Differential Coupling of Smooth and Skeletal Muscle Pyruvate Kinase to Creatine Kinase†

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ABSTRACT: The interaction of pyruvate kinase from skeletal (SKPK) and smooth (SMPK) muscle with MM-creatine kinase (MMCK) and BB-creatine kinase (BBCK) was assessed using temporal absorbance changes, variations in absorbance at different wavelengths, concentration dependence, association in an electric field, and PK kinetic activity. SKPK exhibits a time course of absorbance increase in the presence of MMCK with a time constant of 29.5 min. This increase occurs at all wavelength from 240 to 1000 nm. At 195 nm, the combination of SKPK and MMCK produces a decrease in absorption with electric fields of both 0 and 204 V/cm. The change in SKPK−MMCK is saturable. SKPK activity is significantly increased by the presence of MMCK in solutions of 0−32% ethanol. These results indicate specific SKPK−MMCK interaction. SMPK and BBCK did not exhibit similar coupling when the BBCK concentration dependence of absorbance or SMPK activity in solutions of 0−32% ethanol was determined. Both MMCK and BBCK increased SKPK activity; neither MMCK nor BBCK increased SMPK activity. The ability to form diazymatic complexes with creatine kinase appears to reside in SKPK. This coupling may account for the increased flux through PK without significant substrate changes seen during skeletal muscle activation. This coupling will not occur in smooth muscle.

There have been several reports documenting the physical and kinetic coupling of skeletal muscle pyruvate kinase (SKPK)[1] and MM type creatine kinase (MMCK) (*1*, *2*). This coupling was first noted using nuclear magnetic resonance saturation transfer kinetics, in which magnetically saturated phosphate was shown to exchange between phosphoenolpyruvate (PEP) and phosphocreatine (PCr) (*1*). This finding was subsequently supported by experiments showing that MMCK increased the ethanol solubility, the pH-dependent water solubility, and the enzymatic activity under low-Mg^{2+} conditions of SKPK (*2*). It was also shown that the saturation transfer occurred at high ionic strengths (*2*).

The experiments reported here sought to expand our knowledge of PK−CK coupling. These experiments tested the time course of the formation of the SKPK−MMCK complex, its frequency dependence, its concentration dependence, and the effect of CK on the activity of PK in water and ethanol environments. Further, the effect of CK on smooth muscle creatine kinase (SMPK) was also measured. Smooth muscle glycolysis, of which PK is a constituent enzyme, has been shown to exhibit complex coupling behavior, particularly with membrane ion pumps (*3*, *4*). By isolation and testing of the coupling of SMPK with either the primary smooth muscle CK enzyme, BBCK, or the MMCK form that is present only in small amounts in smooth muscle (*5*), the breadth of PK−CK coupling was tested. The flux through skeletal muscle glycolysis increases greatly under energetic stress without large changes in either PEP or ADP concentrations (*6*). PK−CK coupling may be part of a mechanism controlling this flux independently of free PEP and ADP concentrations. The results provide insight into the regulation of energy metabolism. If both SKPK and SMPK couple to CK, then this coupling may be a general property of kinases; conversely, if SKPK couples but SMPK does not, the physical and kinetic coupling of PK and CK in skeletal muscle may produce a significant energetic advantage not present in smooth muscle. Our results will show that it is this second model that best fits the experimental results.

MATERIALS AND METHODS

Protein Solutions. SKPK, MMCK, and BBCK were obtained from Sigma Chemical Co. SKPK and MMCK were from rabbit skeletal muscle. BBCK was from rabbit brain. SMPK was purified from beef bladders. In the mixing experiment for the time course measurement, we used a solution with final concentrations of 3.5 mg/mL MMCK and SKPK in 50 mM KCl, with the absorbance monitored at 340 nm in a spectrophotometer. In the spectrum experiments whose results are depicted in Figure 1, we used individual SKPK and MMCK solutions at concentrations of 1.5 mg/mL each. For the combined protein measurement, equal aliquots of the individual solutions were combined for final concentrations of 0.75 mg/mL for both SKPK and MMCK. The frequency was changed manually and the absorbance

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[1] Abbreviations: BBCK, brain type creatine kinase; CE, capillary electrophoresis; CK, creatine kinase; Cr, creatine; ETS, electron transport system; MMCK, muscle type creatine kinase; PCr, phosphocreatine; PEP, phosphoenolpyruvate; PK, pyruvate kinase; Pyr, pyruvate; SKPK, skeletal muscle pyruvate kinase; SMPK, smooth muscle pyruvate kinase; SN2, type 2 nucleotide substitution; TCA, tricarboxylic acid.

