CREATINE PHOSPHOKINASE: IN VITRO ACTIVITY MODIFICATION AND PROTEOLYSIS WITH CALPAIN.

By

GAVIN. D. ARTHUR

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Department of HUMAN KINETICS.

The University of British Columbia
Vancouver, Canada

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ABSTRACT

The purpose of this study was to determine if the muscle isoform of the energy transmitting kinase; Creatine Phosphokinase (CPK - EC N^{o} 2.7.3.2) MM-isozyme, is a substrate for calcium activated neutral protease (CANP - EC N^{o} 3.4.22.17). CPK activity was measured under three different conditions: (1) control assay; (2) with 5mM Ca^{2+},($5x10^{-3}$ Ca^{2+}); (3) with 5mM Ca^{2+} and a range of CANP amounts from 10 to 100ug. 5mM Ca^{2+} consistently caused significant inhibition of the CPK activity to 36% of control ($p<0.05$). In the presence of 5mM Ca^{2+} and 10ug of CANP, CPK activity was not significantly different from the control activity. With 27ug CANP there was a slight but significant activation of CPK to 123.18 + 12.9% above the control activity ($p<0.05$). As the amount of CANP present was increased to 54, 67, 84 and 100ug, the CPK activity was reduced to 56.96 + 0.31%, 50.46 + 2.65%, 36.06 + 0.5%, and 2.08 + 2.56% respectively.

SDS-PAGE showed that significant proteolysis of CPK occurred with a range of CANP from 10 to 30ug. Densitometric scanning of the CPK band and the 28kDa CANP subunit showed that proteolysis of CPK was dependent on the amount of CANP present. The proteolysis of CPK resulted in the formation of two large fragments. The molecular weight of these proteolytic fragments were estimated to be 38 and 35kDa. The results of this study show that CPK is a substrate for CANP in vitro and that minor proteolysis results in activation of CPK, while increased proteolysis results in loss of activity.

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INTRODUCTION

Statement of the problem

Calcium activated neutral protease, also called calpain or CANP, and its specific inhibitor calpastatin is a non lysosomal proteolytic system found in mammalian cells. CANP is a cysteine protease which is present in a wide variety of tissues including mammalian skeletal muscle and has a pH optimum from 7 to 8. CANP is known to exist as two isozymes, both isoforms having a large subunit (80-82kDa) which contains four distinct domains. Domain four at the COOH terminal has a calmodulin like binding domain and this region appears to define the Ca^{2+} dependance of CANP. Each of the isoforms has an absolute requirement for Ca^{2+}, uCANP requires uM Ca^{2+} and mCANP requires mMCa^{2+} concentrations to become active. Both have identical small subunits of 28kDa which are believed to have regulatory roles. CANP undergoes autolytic activation to become fully active and has attracted a great deal of attention recently due to its likely role as a regulatory or processing protease in response to increased intracellular Ca^{2+} concentrations. CANP seems ideally suited for this role because CANP substrates are not degraded but are modified by limited proteolysis. Included among the substrates known to date for CANP are:

Muscle proteins like desmin and C-protein. Cytoskeletal proteins such as fodrin, viementin and neurofilaments. Enzymes, including protein kinase C, phosphorylase b kinase and tyrosine hydroxylase.

CANP's restricted proteolytic activity on its enzyme substrates has been suggested to increase their activity, indeed CANP was once known as "kinase activating factor". CANP mediated proteolysis may be facilitated by binding of the CANP to specific amino acids on its substrates, (Wang, Roufogalis, & Villalabo, 1989b). These sequences are called "PEST" sequences and many of CANP's substrates carry these sequences and CANP cleavage seems to occur at locations at or near these sequences.

Situations where CANP is found to be active within the cell include: Duchenne muscular dystrophy, where CANP activity has been shown to be increased ten fold, (Nagy & Samaha,

1986). In the same condition a 5-6 fold and 3-5 fold increase was measured for mCANP and uCANP respectively, (Reddy, Anandavalli, & Anandaraj, 1986). After prolonged running exercise the activity of CANP is elevated in rat skeletal muscle, (Belcastro, 1993).

As CANP is a Ca^{2+} activated protease then it is not surprising that common to the conditions where CANP is active within the cell are elevated intracellular Ca^{2+} levels. Muscles affected by Duchenne muscular dystrophy show increased intracellular Ca^{2+} levels and prolonged exercise leading to fatigue results in a Ca^{2+} imbalance and a Ca^{2+} overload within the cell, (Croall & DeMartino, 1991). During many types of muscle dysfunction there are disturbances in the enzyme creatine phosphokinase, CPK (E.C.No 2.7.3.2.) Again as an example is Duchenne muscular dystrophy where CPK disturbances are present and the release of CPK into the plasma is actually used to screen for the disease in newborns. In prolonged exercise the CPK system is disturbed and CPK release in this case is used as a marker of muscle damage. In addition to being disturbed during periods when intracellular Ca^{2+} levels are elevated and CANP activity is increased, the MM-CPK enzyme contains "PEST" like sequences that are thought to be recognition sites for CANP. Considering all this, the hypothesis that CPK will be a substrate for active CANP in vitro and proteolysis of CPK will be concurrent with increased CPK activity, seems warranted.

To test this hypothesis the following objectives were proposed.

1: Incubation of CPK with CANP to measure any changes in the activity of CPK.

