It Is Diprotonated Inorganic Phosphate That Depresses Force in Skinned Skeletal Muscle Fibers

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The increases in the intracellular concentrations of inorganic phosphate and hydrogen ion accompanying fatigue of skeletal muscle appear to be the most important metabolic changes associated with the decrease in contractile force. Experiments on chemically skinned single fibers from rabbit psoas muscle with *p*H ranging between 6 and 7.25 demonstrate that the depression of maximal calcium-activated force by inorganic phosphate correlates nicely with the concentration of the acidic (diprotonated) species. Therefore, in addition to the well-known depressant effect on the contractile machinery of lowering *p*H per se, any decrease of intracellular *p*H associated with fatigue further depresses force production by converting more of the total inorganic phosphate within the cell to the inhibitory diprotonated form.

THE EFFECTS OF INORGANIC PHOSPHATE (P_i) and *p*H on muscle function are important biological issues inasmuch as the intracellular concentration of P_i and H^+ are dramatically elevated during fatigue of skeletal muscle and are closely associated with the force depression observed under these conditions (*1–4*). A number of investigators have reported that P_i (*5–8*) and lowered *p*H (*6, 9*) have direct inhibitory effects on force production by the contractile machinery of muscle cells, and current work suggests that these two metabolic changes may play major depressant roles in fatigue (*7*). Recent studies with ^{31}P nuclear magnetic resonance (NMR) spectroscopy indicate that there is a strong correlation between the diprotonated (acidic) form of P_i ($H_2PO_4^{-1}$) and the decline of force with fatigue in intact skeletal muscle of the frog (*10*). In the study described here we explicitly tested for such effects on the contractile apparatus using skinned muscle fibers. Unlike intact muscle fibers, these preparations are free of the influences of cellular metabolism and excitation-contraction coupling and thus permit complete control of the solution bathing the contractile machinery.

As shown in Fig. 1B, at *p*H 7 force decreases monotonically with total P_i concentration ($[P_i]$), an effect similar to that observed by others (*5–8*). At near-neutral *p*H, phosphate exists almost exclusively in either monoprotonated (HPO_4^{-2}) or diprotonated ($H_2PO_4^{-1}$) forms whose ratio depends on the proton concentration. Thus, at fixed *p*H, increasing total $[P_i]$ increases both charged forms proportionately. To examine the possible effect of either of the charged species one must change *p*H as well as total $[P_i]$. However, alteration of *p*H affects maximal force in and of itself (Table 1) and must be factored out to examine the additional effects of P_i (Fig. 1A). For simplicity, we assume that the effects of *p*H and P_i are multiplicative and that the sensitivity of the contractile apparatus to phosphate is independent of *p*H. When the effect of *p*H per se is extracted, the strong correlation between total $[P_i]$ and force is lost (Fig. 1B; note the force at 30 m*M* total $[P_i]$). Figure 2 shows that the decline in force correlates nicely with the concentration of $H_2PO_4^{-1}$ (slope, −2.72% per millimolar $H_2PO_4^{-1}$ ± SE of slope, 0.11), and Fig. 3 shows that there is no apparent linear relation with the concentration of HPO_4^{-2}.

Increases in total intracellular $[P_i]$ up to about 26 mmol per kilogram of whole muscle [39 mmol per liter of fiber water (*11*)] have been reported for intact, fatigued skele-