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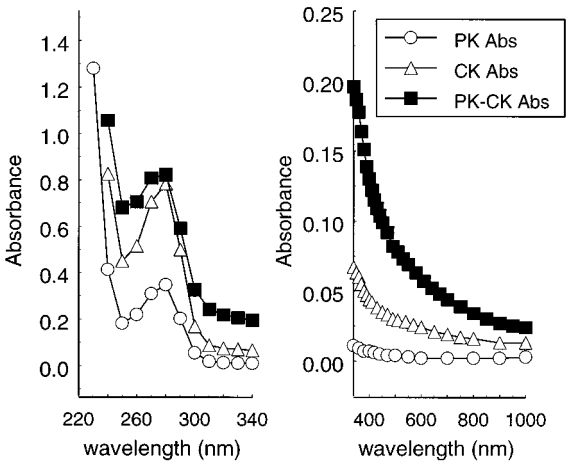

FIGURE 1: Spectrum of solutions of SKPK (○), MMCK (△), and a combination of SKPK and MMCK (■). All solutions contained 1.5 mg/mL. The combination solution contained 0.75 mg/mL for each enzyme and was prepared by mixing equal aliquots of the one-enzyme solutions. The frequency was changed manually and the absorbance compared with that of a reference cuvette containing no protein. The changes on absorbance are not uniform across the spectrum, indicating that the total absorbance change is due to specific protein absorbance changes as well as changes in light scattering.

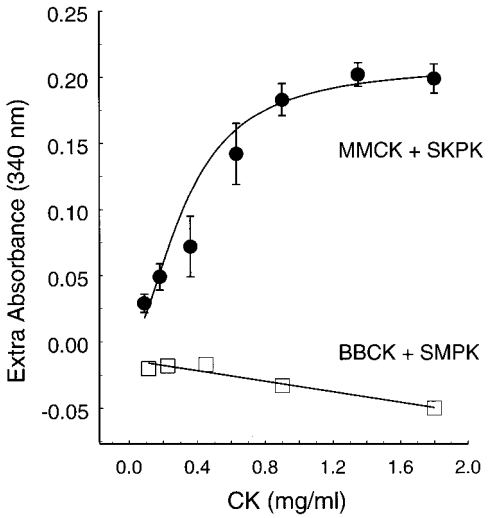

FIGURE 2: Absorbance difference produced by additions of CK to 0.4 mg/mL PK. The extra absorbance is the difference between the combined solution and the individual solutions of CK and PK. For the skeletal enzymes (●), SKPK and MMCK, the values are the mean ± the standard deviation. For the smooth muscle enzymes (□), SMPK and BBCK, the values are the mean values of multiple measurement of single solutions.

compared with that of a reference cuvette containing no protein. In the combined protein experiments whose results are depicted in Figures 2−4, we used buffers to match the buffer at the end of the SMPK isolation and to produce a pH of 6.2, at which the skeletal PK−CK coupling is maximal (*2*). CK solutions were made up in 20 mM Na_2HPO_4, and the PK solutions were made up in 10 mM diammonium citrate, the final buffer in the SMPK isolation. The experimental solutions were mixed so as to maintain final concentrations of 9.5 mM phosphate and 5.3 mM citrate at pH 6.2. Regardless of the concentrations of the proteins that were present, the final concentrations of phosphate and citrate were always the same. In Figure 2, the concentration of PK was 0.4 mg/mL, with the concentration of CK altered as