2: Incubation of CPK with CANP to measure any changes in the enzyme kinetics of CPK.

3: Visualization of any proteolysis of CPK by CANP on SDS-PAGE gels.

Physiological Significance

Any role for CANP mediated modification of CPK could well be significant because CPK plays a major role in energy transmission within the muscle cell. It has been shown to be present at specific sites of energy consumption within the cell such as; the M-line of skeletal muscle, (Turner, Walliman, & Eppenberger, 1973); at the Ca^{2+} ATP'ase on the sarcoplasmic reticulum and Na/K ATP'ase on plasma membranes, (Baskin & Deamer, 1970). CPK is thought therefore to be able to supply the energy required for muscle contraction, Ca^{2+} pumps and Na/K pumps. CPK is also localized at sites of energy production within the cell. CPK is found at the outer side of the inner mitochondrial membrane and also with the complex of glycolytic enzymes at the I-band, (Walliman & Eppenberger, 1985). It does seem that the energy producing and energy consuming sites in the cell rely on these pools of CPK for proper cellular energy transmission, (Walliman & Eppenberger, 1985). Yet there are many conditions where the CPK system is disturbed and if CPK within the cell is a substrate for CANP, then its proteolysis by CANP could be of significance in understanding the events during many types of muscle dysfunction.

Therefore the aims of this study were to determine if CPK was a substrate for CANP in vitro and to determine the effect of CANP on both CPK activity and structure.

Definition of terms

CPK = Creatine Phosphokinase

CANP = Calcium Activated Neutral Protease

Ca^{2+} = Calcium

PCr = Phosphocreatine

SDS-PAGE = Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis

kDa = kilo Daltons

ug = micro grams (10^{-6} grams)

uM = micro molar (10^{-6} molar)

mM = milli molar (10^{-3} molar)

PEST sequence = Amino acid sequence with: Proline (P); Glutamic acid (E); Serine (S); and Threonine (T). PEST scores are determined using the PEST-Find computer programme, (details in Rogers, Wells, & Rechsteiner, 1986).

CPK activity is expressed as Sigma units. One Sigma unit of CPK activity will phosphorylate one millimicromole (nanomole 10-^{9}M) of creatine per minute at 25^{o}C. In this assay the reaction proceeds in the direction of creatine production. For practical purposes empirical conversion in this assay seems warranted. To convert Sigma units to international units of activity (IU = u/mol/min/L) the following regression equation can be used:

IU = 8.3344 X + 0.9. Where X = Sigma units of activity.

Assumptions and Limitations

Results of this study are limited to in vitro conditions.

Ratios of CANP to CPK may not be strictly comparable to those found within the cell.

The pH of the CPK activity assay was constant at 7.5, this is optimum for CANP but above the optimum for CPK, pH is not held constant within living muscle

The temperature was held constant at 37^{O}C during all of the assays. Temperature is not constant within living muscle.

Observations of this study are limited to the mM requiring form of CANP, (mCANP) only. To date no difference has been found between u &mCANP substrates.

Only the MM CPK isozyme was studied.

The BB, MB, and MiMi isozymes of CPK may respond differently to mCANP.

Chapter two

LITERATURE REVIEW

CANP: Discovery

The Calcium activated neutral protease, (CANP), is one of the few well characterized non lysosomal proteolytic systems in mammalian cells. This neutral protease, which shows an absolute requirement for Ca^{2+} for its activity, was discovered by Guroff in 1964 in the soluble fraction of rat brain homogenate, (Guroff, 1964). The second demonstration of CANP was when a protein factor was found in rabbit skeletal muscle that would activate phosphorylase b kinase in the presence of Ca^{2+}, (Meyer, Fisher, & Krebs, 1964). It was named "kinase activating factor". Subsequently, this kinase activating factor was shown to be the same intracellular Ca^{2+} dependent protease that Gurrof had documented, (Huston & Krebs, 1968). A specific inhibitor of calpain was discovered in the late seventies, (Nishiura, Tanaka, Yamoto, & Murachi, 1978). The existence of this specific Ca^{2+} protease inhibitor was also shown by Waxman & Krebs in 1978. This endogenous specific inhibitor protein, now called "Calpastatin", indicated the CANP system to be specifically regulated and controlled within the cell. The discovery of a low Ca^{2+} requiring form of CANP in dog cardiac muscle by Mellgren in 1980 also increased the likelihood that the CANP system was complex and closely regulated, (Mellgren, 1980) Further work by Murachi and co-workers in 1981 also showed that the two CANP's had distinctly different Ca^{2+} requirements and that calpastatin was inhibitory toward both "the micromolar Ca^{2+} requiring CANP, (uCANP or CANP1) and the millimolar Ca^{2+} requiring CANP (mCANP or CANP2)" , (Murachi, Tanaka, Hatanaka, & Murakami, 1981). It seemed that the two forms of CANP and the specific inhibitor protein constituted an intracellular regulatory system which was regulated by Ca^{2+} levels. The two different calcium sensitive forms of CANP and their specific inhibitor differ from tissue to tissue, both in relative and absolute amounts. It was thought likely that these different amounts could well reflect a different physiological role for CANP

in each cell or tissue containing it. The increasing interest in the CANP system resulted in the analysis of CANP structure in the 1980's.