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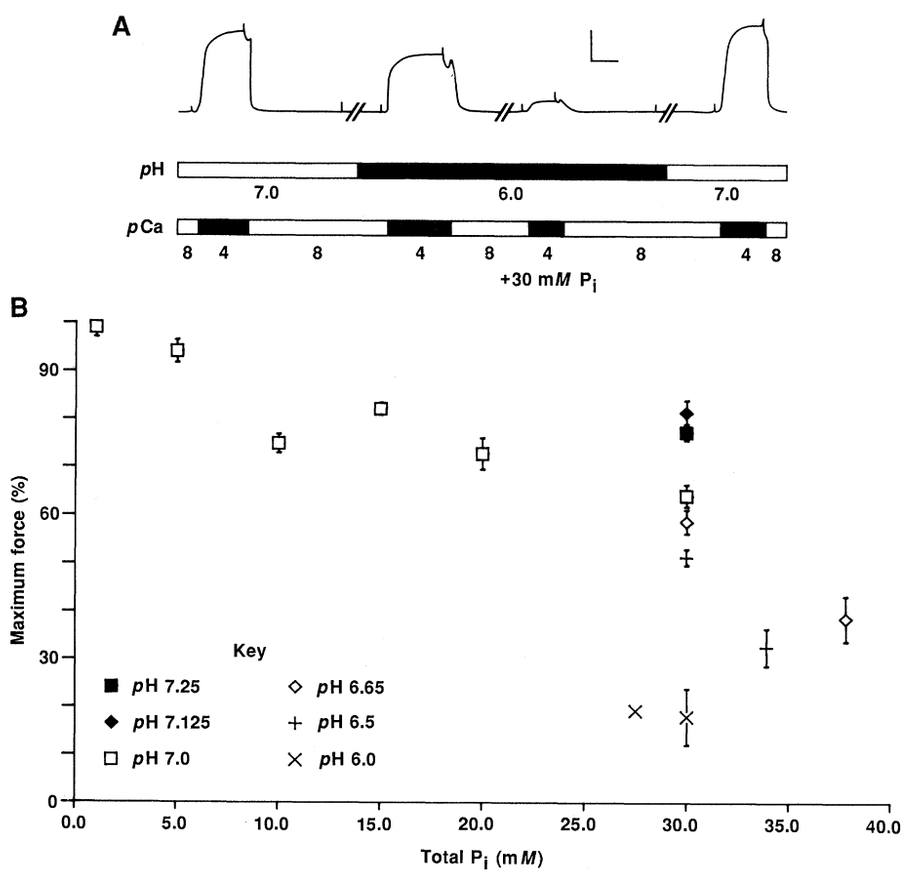
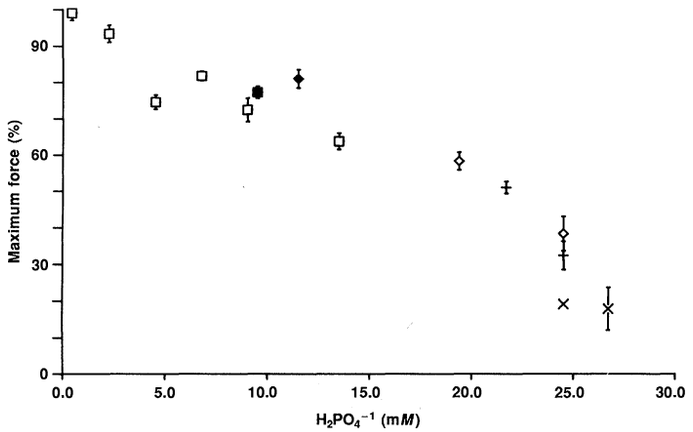


Fig. 1. Effects of total $[P_i]$ on maximal Ca^{2+}-activated force (*p*Ca 4) of skinned muscle fibers. Single fibers from rabbit psoas muscle were skinned in relaxing solution (*p*Ca > 8) containing 0.5% purified Triton X-100. The skinned fibers were sequentially bathed in a series of solutions containing 1 m*M* Mg^{2+}, 1 m*M* magnesium adenosine 5′-triphosphate, 5 m*M* EGTA, 20 m*M* imidazole, 15 m*M* phosphocreatine, 0 to 37.8 m*M* P_i, and 47 to 119 m*M* KCl to maintain ionic strength at 0.2*M*; *p*H 6 to 7.25, *p*Ca either > 8 (no added $CaCl_2$) or 4, and creatine kinase, 3 U/ml. All experiments were performed at room temperature (about 22°C). For further technical details see (*21*). To control for deterioration of the fibers, each test contraction was compared to the mean of two bracketing control (*p*H 7, 0 P_i) contractions. The inhibition of force due solely to phosphate at any *p*H (see Fig. 1A) was taken to be the ratio of a test contraction with and without phosphate. (**A**) Typical record of effect of P_i on maximal force of skinned psoas muscle fiber (No. 2-851030, diameter 50 μm). Calibration bars show 50 mg and 10 seconds and spikes on record are solution exchange artifacts. For concision, the record shown is not continuous but has been truncated at the interrupted lines. (**B**) Influence of total $[P_i]$ for *p*H ranging from 6 to 7.25. Data in this and other figures are means of at least five fibers. Vertical bars show standard error of mean.



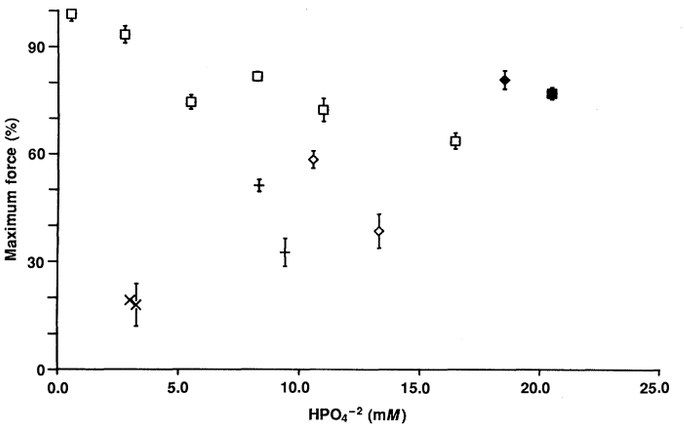


Fig. 2 (left). Correlation of phosphate-induced depression of maximal Ca^{2+}-activated force with concentration of $H_2PO_4^{-1}$ in skinned skeletal muscle fibers. Concentration of $H_2PO_4^{-1}$ calculated from total [P_i] with *p*K of 6.79 (*16*). Symbols as in Fig. 1B. **Fig. 3 (right).** Lack of correlation between phosphate-induced depression of maximal Ca^{2+}-activated force and concentration of HPO_4^{-2} in skinned skeletal muscle fibers. Symbols as in Fig. 1B and calculations as in Fig. 2.