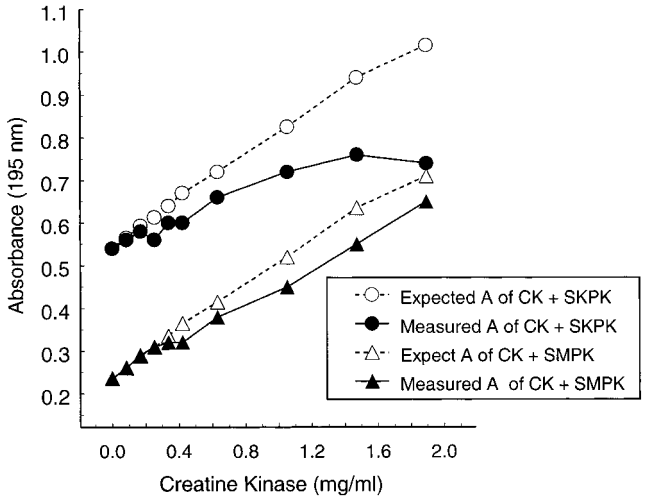

FIGURE 3: Absorbance produced by additions of MMCK to SKPK and SMPK. The expected absorbance is the sum of individual measurements of MMCK and either SKPK (○) or SMPK (△). The measured absorbance is the absorbance produced by solutions containing both MMCK and SKPK (●) or MMCK and SMPK (▲). The decrease in absorbance produced by MMCK and SKPK at 195 nm is in contrast to the increases in absorbance produced by these proteins at higher wavelengths, shown in Figure 1. This is further evidence of specific protein interaction.

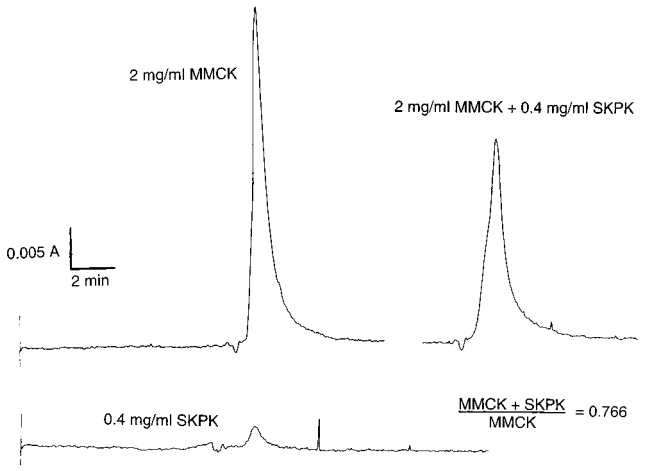

FIGURE 4: CE electropherograms of solutions of SKPK and MMCK and a combined solution of MMCK and SKPK at 204 V/cm. On the left are independent SKPK and MMCK runs. The origin on the time scale is marked by the recorded trace. Almost the entire SKPK peak overlaps with the MMCK peak. On the right, the combination run shows a lower than expected peak. The run time is the same as that for MMCK. This dynamic experiment demonstrating the reduction in absorbance at this wavelength confirms the similar results from the static absorbance measurement depicted in Figure 3.

shown. For each CK concentration, the extra absorbance was calculated by subtracting the absorbances of the individual PK and CK solutions from the absorbance of the combined PK−CK solution. The solutions in Figure 3 used SKPK or SMPK separately or combined with MMCK, were injected into a capillary of volume 7.7 μL (*7*) in a CE unit, and were detected at 195 nm. Thirty microliters of a solution was drawn into a syringe, and 20 μL was injected, ensuring complete filling and washout of the capillary with minimal injection artifact. The expected absorbance is the sum of the absorbances of the separate protein solutions, and the measured absorbance is that of the combined PK−CK

solutions. In experiments using ethanol, the ethanol concentration was determined by v/v addition from a 95% ethanol stock.

Isolation of SMPK. The bladders were collected from Bain's Packing and Refrigeration (Howell, MI) at the time of slaughter. Bladders were removed from the beef 5 min after death, cut open, rinsed, and stored on ice. The isolation was started on the same day as the tissue collection. Bladders were cut into strips and homogenized in 100 mM imidazole (pH 7.4). The homogenate was centrifuged. All centrifugations were carried out at 25000*g* at 4 °C for 30 min. To the supernatant was added an equal volume of 15% PEG, and the solution was centrifuged. The pellet was resuspended in 100 mM imidazole (pH 7.4) at 21.5 °C (room temperature) for 1 h. The solution was centrifuged and the pellet discarded. The supernatant was filtered through a Bio-Rad Bio-Gel A-1.5m column with bed dimensions of 2.5 cm (internal diameter) × 50 cm (length). SMPK eluted with 10 mM diammonium citrate at pH 5.3. Fractions of 4.3 mL were collected, and four or five fractions with the greatest PK activity were pooled. The pooled eluent was loaded onto a Bio-Rad CM Bio-Gel A column with bed dimensions of 1 cm (internal diameter) × 8.5 cm (length). SMPK eluted in 1.0 mL fractions with 10 mM diammonium citrate at the front of a 0 to 1 M NaCl gradient. From 32.1 units/g tissue wet weight of pyruvate kinase activity, this procedure produced SMPK with 390-fold purification from 0.0875 unit/mg of protein to 34.4 units/mg of protein activity.