Structure

The two isoforms of CANP both have a large (80-82kDa) subunit and a small (29kDa) subunit. The larger subunit of the CANP's are genetically different proteins while the small subunits are from the same gene, (Suzuki et al, 1988). The large uCANP & mCANP subunits have the same fundamental structures and share 50% sequence identity. Suzuki and co-workers elucidated the primary structure of the 80 kDa protein and showed it to have four main domains, (Ohno, Emori, Kawasaki, Kisaragi, & Suzuki, 1984). The 29kDa subunit has two main domains, see figure 1.

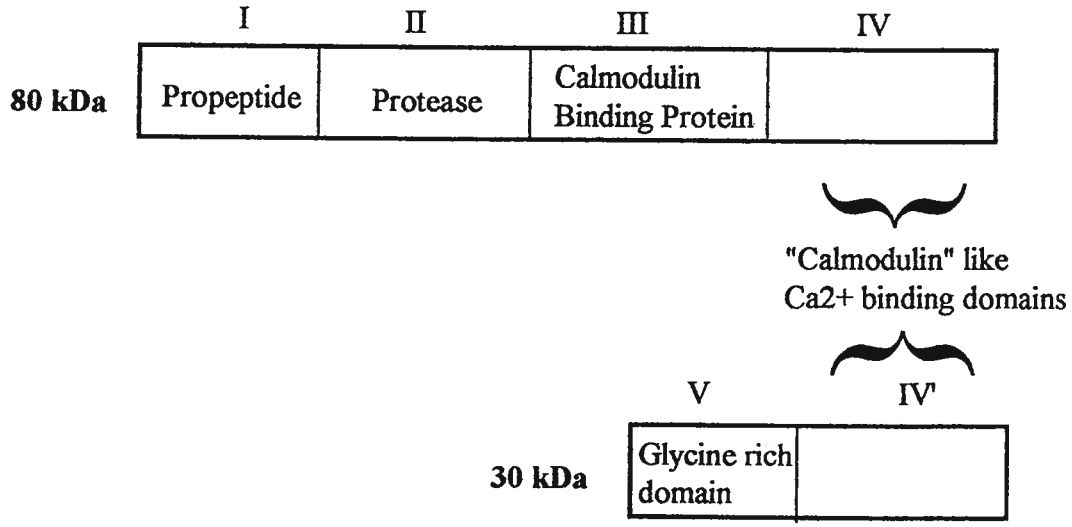


Figure 1: Schematic diagramme of the large and small CANP subunits

Domain two shows a high sequence homology with papain, cathepsins B, H & L. Domain four of each subunit has calmodulin like calcium binding domains, of these four EF hand structures, the first and fourth are active calcium binding sites. A possible reason that CANP requires Ca^{2+} for activity is that domain four interacts with domain two and inhibits its proteolytic ability. When Ca^{2+} binds to the enzyme, domain four undergoes a conformational change which removes this interference with domain two. Participation of calmodulin binding domains or proteins can usually be seen in Ca^{2+} and calmodulin activation of enzymes. Domain three of the CANP molecule is thought to act as a calmodulin binding region and to interact with the Ca^{2+} bound domain four to render domain two free and active. The u and mCANP 80kDa subunits appear to have essentially the same domain two protease, however they do differ in domain four, which is the domain that defines the Ca^{2+} sensitivity of the protease. Domain one can also inhibit the intrinsic protease activity but it is removed during autolytic activation. Two kinds of calpastatin have been found, the most prominent is a 73kDa protein. A 43kDa calpastatin has been found in erythrocytes. The large calpastatin molecule contains four repeating slightly homologous domains of about 140 amino acids. Each of the four repeating domains has inhibitory ability, therefore theoretically one large calpastatin molecule can inhibit up to four CANP molecules, (Maki, Hatanaka, Takano, & Murachi, 1990).

Activation

With Ca^{2+} ions playing a major role in mediating intracellular signalling and regulation through various calcium binding proteins etc, then the question was, "how is this Ca^{2+} dependant intracellular protease activated and its activity regulated within the cell" ? Incubation of CANP in the presence of Ca^{2+} and a caesin substrate initially results in modifications of both the CANP subunits. This modification of the subunits occurs before any proteolysis of the substrate occurs. CANP is therefore an inactive pro-enzyme, (Saido et al, 1992). During the activation process, a 76kDa fragment is formed from the large 80kDa

sub-unit and an 18kDa fragment is formed from the small 30 kDa sub-unit. The N-terminal regions of both the sub-units have been shown to change during this period of autolysis. The large sub-unit is modified first and modification of the small sub-unit follows just prior to the appearance of proteolytic activity. The N-terminal region of the 80kDa sub-unit must be modified before proteolytic activity occurs. Modification of the small sub-unit apparently has no effect on the proteolytic ability of the CANP but seems essential for its interaction with phospholipids, (Suzuki et al, 1988). Because the activation rate of CANP has been shown to be independent of the CANP concentration studied, it is suggested that the autocatalytic method of CANP activation is most likely an intramolecular event rather than an intermolecular one, (Inomota, Kasai, Nakamura, & Kawashima, 1988).

