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SHORT COMMUNICATION

ARGININE KINASE AND CREATINE KINASE APPEAR TO BE PRESENT IN THE SAME CELLS OF AN ECHINODERM MUSCLE

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Phosphagens are phosphorylated guanidine compounds which, *via* the catalytic action of corresponding phosphagen kinases, function as temporal and possibly spatial ATP buffers (Meyer *et al.* 1985) in cells capable of dramatic metabolic transitions such as muscle, neurons and spermatozoa. At least eight different phosphagen kinases have been identified and these enzymes are widely distributed throughout the animal kingdom (see excellent reviews by Van Thoai, 1968; Watts, 1968).

One puzzling feature about the distribution of phosphagen kinases is the occasional presence of more than one phosphagen type in a given tissue, the so-called 'pluriphosphagen' phenomenon (Robin, 1964). For instance, many species of polychaetes, echinoids and tunicates contain two or more phosphagen systems in their muscles (Needham *et al.* 1932; Yudkin, 1954; Robin, 1964, 1980; Van Thoai *et al.* 1964; Watts, 1975). The situation in sea urchin lantern muscles is quite striking in that certain species contain both arginine kinase (AK) and creatine kinase (CK), while others, all relatively primitive echinoids, contain AK only (Yudkin, 1954; Morrison *et al.* 1967; Ratto *et al.* 1989). All asteroids and ophiuroids contain CK in muscle, while the more primitive echinoderm classes, holothuroids and crinoids, contain AK only (Ratto *et al.* 1989). The distribution of phosphagen kinases in the echinoderms shows that CK is present in the more advanced forms, implying some kind of evolutionary trajectory. As Ratto *et al.* (1989) put it '... echinoderms are displaying a transition from one enzymatic system to another, and that simultaneous expression of both enzymes is in fact a still picture of a long-term evolutionary process'.

Recently, we showed that the phosphagen kinase reactions are not thermodynamically equivalent (Ellington, 1989). That is, the apparent equilibrium constant for the AK reaction (K'_{apk}=[arginine][ATP]/[arginine phosphate][ADP]) is only 13 % of K'_{cpk}. The K' values for the lombricine, glycocyamine and taurocyamine kinase reactions are intermediate (Ellington, 1989). These differences have

Key words: *Strongylocentrotus purpuratus*, arginine kinase, creatine kinase, muscle, echinoderm.

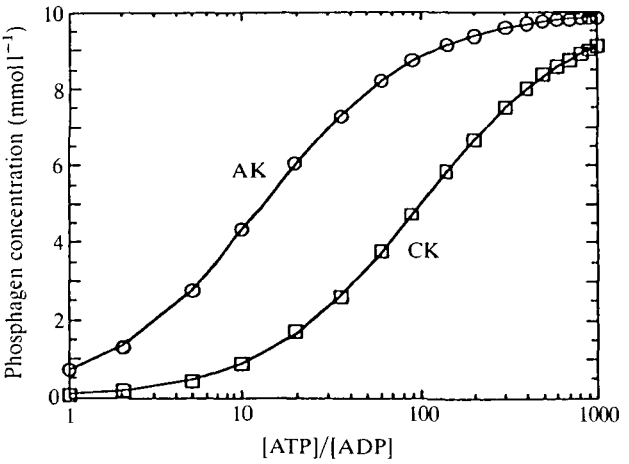

Fig. 1. Equilibrium concentrations of creatine phosphate and arginine phosphate at various [ATP]/[ADP] ratios based on the thermodynamic properties of the CK and AK reactions (Ellington, 1989). Total creatine (creatine phosphate+creatine) and arginine (arginine phosphate+arginine) pools were both set at 10 mmol l^{-1}.

important functional consequences, as is evident from Fig. 1 where the equilibrium concentrations of phosphagen in the AK and CK reactions are plotted against the [ATP]/[ADP] ratio. The steep portion of the CK equilibrium curve lies in a much higher range of [ATP]/[ADP] ratios than that of AK (Fig. 1). Thus, the CK reaction functions as a much more effective ATP buffer system than the AK reaction (Ellington, 1989) in that it can buffer ATP at much higher [ATP]/[ADP] ratios.

Given the dramatic thermodynamic differences between CK and AK, it is hard to rationalize the presence of both reactions in the *same* cells. Watts (1975) suggested that AK and CK might be present in different cells in echinoid lantern muscle. We chose to investigate this possibility further in lantern protractor muscle of the Pacific coast sea urchin *Strongylocentrotus purpuratus* (obtained from Marinus, Long Beach, California). Crude, cell-free extracts of protractor muscle, assayed using standard spectrophotometric assays (Ellington, 1989), contained similar CK and AK activities, 45.42±7.73 and 34.01±4.19 μmol min^{-1} g^{-1} wet mass, respectively, at 25 °C (mean±1 S.D., N=4). The CK/AK activity ratio was 1.327±0.070. Cellulose acetate electrophoresis showed that these two activities were catalyzed by *different* proteins (bands were more than 2 cm apart on a standard gel).