Capillary Electrophoretic SKPK−MMCK Coupling. The CE measurement of kinase coupling was made using an ISCO model 3850 electropherograph. Coupling is assessed by injecting both molecules separately or together and measuring the concentration-dependent size of a new peak or a change in the size of one of the peaks (*8*). The solutions were vacuum injected for 5 s into a 98 cm long, 100 μm diameter fused silica glass capillary. The samples were driven using 20 kV toward ground using the 9.5 mM phosphate/5.3 mM citrate solution as the carrier buffer, producing a current of 43 μA. The detection window was at 68 cm from the injection site, and the absorbance at 195 nm was recorded on a strip chart recorder. The relative size of the peaks was compared using their areas.

PK Activity Measurement. PK activity was measured as a linked assay with lactate dehydrogenase (LDH). The activity assays were carried out in 4 mL cuvettes using a Spectronic 21D set at 340 nm for measurement of changes in NADH absorbance. The assay was prepared by adding 2.7 mL of a solution of 50 mM imidazole, 120 mM KCl, and 62 mM $MgSO_4$, setting the absorbance to zero, and adding 0.3 mL of a substrate solution containing 1.5 mM ADP, 1.5 mM PEP, and 240 μM NADH, followed by the addition of 10 μL (0.0135 unit) of LDH solution. The change in baseline was not significant even when the solutions were allowed to stand for 30 min. The assay was started by adding 10 μL of a PK sample solution and the change in absorbance recorded. An enzyme activity unit is defined as the amount of enzyme that converts 1 μmol of substrate per minute for the isolation, and one absorbance unit per minute for the complexing experiments. The assays were carried out in a volume of 3 mL at pH 7.4 and 37 °C. Statistical differences were calculated using a two-tailed *t* test with differences considered significant at the $p < 0.05$ level.

RESULTS

The time course of the mixing of SKPK and MMCK at 340 nm was determined. The sample had the two kinases mixed together, and the absorbance exhibits a steady increase with an exponential time constant of 29.5 min ($R^2 = 0.90$). The sample was mixed twice during the absorbance measurement. There was no change in the absorbance following either mixing.

The spectrum of the SKPK−MMCK complex is shown in Figure 1. The combined PK−CK solution is a mixture of the two separate PK and CK solutions. The expected absorbance would be between the individual PK and CK absorbances. Over a broad spectrum, the combined kinases yield an absorbance far greater than the predicted absorbance. At wavelengths below 240 nm, there is a large increase in all absorbances. When the concentration dependence of the extra absorbance was measured at 340 nm (Figure 2), increasing concentrations of MMCK with 0.4 mg/mL SKPK reached a plateau. Figure 2 also exhibits the extra absorbance at 340 nm of SMPK as the concentration of BBCK increases. This pair does not exhibit a similar increase in absorbance as that seen with SKPK and MMCK.

Figure 3 shows a concentration-dependent decrease in the absorbance of the combined proteins when compared with the absorbance expected from the individual measurements of SKPK and MMCK. When MMCK was combined with SMPK, no similar concentration-dependent change in absorbance occurred. Thus, neither BBCK (Figure 2) nor MMCK (Figure 3) was able to significantly alter the absorbance of SMPK. At 195 nm, the combination of SKPK and MMCK exhibits a lower than expected absorbance (Figure 3), while above 230 nm, the combination exhibits a higher than expected absorbance which is especially marked around 250 nm and above 300 nm (Figure 1).