Regulation

The calcium sensitivity of native or "pro-canp" in vitro increases significantly when pre-incubated with Ca^{2+}. This Ca^{2+} sensitivity is generally recognized to be the Ca^{2+} required for autolysis and not the Ca^{2+} level required for proteolytic activity. The autolysed CANP requires lower Ca^{2+} for proteolytic activity and therefore may be active at near physiological Ca^{2+} levels. The question is then: how does CANP become active in vivo if it requires higher than physiological Ca^{2+} levels for activation in vitro ? Coolican and Hathaway in 1984 first reported that phospholipids could significantly lower the Ca^{2+} concentration required for autolytic activation of CANP. This possible mechanism for reducing the Ca^{2+} required for CANP activation was further investigated, (Suzuki, et al, 1988 also, Saido, Mizuno, & Suzuki, 1991). Biological disulphides were reported to reduce the Ca^{2+} required by CANP for its activation, this was suggested as a possible physiological mechanism that could play a role in the regulation of CANP activation, (Sacchetta, Santarone, & Dicola, 1990). Acidic phosholipids were found to lower the Ca^{2+} concentration required for autocatalytic activation, the Ca^{2+} level required was lowered with increased phosphate groups on the phospholipid. With phospatidylinositol-4-phosphate (PIP)

or phosphotidylinositol-4,5-bisphosphate (PIP2), then autocatalytic activation occurred at 10^{-6} to 10^{-7} M Ca^{2+}, a plausible physiological Ca^{2+} concentration. The phospholipids PIP3 or PIP4, recognized as precursors or storage forms of 2nd messenger, may be more effective in assisting the activation of CANP. The amount of PIP2 shown to reduce the Ca^{2+} levels required were within the levels normally found at typical biological membranes. Pro-CANP has been shown to be able to translate to the plasma membrane from the cytoplasm in the presence of uM Ca^{2+} and autocatalytic activation could then occur at the membrane in vivo in rat liver. This possible activation of CANP at biological membranes would suggest that CANP substrates could be membrane associated proteins. However it does not preclude the release of the activated CANP from the membrane to act on cytosolic protein substrates, or that it could be activated at sites other than biological membranes, (Mellgren, 1987). There is evidence to suggest that the specific CANP inhibitor protein calpastatin could be associated with the sarcolemma and possibly the sarcoplasmic reticulum, (Lane, Mellgren, & Mericle, 1985 also Melgren, Lane, & Kakar, 1987). Considering the suggested method of CANP activation at the membrane, then a membrane associated calpastatin is an attractive idea. The calpastatin would be ideally placed to modulate the autoactivation of the CANP and so possibly regulate its action on membrane bound substrates. It was proposed that when the CANP to calpastatin molar ratio is greater than 1:1 then the calpastatin could be removed from its membrane sites, leaving the membrane bound protein substrates open to proteolytic modification by the CANP. The role of the membrane bound calpastatin could therefore be to act as a protective buffer against any brief exposure to elevated Ca^{2+}. Intuitively it makes sense to have this intracellular proteolytic system under some kind of tight spatial control to ensure no unwanted cleavages of substrates within the cell. Examples of some membrane associated events include: The fact that uCANP can be translocated to both the plasma and granule membranes as the inactive "proCANP" and then be activated at the membrane during thrombin stimulated platelet activation, (Kuboki, Ishii, & Kazama, 1990). Another example is the long term potentiation produced from repetitive stimulation of

hippocampal neurons resulting from post synaptic activation of uCANP. A suggestion as to how this occurs is that the pre-synaptic release of glutamine, opens the post synaptic Ca^{2+} channels and the resulting Ca^{2+} influx causes activation of uCANP and then hydrolysis of the membrane cytoskeletal attachment protein fodrin. It is thought that this post synaptic remodelling makes latent glutamate receptors expose themselves to subsequent release of neurotransmitters, (Lynch & Baudryn, 1984). Further evidence for a CANP involvement in this process is that the cysteine protease inhibitor Leupeptin can prevent the increase in glutamate receptors.

Another example of a membrane location for CANP activity is the CANP dependent activation of Protein Kinase C (PKC). CANP has been shown to be able to proteolyze PKC, the rate of cleavage is enhanced by the addition of phospholipid and diacylglycerol. The proteolysis of the 80kDa PKC produces an active 50kDa enzyme fragment requiring neither Ca^{2+} or phospholipid for activity, (Murray, Fournier, & Hardy, 1987 also Melloni et al, 1986). A model of CANP activation and regulation that explains all of the data to date has not been agreed upon.

The membrane activation hypothesis is an attractive one and has been given general widespread acceptance. However it does not accommodate some other experimental findings. Except for the Ca^{2+} required for the proteolytic activity of CANP after autolysis then the Ca^{2+} levels required by CANP for autolysis and proteolysis are higher than the 100-800nM free Ca^{2+} found in vivo (0.1 - 0.8 uM). It may be possible to suggest mechanisms that allow physiological Ca^{2+} levels to initiate autolysis of CANP which requires Ca^{2+} levels of 1-5uM in the presence of phospholipid. An example of this could be transient large increases in Ca^{2+} locally at sites such as Ca^{2+} channels. Immunolocalization studies show CANP and calpastatin are distributed throughout the interior of cells with no preferential location near the plasma membrane, (Kumamoto, et al 1992). Several studies have shown that CANP binds to proteins and not the phospholipids in membrane vesicles, (Kuboki et al, 1990). It has been shown that Ca^{2+} levels required for either u or mCANP

autolysis were not changed by the presence of inside out erythrocyte membrane vesicles, (Zalewska, Thompson, & Goll, 1991). No changes in the ratio of cytosolic to bound CANP was found despite increased overall CANP activity in exercised rat skeletal muscle, (Belcastro, 1993).