tal muscle. From NMR studies of intact muscle it has been suggested that it is the diprotonated form ($H_2PO_4^{-1}$) of P_i that depresses force by direct action on cross-bridges (*10*). Work with intact cells, however, is subject to the criticisms that (i) the intracellular milieu is not well controlled, and (ii) force production reflects excitation-contraction (E-C) coupling processes as well as cross-bridge activity. Neither are germane to skinned fibers where the composition of the bathing medium is under strict experimental control and E-C coupling is circumvented. Thus, in our experiments, the effects of P_i and *p*H on force are those exerted solely on the contractile apparatus, and they strongly support the suggestion that maximal force produced by the cross-bridges is, indeed, dependent on $H_2PO_4^{-1}$ and not on HPO_4^{-2} or total P_i.

In addition, there is a strong quantitative similarity between our findings and those of Dawson and co-workers (*10–12*) as to the slope of the relation between force and $H_2PO_4^{-1}$. In their experiments with intact gastrocnemius muscle from frog, the relation is linear, with zero force attained at about 18.5 mmol per kilogram of whole muscle (*10, 12*), which converts to about 28 mmol per liter of cell water (*11*). This is similar to our data for skinned fibers from rabbit psoas (Fig. 2), which approach zero at around 30 to 35 m*M*. Moreover, unpublished ^{31}P NMR data from human adductor pollicis in vivo during voluntary, fatiguing contractions also show a strong correlation between maximal voluntary force and $H_2PO_4^{-1}$ with a zero force intercept between 30 to 40 m*M* (*13*). This finding suggests that the sensitivities of cross-bridges to $H_2PO_4^{-1}$ in amphibia and mammalia are much the same.

Table 1. Influence of *p*H on maximal Ca^{2+}-activated force (*p*Ca 4) of skinned rabbit psoas muscle fibers. The composition of the bathing solution is described in the legend to Fig. 1. Data normalized to maximum force at *p*H 7.

*p*H	Maximum force (% ± SEM)	Fibers (*n*)
7.25	120.5 ± 1.4	6
7.125	114.7 ± 2.8	6
7.0	100	76
6.65	95.3 ± 1.4	12
6.5	86.2 ± 1.4	10
6.0	67.7 ± 2.2	6

The effect of P_i on maximum force can be explained by the finding of Hibberd and co-workers (*8, 14*) that P_i appears to reverse the putative force-producing step of the cross-bridge cycle (that is, P_i release from the attached cross-bridge) by shifting the distribution of cross-bridges toward those states with a full complement of bound products [adenosine 5′-diphosphate (ADP) and P_i]. This shift would lead to a decrease in the instantaneous number of force-producing bridges [however, see (*15*)]. If this view is correct, the observation that maximal force produced by the cross-bridges depends on $H_2PO_4^{-1}$, and not on HPO_4^{-2} or total P_i, implies that it is $H_2PO_4^{-1}$ that is released during the force-producing step of the cross-bridge cycle.

Since the *p*K for the reaction $HPO_4^{-2} + H^+ \leftrightarrow H_2PO_4^{-1}$ is near the normal intracellular *p*H (pH_i) (*16, 17*), a decrease in pH_i would convert more of the intracellular P_i to $H_2PO_4^{-1}$. Therefore, the decline of pH_i normally seen with fatigue, which can approach a full *p*H unit (*1–4, 18*), will inhibit force not only directly (*6, 9* and Table 1) but indirectly as well, by increasing the fraction of total P_i in the $H_2PO_4^{-1}$ form. Thus the combined effects of increased intracellular [P_i] and decreased pH_i observed under these conditions will result in a dramatic depression of maximal force production by the contractile apparatus (*19*). These effects will be additive with the inhibition of E-C coupling that is likely to occur with fatigue (*20*).