A method for measuring the interaction of pairs of molecules using capillary electrophoresis has recently been developed (*8*). This technology was applied to the SKPK−MMCK combination (Figure 4). Using the same mixing conditions that produced the change in absorbance, the combined proteins were compared with electrophoretic runs of the individual proteins at 195 nm. Both individual proteins required similar amounts of time to reach the detection window. Figure 4 shows typical runs where the MMCK- and SKPK-independent run times can be compared. If two compounds run in the same place, and do not interact, their absorbance will be additive. This is not the case with MMCK and SKPK. As seen in Figure 4, the combined proteins exhibit a marked decrease in absorbance compared with the individual runs. The mixed proteins produced an absorbance area far smaller than even the MMCK peak alone. This finding is consistent with the absorbance change produced by the SKPK−MMCK combination at 195 nm in zero electric field (Figure 3). When the concentration dependence of the combination compared with the individual CE runs was measured, the absorbance of the combined proteins compared with the MMCK peak was consistently smaller using CK concentrations ranging from 0.1 to 2.0 mg/mL. The decreased absorbance was more pronounced as the CK concentration increased.

Figure 5 shows the effect of increased ethanol concentrations on the activity of PK in the presence and absence of

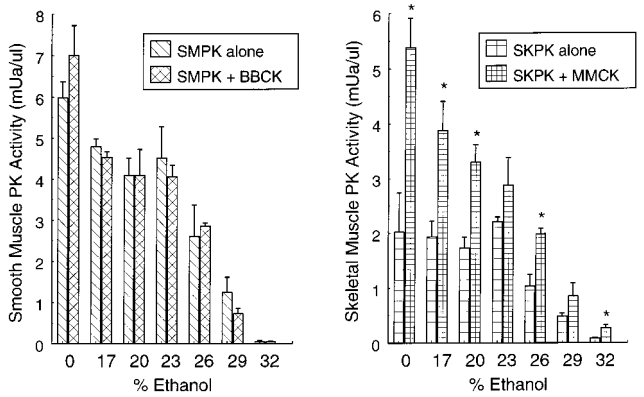

FIGURE 5: Comparison of smooth muscle (left diagram) and skeletal muscle (right diagram) PK activity in the presence and absence of BBCK (for smooth muscle) and MMCK (for skeletal muscle) over a range of ethanol concentrations. The asterisk denotes a significant difference at $p < 0.05$ between PK with CK and PK alone. The solutions were mixed and allowed to stand for several hours before centrifugation. The supernatant was then drawn off and the activity measured. The skeletal proteins produce activity changes not seen with the smooth muscle proteins.

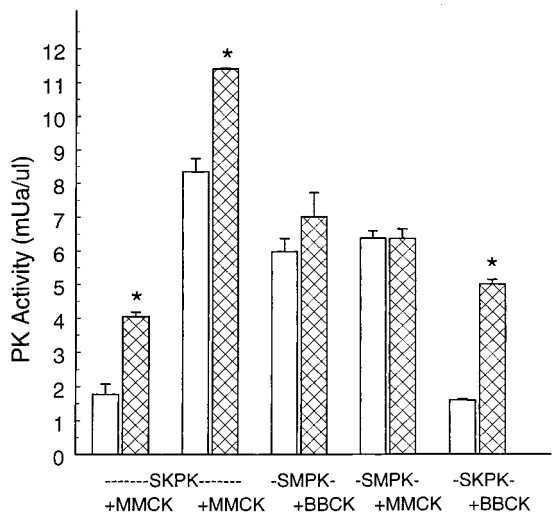

FIGURE 6: Effect of MMCK and BBCK on the enzymatic activity ± SD of both SKPK and SMPK. The asterisk denotes a significant difference at $p < 0.05$ between PK with CK and PK alone. The experiment was conducted like the ethanol experiments. No ethanol was added, but the activities were measured several hours after mixing. SKPK was used at two different concentrations to span the SMPK activity. Both enzymes were in their linear range. The increased activity seen with SKPK but not SMPK is therefore not due to the activity range of the PK. Note that BBCK, which will not see SKPK in vivo, is able to produce a large increase in SKPK activity.

the CK normally present with the particular PK. While CK has a high ethanol solubility (*2*, *5*), PK precipitates in the ethanol range used in the experiments whose results are depicted in Figure 5. For the smooth muscle proteins, the SMPK activity fell as the ethanol concentration increased. The presence of BBCK had no effect on SMPK activity at any ethanol concentration, including zero ethanol. In contrast, MMCK caused a substantial and significant increase in SKPK activity over a wide range of ethanol concentrations, including zero ethanol.