Immunolocalization studies show that in addition to plasma membranes, CANP will bind to a whole variety of subcellular structures eg; myofibrills and Z-disks. It could be that some feature of the binding of CANP to its substrate is required to lower the Ca^{2+} required for autolysis, (Melloni & Pontremoli, 1989). However work by Barret, Goll, and Thomson (1991) suggests that in general the substrate has no effect on the Ca^{2+} required by the CANP. The specific role of calpastatin is also unclear in the membrane activation hypothesis. Calpastatin completely inhibits both autolyzed and unautolyzed u and mCANP and does so at Ca^{2+} concentrations below those required for either autolysis or proteolytic activity, (Kapprell & Goll, 1989). Also puzzling is the fact that most cells contain enough calpastatin to inhibit all the CANP that immunolocalization studies show it to be co-localized with. Therefore cells must have a means for regulation of the inhibition of CANP by calpastatin if CANP is to have any role in the cell.

A simple and direct explanation for the various contradictory pieces of evidence regarding the actual and required Ca^{2+} levels could be that there is some other piece to the CANP puzzle. This "piece" could take the form of an "activator" that could enhance the affinity of the CANP-proteolysis binding sites so that Ca^{2+} can bind at physiological concentrations. This could involve a kinase or phosphatase that is activated by a physiological Ca^{2+} influx, the activator may then phosphorylate or de-phosphorylate the CANP, thereby increasing the Ca^{2+} affinity. Such a factor may be analogous to Calmodulin eg; can bind calcium at physiological Ca^{2+} then bind to CANP and change its Ca^{2+} affinity. A 40 kDa protein has been reported in neutrophils and muscle extracts which lowers the Ca^{2+} required by mCANP 50 fold but does not affect the Ca^{2+} required by uCANP. This protein also prevented the inhibition of CANP by calpastatin, (Pontremoli et al, 1990). Another protein

factor has been reported which seems to modify CANP activity, (Shiba et al, 1992). This so called "CANP activator" was purified from human platelets and could increase CANP activity two fold although it did not alter the Ca^{2+} sensitivity of the CANP.
Since the process of CANP activation and regulation can not be explained in a way to encompass all the pieces of evidence to date, it may be that CANP activation and regulation differs depending upon the location and characteristics of the substrate being targeted.

Substrate characteristics

By knowing which proteins are substrates for CANP, then the physiological role(s) of the CANP system should become clearer. Proteolysis of a particular substrate may be dependent on the metabolic state of the tissue and therefore the proteins within the cell. For example, ATP depletion has been shown to stimulate CANP dependent protein breakdown in chick skeletal muscle, (Fagan, Wajnberg, Culbert, & Waxman, 1992). Certain conditions could also make CANP substrates resistant to proteolysis. Covalent modifications of substrates and oxidation reduction status of proteins are suggested to allow targeting by CANP in skeletal muscle, (Belcastro, 1993). Phosphorylation of actin binding protein by cAMP dependent protein kinase increases its resistance to proteolysis by CANP, (Zhang, Lawrence, & Stracher, 1988). Another example is the microtubule associated protein tau, when phosphorylated by cAMP-dependent protein kinase its proteolysis by CANP is significantly reduced compared to un-unphosphorylated tau, (Litersky & Johnson, 1992). These authors suggest a role for phosphorylation in regulating the degradation of tau, and that abnormal phosphorylation may result in a resistant population of tau which could lead to the formation of paired helical filaments found in people with Alzheimer's disease.
A wide range of substrates for CANP have been demonstrated in vitro and despite the advances in knowledge about the structure and the enzymatic properties of purified CANP, its physiological roles are still poorly understood. It is clear that CANP does not act just as a general protease, CANP has specific substrates which it cleaves in a specific and usually

limited fashion. The two different Ca^{2+} requiring isozymes will proteolyze the same proteins in vitro, (Wang et al, 1989b).

Myofibrillar substrates

Initially the CANPs were suggested to be involved in the turnover of myofibrillar proteins, (Reville, Goll, Stromer, Robson & Dayton, 1976). Contractile proteins are known to be co-localized with the CANPs and remodelling or metabolic turnover of these contractile proteins is thought to be one of CANPs main physiological functions. Initial studies on the effect of CANP on muscle myofibrillar proteins showed that mCANP was able to cleave desmin and troponin T rapidly. Troponin I, tropomyosin and C-protein were cleaved more slowly, while actin, myosin and troponin-C were resistant to modification by CANP, (Reddy, Anandavalli, & Anandaraj, 1986). Activation of CANP under physiological conditions during prolonged running exercise in rat skeletal muscle was shown by Belcastro in 1993. The myofibrils from the exercised animals showed increased rates of CANP induced degradation of desmin, vimentin, C-protein, and the removal of alpha-actinin. This study showed that physiologically induced modifications of these CANP substrates due to exercise made them more succseptable to CANP cleavage, (Belcastro, 1993).

The CANPs have also been attributed with the role of disassembly of cell cytoskeleton, especially at sites where the cytoskeletal proteins are attached to the plasma membrane, (Ek & Helden, 1986 also Kay, 1983). This ability of the CANPs to influence the cytoskeletal proteins has been well studied in platelets. The activation of platelets is accomplished by an increase in intracellular Ca^{2+} concentration and this is followed by the specific cleavage of filamen, talin and spectrin, (Barret et al, 1991).

