-chemical properties of macromolecules in a salt-specific way, dating back to the initial work of Hofmeister on lyotropic salts (Hofmeister, 1888, 1891; see also von Hippel and Schleich, 1969). In accord with our data in skinned fibers, Geeves and Goldmann (1990) found that an increase in KCl concentration from 0.1 to 0.5 M reduced the overall affinity of actin for myosin subfragment-1 to a greater extent than similar elevation of the potassium salt of acetate or propionate. They suggest that chloride destabilizes the attached cross-bridge state by increasing the rate constant of detachment of myosin from actin.

In agreement with the results of Bello et al. (1956) and Robinson and Jencks (1965) on collagen structure and enzyme function, respectively, the present results indicate that ion- and salt-specific effects are not simply related to size or solvation radius of the ion(s) in solution. Though fibers bathed in solutions containing large cations, e.g., choline and TMA, generated greater force than those bathed in solutions containing small cations, e.g., potassium and sodium, such differences are not large. In addition, other studies have indicated that both large cations, such as tetraethyl- and tetrapropylammonium (Gordon et al., 1973), and small ions, such as lithium (Tonomura et al., 1962), are deleterious to force generation of skinned fibers, some irreversibly so. Similarly, no simple relationship is seen between anion size and F_{max}.

In agreement with the preferential binding theory (and our anion series), Bello and Vinograd (1956) and Bello et al. (1956) ranked $MeSO_3^-$ < chloride < nitrate in their capacity to bind to and decrease the stability of the collagen helix. In addition, Brahms and Brezner (1961) demonstrated from electrophoretic mobility that anions

bind to myosin in the following order: acetate < chloride < thiocyanate < nitrate. A number of studies (Collins and Edwards, 1971; Caille and Hinke, 1973; Elliott, 1980; Naylor et al., 1985) have used microelectrode recordings to infer that chloride binds to the myofilaments, and that chloride, but not propionate, binds to barnacle myofilaments (Clark et al., 1981). Stafford (1985) localized one site of action of anions on the cross-bridge. Using both papain digestion and helix-coil transition temperatures to study the stability of skeletal muscle myosin, he concluded that chloride, but not acetate, destabilized a domain near the junction between light meromyosin and subfragment-2.

As concerns cations, sodium and potassium have been proposed to bind to the myofilament lattice in vitro (McLaughlin and Hinke, 1966; Caille and Hinke, 1973), with direct evidence of potassium binding to the myofilaments shown by Tigyi et al. (1981) using $^{42}K^+$ and radioautographic techniques. Seidel (1969) found no evidence that large cations such as cesium and TMA bind to myofilaments.

Thus, there is considerable evidence that ion binding, with subsequent protein destabilization, might explain many of the ion-specific effects on F_{max}, fiber swelling, and protein extraction we have observed. However, other effects must be postulated to explain the deleterious influence of propionate on F_{max}. While propionate extracts appreciably less protein than chloride and is not known to bind to the myofilaments (Clark et al., 1981), similar to acetate and $MeSO_3$, it affects F_{max} to a degree similar to chloride. Therefore, propionate must be affecting myofilaments through another mechanism. It may be that while ions such as propionate do not bind to the myofilaments, they may alter F_{max} through other mechanisms such as electrostatic alteration of cross-bridge function or alterations of solvent–solute interactions.

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