Given the nearly equivalent CK and AK activities present in *S. purpuratus* lantern protractor muscle, we next chose to localize CK activity in tissue sections by indirect immunofluorescence using polyclonal anti-human CK (Ventrex, Portland, Maine, lot no. HLP53–0179). It was first necessary to assess the specificity of the antibody preparation with respect to protractor muscle proteins. A crude muscle extract was subjected to denaturing, SDS–polyacrylamide ge

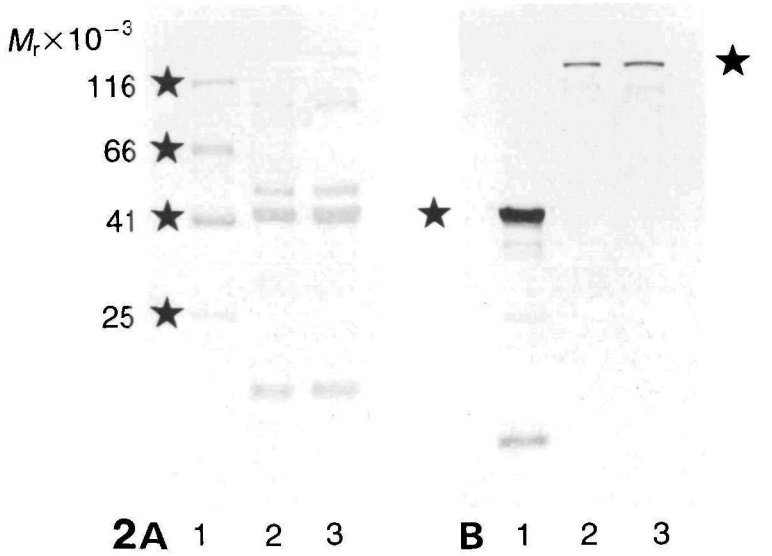

Fig. 2. Transfer blots on nitrocellulose membranes stained for general protein (A) and immunoreactivity with anti-human CK antisera (B). (A) Lane 1 contains relative molecular mass standards (positions indicated by stars) of β-galactosidase (116×10^3), bovine serum albumin (66×10^3), rabbit CK (41×10^3) and chymotrypsinogen (25×10^3); lanes 2 and 3 contained 2.5 and 5 μl, respectively, of an extract of *Strongylocentrotus purpuratus* muscle. (B) Lanes 1–3 were identical in composition to lanes in A. Stars indicate positions of intense immunoreactivity in lane 1 and lanes 2 and 3 (B). Muscle tissue was extracted in 15 mmol l^{-1} Tris/HCl, 15 mmol l^{-1} Hepes, 150 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$ and 0.05 % nonidet 40 (pH 8). Homogenates were 10 % (w/v).

electrophoresis (PAGE) according to Laemmli (1970) using a Hoefer mini slab gel device. The proteins were then transferred to a nitrocellulose membrane using a BIORAD Mini Transblot device. The resulting blot was stained with the reversible stain Ponceau S to reveal proteins (Fig. 2A). The blot was then incubated in 10 % non-fat dry milk in 10 mmol l^{-1} Tris/HCl (pH 7.4), 150 mmol l^{-1} NaCl and 0.05 % Tween 20 (Tris-buffered saline, TBS) for 45 min followed by three 8 min wash cycles with TBS. The blot was then incubated for 60 min with 10 % non-fat dry milk and TBS containing a 1:2000 dilution of the rabbit anti-human CK antisera. Following three wash cycles, the blot was incubated for 60 min with 10 % non-fat dry milk and TBS containing a 1:5000 dilution of goat anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, Indiana). After three wash cycles, the blot was incubated in alkaline phosphatase reaction mixture consisting of 25 mmol l^{-1} Tris/HCl (pH 9.5), 25 mmol l^{-1} NaCl, 100 mmol l^{-1} $MgCl_2$, 165 μg ml^{-1} 5-bromo-4-chloro-3-indolylphosphate and 330 μg ml^{-1} nitroblue tetrazolium. After development of dark blue bands, the

reaction was stopped by placing the blot in 20 mmol l^{-1} Tris/HCl (pH 8) containing 4 mmol l^{-1} EDTA.

The immunoblots (Fig. 2B) show that the rabbit anti-human CK reacts strongly against rabbit CK (lane 1) and against a band in the lantern protractor muscle extract (lanes 2 and 3). This immunoreactive band ran somewhat slower than β-galactosidase, which has a relative molecular mass of 116×10^3. Echinoderm CKs range in relative molecular mass from 140×10^3 to 155×10^3 (Ratto *et al.* 1989). Given the position of the immunoreactive band in our lantern muscle extracts (Fig. 2A,B), and by comparison with the immunoblots of Ratto *et al.* (1989), it is clear that the anti-human CK antisera reacts specifically with CK in *S. purpuratus* lantern muscle.