Enzyme substrates

Specific cleavage by CANP also activates or alters a number of different enzymes. An unknown protein factor, later shown to be CANP, was found to activate phosphorylase-b

kinase in the presence of Ca^{2+}, (Myer et al, 1964). This unknown factor was given the name "kinase activating factor". Usually when a kinase or phoshaptase is proteolysed by CANP the remaining large fragments retain their activity, but they are usually now free from the controls that regulated the intact enzyme. An example of this is protein kinase C, (PKC), which is cleaved from an 80kDa protein to a 50kDa protein which no longer needs the co-factors that the 80kDa protein requires for activity, (Murray et al, 1987). Phosphorylase kinase is also activated by mCANP proteolysis, this occurs independently from its normal mode of activation by cyclic AMP and phosphorylation. Another substrate that CANP converts to an irreversibly active form is myosin light chain kinase, which usually requires calmodulin and Ca^{2+} for activation. Some other enzymes which are activated by CANP activity are; Protein activated kinase II, which is converted to an active form by uCANP (Perrisic & Traugh, 1988); as is the D form of glycogen synthase, (Hiraga & Tsuiki, 1986). Calcineurin, a calmodulin dependent protein phosphatase was also found to be a substrate for CANP and was converted to an active, calmodulin independent form. Its activation was concurrent with the formation of a 45kDa protein after cleavage of the original 60kDa protein by uCANP, (Wang et al, 1989a). A specific cleavage by CANP on tyrosine hydroxylase also results in the formation of a large, (56kDa), fragment from the 60kDa original. This removal of the N-terminal end of the tyrosine hydroxylase resulted in a slight but significant activation of the enzyme. Although the list of enzymes that are substrates for CANP is growing, only selected enzymes are substrates for CANP. Obviously these enzymes have some common feature to set them apart for recognition by CANP.

PEST sequences

What makes certain proteins recognizable substrates for CANP ? Rogers and co-workers examined the amino acid sequences of ten proteins with intracellular half lives of less than 2 hours. Common to each protein was at least one region rich in proline (P), glutamic acid (E), serine (S) and thereonine (T), now known as "PEST" regions. The analysis of another

group of 35 longer lived proteins showed that only 15 of these contained these so called PEST regions. Two types of PEST regions have been defined, determined on how closely related they are to the above amino acid sequence. These scores can be greater than zero or "strong pest regions", or they can be between -5 and zero, or "weak pest regions". Of the group of 35 longer lived proteins, only 3 of them contained strong PEST regions. Rogers suggested that as well as conferring the property of rapid degradation to these proteins, the negatively charged and phosphorylatable PEST regions could bind Ca^{2+}. This localized increase in Ca^{2+} concentration could possibly activate CANP, (Rogers et al, 1986). PEST regions are very hydrophilic and are thought to form surface loops on the protein, these would be easily accessible to CANP, which would then cleave the protein nearby, (Rechsteiner, Rogers, & Rote, 1987). The fact that many calmodulin binding proteins are known to be substrates for CANP prompted a search for PEST like regions in these proteins, (Wang et al, 1989b). The substrates for CANP generally showed the presence of PEST like regions located at or near the site of cleavage by the CANP. These included; Insulin receptor substrate-1 which contains PEST like sequences and is degraded by CANP from 170kDa to 90 & 79kDa fragments, (Smith, Bradshaw, Croall, & Garner, 1993). Plasma membrane Ca^{2+} ATP'ase, where the C-terminal end of the protein showed PEST like regions near the sites of CANP cleavage, (Wang, Roufogalis, & Villalabo, 1988). u&m CANP also cleave alpha-fodrin (240kDa) selectively to produce a specific fragment (150kDa). It as been shown that location of the CANP cleavage on the fodrin is in the same area as the only PEST like region in the protein. The CANP cleavage site on protein kinase C has also been found to be in the same area as the region containing the pest like sequence. Another enzyme substrate for CANP proteolysis is HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis. It contains two PEST like sequences at approximately 40 and 47kDa from the N-terminal, (Chin et al, 1984). The CANP cleavage sites are found at approximately 35 and 49kDa from the N-terminus, (Liscum, Finer-Moore, Stroud, Luskey, Brown, & Goldstein, 1985).

The degradation rates of proteins may also depend on their association with cellular structures, membranes, filaments etc. This binding to intracellular sites could mask the sites recognized by intracellular proteolytic agents. It is likely that CANP localization can give some clues to its role.

Examples of CANP activation in skeletal muscle

CANP has been shown to be activated in a number of "physiological" situations. An example where CANP has been shown to be active is in dystrophic muscle, where there are increased rates of protein degradation and increased levels of CANP activity. Increased measurable CANP activity of 3-5 fold for uCANP and 5-6 fold for mCANP in muscle from Duchenne muscular dystrophy patients has been reported, (Reddy et al, 1986). A ten fold increase in CANP activity was found in the same condition by Nagy & Samaha in 1986. Skeletal muscle CANP activity was increased and the calcium required for 50% of maximal activity (pCa_{50}), was reduced after prolonged running exercise, (Belcastro, 1993). Cardiac CANP activity was increased by 60 minutes of level running in rats, (Arthur & Belcastro, 1994) abstract.?. In skeletal muscle of mice with diabetic amyotrophy, intracellular Ca^{2+} levels and CANP activity were both elevated, (Kobayashi, Fujihara, Hoshino, Kimura & Kimura, 1989). Common to many situations where CANP activity is increased are disturbances in the localization of the enzyme creatine phosphokinase (CPK), In Duchenne muscular dystrophy the plasma levels of CPK are often two orders of magnitude greater than normal and elevated levels of CPK in the blood are used as a marker for the disease in newborns. During episodes of malignant hyperthermia, (MH), that occur in susceptible individuals after exposure to depolarizing muscle relaxants or anaesthesia, there are muscle contractures, rigidity and disturbances of CPK in the muscle, (Lang, 1981). Elevation of CPK levels in the serum were once used to screen for MH, (Gronert, 1980). Disturbances in CPK from cardiac muscle after coronary damage has been extensively studied and the plasma levels of CPK are used as an indicator of infarct size, (Roe, 1977 also Jones, Jackson,