The influence of each CK on the activity of each PK was measured and is reported in Figure 6. Similar to the findings in Figure 5, MMCK produced a significant increase in SKPK activity from original concentrations giving 2 and 8 milliunits of absorbance/μL. The activity of these SKPK experiments bracketed the comparison of SMPK using 6 milliunits of absorbance/μL with and without BBCK present. BBCK, also similar to the data in Figure 5, did not produce a significant increase in SMPK activity. Likewise, MMCK, present in small amounts in smooth muscle (*5*), did not produce any change in the activity of SMPK. In sharp contrast, BBCK was able to significantly increase the enzymatic activity of SKPK. BBCK and SKPK are never present in the same muscle cells.

DISCUSSION

The experiments here consistently demonstrate that SKPK coupled with MMCK, showing the time course of the interaction, its frequency dependence, its concentration dependence in multiple conditions, and its influence on PK kinetics. In all the experiments presented here, including CE experiments, we used solutions that had been made more than 1 h before the experiment. Conversely, the time course data only show the effect for absorbance.

The change in absorbance in the presence of both SKPK and MMCK also indicates the nature of the complex. The change in absorbance can be seen throughout the spectrum and is increased through most of the spectrum. The data suggest that there are effects of scattering and absorbance by the complex. Since solutions become cloudy, light scattering is expected. The specific changes in light absorption are also important; the increase in measured absorption is not smooth and can best be seen around 250 nm and above 310 nm, and a decrease is measured at 195 nm (Figure 3). The reversal of the absorbance change is indicative of specific protein conformational changes rather than light scattering alone. The plateau phase in Figure 2 is consistent with there being a limited number of binding sites for MMCK on SKPK. Because MMCK is a dimer and SKPK is a tetramer, estimation of a binding constant from experiments such as that in Figure 2 is tenuous, and was not made. Since the saturation of 0.4 mg/mL (monomer concentration of 7 μM) SKPK is fairly complete at 1.35 mg/mL (monomer concentration of 33 μM) MMCK, a 1:1 monomer:monomer binding is a strong possibility. Since SKPK is a tetramer and MMCK is a dimer, a 1:1 monomer binding may lead to polymers forming. The potential for multiple binding on each protein makes direct calculation of their stoichiometry beyond the range of these experiments. The rate constants of the dissociation, the steric effects of the complex on further binding, and the allosteric effects of complex formation on subunits not directly involved in the binding determine the equilibrium state of the complex in the experiments. In vivo, the presence of membranes, other glycolytic enzymes, and the level of glycolytic flux may also be important in determining the stoichiometry. The reversal of the absorbance change, i.e., the complex absorbance decreasing at 195 nm, indicates a specific interaction must be occurring. The decrease at 195 nm would occur when a specific group is involved in complex formation. This is also consistent with other proteins being unable to form similar complexes (*2*), and with SMPK being unable to form complexes, as reported here. The ability of MMCK to saturate the absorbance of PK at 340 nm is a further indication of the specific nature of the complex formation. In any case, the presence of SKPK

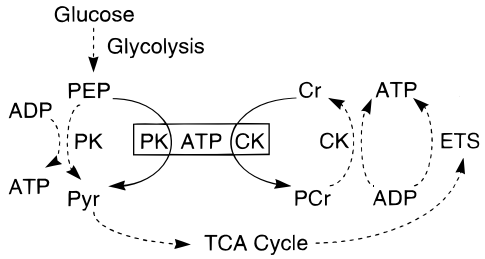

FIGURE 7: Model for the transfer of phosphate energy from PEP to PCr by way of a diazymatic complex of SKPK and MMCK. The model shows the possible transfer of ATP via the PK−CK complex as well as the classical release of ATP in solution. Complex formation provides a means of glycolytic product removal leading to increased flux during high energy utilization. Also, the PK−CK complexes may be used to maintain PCr and hence ATP in particular pathways.

with either CK will produce a change in absorbance which will make estimates of protein concentration from the absorbance difficult and tenuous.