Lantern muscle was fixed for immunofluorescence while still attached to the calcareous test. This was accomplished by cutting away the test except for a 1 cm wide portion surrounding the jaw apparatus. The attached muscle was fixed for 6 h at 4°C in 4% paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer (PB) (pH 8). The fixed muscle was dissected from the test and cryoprotected by serial incubation in 10%, 20% and then 30% sucrose in 0.1 mol l^{-1} PB (pH 8). Subsequently, 10 μm thick frozen cross sections were prepared and placed on glass slides (stored at −60°C).

Indirect immunofluorescence was then conducted. Sections were first incubated in 10% non-fat dry milk, 3% normal donkey serum and 0.4% Triton X-100 in 0.1 mol l^{-1} PB (pH 7.2) (block solution) for 60 min at 4°C followed by three 8 min wash cycles with 0.1 mol l^{-1} PB (pH 7.2). The slides were then incubated in 1:100 dilution of the rabbit anti-human CK antiserum in block solution for 2 h at 4°C followed by three 0.1 mol l^{-1} PB (pH 7.2) washes. Slides were then incubated in 1:50 dilution of donkey anti-rabbit IgG–fluorescein conjugate (Jackson Immunoresearch Lab, West Grove, Pennsylvania, lot no. 13985) for 60 min followed by three washes. Sections were stabilized with a mounting medium consisting of 90% glycerol, 10% 0.01 mol l^{-1} PB, 0.15 mol l^{-1} NaCl and 0.1% paraphenylenediamine. Fluorescence microscopy was conducted using a Zeiss microscope and photographs were taken using Kodak Tri-X film.

Sections that had been incubated with block solution containing rabbit anti-human CK antisera showed uniform fluorescence when incubated with donkey anti-rabbit IgG conjugated with fluorescein (Fig. 3A,B). In contrast, sections incubated with block solution only followed by the fluorescein–antibody conjugate showed minimal fluorescence (Fig. 3C,D). These data strongly suggest that CK is present in *all* protractor muscle fibers. Furthermore, given the similar CK and AK activities in this tissue, it is highly likely that CK and AK coexist in some, if not all, of the cells.

If CK and AK are present in the same compartment and catalyze near-equilibrium reactions, and the total creatine and arginine pools are similar, then at physiological [ATP]/[ADP] ratios, arginine phosphate (AP) should be present at higher levels than creatine phosphate (CP) (Fig. 1; also see Ellington, 1989). We chose to investigate this possibility by looking at phosphagen levels in superfused