& Edwards, 1983). CPK disturbances are found with prolonged running exercise, which also increases CANP activity. Denervation or fasting and refeeding increases the concentration of CANP and calpastatin in rat soleus muscle, and serum CPK levels are also elevated, (Kumamoto et al, 1992). The disturbance and release of CPK has long been used as a marker of muscle damage, (Linjen et al, 1988 also Amelink, Bar, Van DerKalen, & Wokke, 1990). CPK has also been shown to become unbound from its intracellular locations in isolated muscles stimulated to fatigue, (Guderley, Jean, & Blovin, 1989). Total CPK activity is reduced in the myocardium of cardiomyopathic hamsters, (Khuchua, Ventura-Clapier, Kusnetsov, Grishin & Saks, 1989). Parathyroid hormone induces increases in CPK activity which can be mimicked by synthetic diacyl glycerol or the Ca^{2+} ionophore A23187. It is likely that CANP will be active at these times, (Somjen, Zor, Kaye, Harell & Itzhak, 1987). However CPK activity was unchanged in diabetic amyotrophy while Ca^{2+} levels and CANP activity were elevated, (Kobayashi et al, 1989).

Situations where CPK disturbances are found are usually associated with elevated intracellular Ca^{2+} concentrations which increase the possibility that CANP is active within the cell.

Thus a relationship may exist between the increased CANP activities and disturbances in the CPK system. However no data exists for CPK being a substrate for CANP mediated modification.

Initial study of the amino acid sequence of CPK has shown that CPK does contain some weak pest regions, see figure 2.

CPK: Structure

The enzyme ATP:Creatine N-Phosphotransferase (EC No 2.7.32), commonly known as creatine phosphokinase (CPK), is a dimeric enzyme composed of two 360/380 amino acid monomers and contains two catalytic centers. The molecular weight of the enzyme as

determined in a non denaturing electrophoresis system is approximately 82,600 daltons, made up of two subunits of 41,300 each. The primary sequence of MM-CPK is shown in figure 2. The PEST sequences of the MM-CPK are:-

POSITION From	To	PEST SCORE	COMMENT	SEQUENCE
14	24	-0.21	weak	FSAEEEFPDLS
46	65	-0.4	weak	ETPSGFTLDDVIOTGVDNPG
67	85	-12.48	weak	PFIMTVGCVAGDEESYEVF

```
                                    p
 1  M P F S S T H N K H K L K F S A E E E F P D L S K H N N H M
                                                 p           p
31  A K V L T P E L Y K R L R D K E T P S G F T L D D V I Q T G
61  V D N P G H P F I M T V G C V A G D E E S Y E V F K D L F D
                                  p
91  P V I Q D R H G G Y K P T D K H R T D L N H E N L K G G D D
                  p       p                             p
121 L D P K Y V L S S R V R T G R S I K G Y S L P P H C S R G E
                                                        p
151 R R A V E K L S V E A L N S L E G E F K G R Y Y P L K A M T
181 E Q E Q Q Q L I D D H F L F D K P V S P L L L A S G M A R D
211 W P D A R G I W H N D N K T F L V W V N E E D H L R V I S M
241 E K G G N M K E V F R R F C V G L K K I E E I F K K A G H P
271 F M W T E H L G Y I L T C P S N L G T G L R G G V H V K L P
                  p                       p
301 K L S Q H P K F E E I L H R L R L Q K R G T G G V D T A A V
                  p               p
331 G A V F D I S N A D R L G F S E V E Q V Q M V V D G V K L M
361 V E M E K K L E Q N Q P I D D M I P A Q K
```

Figure 2: The primary sequence of MM-CPK. Sequences recognized as possible PEST sequences are underlined. The active site is in bold and underlined (residues 283 to 289). Phosphate groups are represented with a "**p**" above the appropriate amino acid.

Function

The first event in the activation of the myofibrillar adenosine triphosphatase by Ca^{2+} is hydrolysis of ATP, leading to a local accumulation of ADP and H^{+}, both of which actually serve as substrates for the Lohman reaction by CPK.

CPK catalyses the reversible transfer of a phosphate residue in high energy bonding between adenosine triphosphate (ATP) and Creatine (Cr) as follows:

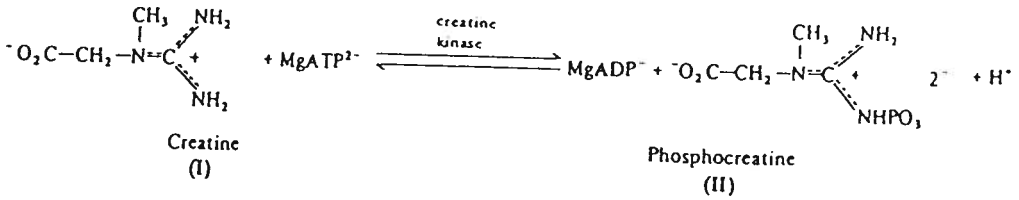


Figure 3: Enzymatic reaction catalyzed by CPK.