The assessment of the complex formation using capillary electrophoresis is very difficult using kinases. Kinases stick to the capillary under conditions when other proteins, including a variety of antibodies, as well as insulin, run through easily. The kinases can pass in a restricted pH range, from 6 to 6.7, which fortuitously is also the range in which the coupling of SKPK and MMCK occurs most strongly (*2*). The concentration of MMCK is not high enough to produce saturation of the SKPK in the 204 V/cm electric field. With a stronger electric field, a higher concentration of metabolites is needed to reach the saturation level (*8*). The demonstration of SKPK−MMCK coupling in an electric field is further evidence of the robust nature of their association.

The concentrations of PEP and ADP in skeletal muscle do not rise sufficiently to account for the increased flux through PK that can occur during muscle activation (*6*). There must be an alternative mechanism assisting flux through PK. MMCK may provide such a mechanism. Experiments here show that MMCK can significantly increase the specific activity of SKPK, using concentrations of proteins that are much lower than the 40 mg/g of MMCK and 29 mg/mL SKPK (*9*) present in skeletal muscle. Control of the specific activity of PK may be a mechanism of flux control. Given the coupling of SKPK and MMCK, shown by the many experiments both here and in previous work, the model of their interaction depicted in Figure 7 is reasonable. Under uncoupled conditions, with no PK−CK interaction, energy in the form of ATP is produced by glycolysis (of which PK is a part), the TCA cycle, and finally the electron transport system (ETS). In skeletal muscle, much of the energy of the phosphate bonds is then stored as phosphocreatine. The reactions responsible for the storage and release are both catalyzed by CK. This path is summarized by the dashed lines in Figure 7. If PK and CK form a complex that can exchange phosphate, a new route is created. Saturation transfer experiments (*1*) showed that bound ATP is an intermediate in the transfer of phosphate from PEP to creatine. This is consistent with the active sites for both kinases having an SN2 type substitution transfer reaction, where the metabolites (pyruvate and adenosine, or adenosine and creatine) must be present for the transfer to occur (SN2), rather than an elimination reaction in which the phosphate remains attached to the kinase without either metabolite present. Since ADP is readily available for the acceptance of the phosphate from PEP, this provides no difficulty. During muscle activation, there will be a significant increase in the level of free creatine, creating a stronger driving force for the transfer of phosphate by a diazymatic (*1*) rather than direct enzymatic reaction. This increased availability of creatine and the activation of SKPK by MMCK may account for the increased flux through PK during activation in the absence of sufficient changes in the concentrations of PEP and ADP (*6*). This new pathway is shown as the solid lines in Figure 7. This transfer pathway may be an extension of glycolytic enzyme coupling (*10*) and an example of the more general concept of metabolic channeling that has grown in recent years (*11*−*13*). Specific binding of BBCK and enolase has been proposed in neurons (*14*). Additional protein complexes may play significant physiological roles.

Because SKPK can couple to BBCK, a molecule it would not be in contact with physiologically, this indicates the special nature of the SKPK molecule. The ability of SKPK to couple would provide a significant evolutionary advantage, since energy flux could now be accomplished more readily. Smooth muscle, with SMPK, would lack the ability to form a PK−CK diazyme, despite having both BBCK and MMCK. The possible formation for other kinase complexes, such as PK and arginine kinase, remains an intriguing possibility. MMCK may have two effects in the presence of ethanol, increased SKPK solubility, noted previously (*2*), and a direct increase in the activity of SKPK. If the MMCK effect on ethanol solubility is indicative of its effect in other hydrophobic environments, the absence of an ethanol effect of BBCK on SMPK may reduce the likelihood of one possible mechanism for the membrane association of SMPK and of other glycolytic enzymes in smooth muscle (*3*, *4*). Since PK has a low solubility in a nonaqueous environment, it is possible that the formation of a SMPK−BBCK or SMPK−MMCK complex, similar to that of SKPK−MMCK (*2*), could increase the PK solubility at the membrane. While this possibility is not entirely ruled out, the ethanol experiments showing no change in SMPK activity, as well as the absence of any evidence of SMPK−CK coupling, make CK-assisted PK association with a membrane less likely.

In summary, there is significant evidence that SKPK can physically and kinetically couple with creatine kinase, either MMCK or BBCK. This coupling is transitory, but can increase the flux through PK by transferring newly formed phosphate bonds to creatine. Smooth muscle PK does not exhibit any of these characteristics.

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