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Cellular Localization of Somatomedin (Insulin-Like Growth Factor) Messenger RNA in the Human Fetus

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The somatomedins or insulin-like growth factors (IGFs) are synthesized in many organs and tissues, but the specific cells that synthesize them in vivo have not been defined. By in situ hybridization histochemistry, IGF I (somatomedin C) and IGF II messenger RNAs were localized to connective tissues or cells of mesenchymal origin in 14 organs and tissues from human fetuses. IGF messenger RNAs were localized to perisinusoidal cells of liver, to perichondrium of cartilage, to sclera of eye, and to connective tissue layers, sheaths, septa, and capsules of each organ and tissue. All of the hybridizing regions are comprised predominantly of fibroblasts or other cells of mesenchymal origin. Because these cells are widely distributed and anatomically integrated into tissues and organs, they are ideally located for production of IGFs, which may exert paracrine effects on nearby target cells.

THE SOMATOMEDINS OR INSULIN-like growth factors (IGFs) are peptide mitogens that are structurally homologous to proinsulin (*1*). In man, two major IGFs, IGF I (somatomedin C) and IGF II, have been characterized by amino acid sequence analysis of peptides purified from serum (*2*) and by isolation of complementary DNAs (cDNAs) encoding precursor forms of these peptides (*3, 4*). Traditionally, the liver has been thought to be the primary site of IGF synthesis, but evidence from a number of studies now suggests that the IGFs are synthesized in many mammalian tissues: (i) immunoreactive IGFs are secreted by cultured explants of a number of mouse organs (*5*) and by monolayers of fibroblasts derived from several human and rat tissues (*6*); (ii) concentrations of immunoreactive IGF I (*7*) and abundance of IGF I messenger RNA (mRNA) (*8*) is regulated by growth hormone in a variety of adult rat tissues; (iii) immunoreactive IGF I can be extracted from multiple midgestation human fetal tissues (*9*); and (iv) mRNA encoding IGF I and IGF II can be detected in a number of fetal and adult rat tissues and human fetal tissues (*10*).

The mitogenic actions of the IGFs on cultured cells from fetal tissues of several mammalian species and the presence of specific IGF receptors in many fetal tissues and cells (*1, 11*) point to a role for the IGFs in fetal growth. These findings and the evidence that IGFs are synthesized in many fetal tissues (*5–10*) support the hypothesis that IGFs act locally to elicit the biologic response (*5, 7*). Information about the cellular sites of synthesis of IGFs in the fetus should provide insight into their potential role as paracrine fetal growth factors and, toward this goal, we have investigated the cellular localization of IGF mRNA in human fetal tissues.

Synthetic oligodeoxyribonucleotides (oligomers) complementary to portions of human IGF I (*3*) and IGF II (*4*) mRNA (Fig. 1, A to C) were used as probes for in situ hybridization histochemistry to localize IGF mRNA in sections of human fetal tissues. The 31-nucleotide (nt) IGF II oligomer and cDNAs encoding human IGF I and II precursors were first tested in Northern blot hybridizations with polyadenylated RNA from human fetal tissues. The IGF II oligomer hybridized with five distinct mRNAs in human fetal liver and muscle that are the same size as mRNAs detected with the IGF II cDNA, but different in size from two mRNAs detected in these tissues with the IGF I cDNA (Fig. 1D). These data, plus observations that the 31-nt IGF II oligomer and a second 39-nt IGF II oligomer that corresponds to a different region of human IGF II mRNA do not hybridize with human IGF I cDNA, provide evidence for the specificity of the IGF II oligomers for human IGF II mRNA. Similarly, the three IGF I oligomers—IGF IA (20 nt), IGF IA (36 nt), and IGF IB (Fig. 1, A and B)—showed no hybridization with human IGF II cDNA or IGF II mRNA, providing evidence that these oligomers are specific for human IGF I mRNA.

Human fetal tissues were collected from prostaglandin-induced abortuses of 16 to 20 weeks gestation with prior approval of the Committee for the Protection of Rights of Human Subjects at the University of North Carolina at Chapel Hill. Sections prepared from fixed human fetal tissues (*12*) were hybridized with IGF IA, IGF IB, or IGF II oligomers labeled at the 5′ end with ^{32}P (Fig. 1). All human fetal tissues tested, except cerebral cortex, showed hybridization with IGF IA, IGF IB, and IGF II oligomers. With each IGF probe and in all tissues showing positive hybridization, the hybridization signal was localized to connective tissues or to cells of mesenchymal origin (Fig. 2 and Table 1). In liver, IGF oligomers hybridized to perisinusoidal cells and showed no detectable hybridization to hepatocytes. Although the precise identity of these hybridizing cells could not be conclusively established with the resolution achieved, perisinusoidal cells are composed of mesenchymal cells, such as endothelial and reticuloendothelial cells, and fibroblasts. In costal cartilage, hybridization of IGF oligomers was to perichondrium and fibrous sheath and not to chondrocytes. In the eye, hybridization was to sclera and not to neuroretina, choroid, or lens. In heart, thymus, lung, skeletal muscle, skin, spleen, kidney, adrenal, stomach, and small intestine, IGF

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