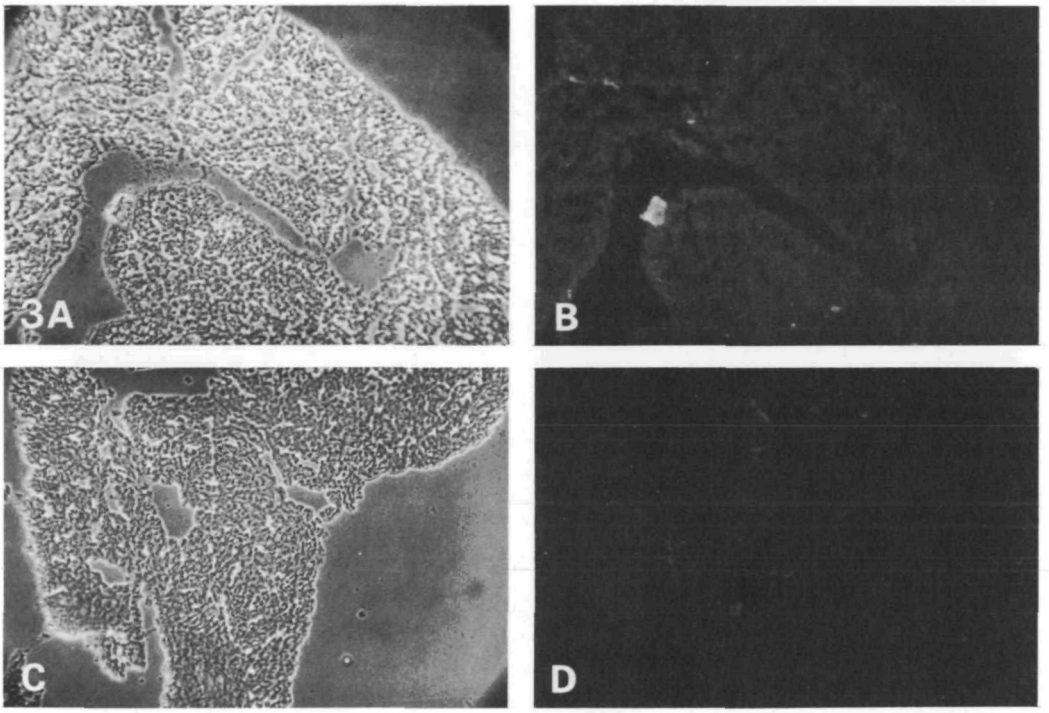

Fig. 3. Photomicrographs of cross sections of *Strongylocentrotus purpuratus* lantern protractor muscle taken using a 16× objective. (A) Section incubated with the primary antibody (anti-human CK) and then the secondary antibody–fluorescein conjugate, viewed in phase contrast. (B) Same section as in A but viewed in fluorescence. (C) Section incubated with block solution and then the secondary antibody–fluorescein conjugate, viewed in phase contrast. (D) Same section as C but viewed in fluorescence. A 3 min exposure time was used in the fluorescence photomicrographs.

in vitro preparations of lantern protractor muscle *via* phosphorus n.m.r. (^{31}P-n.m.r.) spectroscopy. Single muscle bundles were dissected still attached to skeletal components, mounted in a custom-fabricated 5 mm ^{31}P-n.m.r. probe and superfused at 20°C with MBL formula artificial sea water buffered at pH 7.8 with 10 mmol l^{-1} Hepes. Data were acquired at 109 MHz using a Bruker/IBM spectrometer in the Fourier transform mode. Spectra consist of the summation of 750–1200 data acquisitions (3.5 μs pulse width, 5000 Hz sweep width, 2 s pulse interval). Under the above acquisition conditions, CP and AP were both fully relaxed.

^{31}P-n.m.r. spectra of *S. purpuratus* lantern protractor muscles showed typical phosphate resonances [inorganic phosphate (Pi), phosphagens and γ, α- and β-ATP]. Pi levels were quite low and remained essentially constant for up to 4 h, indicating that these preparations were in good physiological condition (data not shown). CP and AP were present in all seven, independent muscle preparations investigated (Fig. 4). However, in contrast to expectations, CP levels were

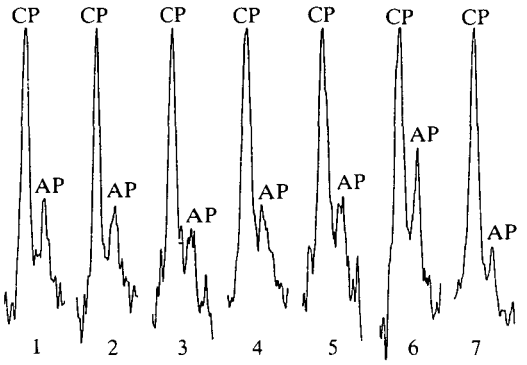

Fig. 4. ^{31}P-n.m.r. spectra of seven different preparations of *Strongylocentrotus purpuratus* lantern protractor muscle showing the portion of each spectrum containing the creatine phosphate (CP) and arginine phosphate (AP) resonances.

consistently higher than AP levels as shown by peak height (Fig. 4). The higher CP levels might be explained by the possibility that the total creatine pool in protractor muscle is much higher than the total arginine pool. Neutralized, perchloric acid extracts of protractor muscles were prepared, brought to 1.5 mol l^{-1} HCl and heated at 100 °C for 3 min to hydrolyze CP and AP. The samples were lyophilized, dissolved in 0.5 ml 100 mmol l^{-1} Tris/HCl (pH 8) and assayed for creatine and arginine by enzyme-linked, spectrophotometric assays (Lowry and Passonneau, 1972) using CK (Boehringer Mannheim) and AK (purified from horseshoe crab, *Limulus polyphemus*, muscle as described by Doumen and Ellington, 1990). The ratio of [total creatine]/[total arginine] was 1.185±0.391 (mean±1 S.D., N=5), indicating that differences in pool sizes cannot explain the much higher CP levels in this system.

On balance, it appears that there is strong evidence for the coexistence of CK and AK reactions in cells of *S. purpuratus* lantern protractor muscle. However, these reactions do not appear to be in equilibrium with each other and the adenylate system. This may be due to the fact that the CK and AK reactions exist in separate microcompartments within the cells or that AK activity is somehow suppressed *in vivo*. We have no evidence for subcellular compartmentation. Alternatively, a subpopulation of muscle cells may exist that has very high AK activity relative to CK activity while the remaining cells contain no AK. However, we consider this unlikely as the indirect immunofluorescence against CK shows no evidence for cellular heterogeneity. In any event, the *functional* significance, if any, of this 'pluriphosphagen' phenomenon remains obscure.

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