Increased availability of ADP, phosphocreatine (PCr), and the lowering of intramyofibrilar pH will all activate CPK in the direction of ATP regeneration. The Km values for PCr and creatine for MM-CPK are 1.7mM and 16mM respectively, (Saks, Chernousova, Gukovsky, Smirnov, & Chazov, 1976). The high affinity of MM-CPK for Mg-ADP (Km=0.08 mM) causes the latter to be trapped and rephosphorylated into ATP by CPK as long as creatine phosphate is present at concentrations of 3-4 mM or higher. The rephosphorylation of ADP to ATP also acts to buffer hydrogen ions and maintain pH levels within the cell. The pH optimum of the CK reaction is between pH 6.5 and 6.6, (Walliman & Eppenberger, 1985).

Distribution & Physiological significance

CPK is the key energy transmitting kinase in the muscle cell and is known to exist in four different isozyme forms. Three of these isozymes are formed by combination of either two homologous subunits, MM-CPK for muscle, BB-CPK for brain or hetrologous subunits, MB-CPK. In skeletal muscle the predominant form of CPK is the MM isoform. CPK was originally thought to be strictly cytoplasmic and therefore soluble. However it became apparent that CPK is also specifically bound at strategically important locations as well. From ten to thirty percent of total CPK activity, depending on muscle type is located on the inner mitochondrial membrane as the fourth isozyme of CPK, MiMi-CPK a specific mitochondrial adenylate translocase isoenzyme. CPK is found at the sites of energy production within the cell, such as oxidative phosphorylation, ATP/ADP translocase and

glycolysis, (Walliman & Eppenberger, 1985). These sites of energy consumption within the cell where CPK is bound include the M-line of skeletal muscle, (Turner et al, 1973), sarcoplasmic reticulum and ribosomes, (Sharov, Saks, Smirnov, & Shazov, 1977), and plasma membranes, (Baskin & Deamer, 1970). The small strategically located sites of CPK are thought to be able to respond quickly to changes in their local environment such as ATP hydrolysis and rephosphorylation of ADP. CrP is thought to move from the sites of energy production to the sites of consumption and Cr moves from the sites of energy consumption to the sites of energy production.

The functional significance of these "pools" of CPK within the muscle cells have been demonstrated by numerous experiments. CPK bound to skeletal muscle sarcoplasmic reticulum maintains a much higher rate of Ca^{2+} ion uptake in the presence of CrP rather than an added ATP regenerating system at the same adenine concentration, (Levitsky, Levchenko, Saks, Sharov, & Smirnov, 1977). Ion transport across the cell membrane can also get its energy supply from CrP via local CPK. The adequacy of the CPK system has been demonstrated using the rate of hydrolysis of ATP, proportion of M-line bound CK, and its ATP regeneration potential. That is, the intra myofibrillar regeneration of ATP by the M-line bound CPK can account for the entire energy required for contraction, (Walliman & Eppenberger, 1985). When ATP containing radio labelled gamma phosphate (5mM) and normal CrP (10mM) was supplied to the myofibrillar ATP'ase, there was very little of the labeled phosphate produced via the muscle contraction. This shows that the phosphate group used to rephosphorylate the ADP formed during the contraction was from the CrP, (Bessman, Geiger, Erickson-Vitanen, & Yang, 1980). The rate of amino acid uptake by isolated polysomes was found to be much higher in the presence of CrP and low ATP (0.05mM), than with an equimolar or even higher ATP concentration or an added ATP regenerating system, (Savabi, Carpenter, Mohan, & Bessman, 1988).

CPK both bound and free at the sites of energy production and consumption could play the key role in energy transmission within the cell. This system is known as the creatine

phosphate shuttle, (Bessman et al, 1980 also Walliman & Eppenberger, 1985 and Savabi et al, 1988). With CPK playing such a key role in energy transmission within the cell, it would be expected that alterations in CPK activity by inhibition, proteolysis or removal, may lead to functional changes within the muscle.

Muscle Dysfunction

Disturbances in the normal functioning of muscle often occurs when the CPK system is perturbed or modified. In several muscle diseases in both humans and experimental animals, including muscular dystrophy and nutritional myopathy, there is a distinct inability of the muscle to retain creatine. It has been demonstrated that CPK changes are a causative factor of muscle dysfunction. Chicks fed with B-guanidobutyric acid which inhibits creatine transport into the muscle show ultrastructural changes within the muscle. These changes include disruption of normal filament organization, Z band streaming, scattering of the sarcoplasmic reticulum and t-tubules, (Laskowski, Chevli, & Fitch, 1981). These changes were not attributed to any loss of a direct structural role of creatine. Removal of the CPK localized at the M-line in skeletal muscle using a low ionic strength buffer, (Walliman & Eppenberger, 1985), resulted in a loss of ability to regenerate ATP and an obvious functional limitation in that the remaining CPK was unable to maintain the supply of ATP and also was unable to buffer the H^+ ion production. The deterioration in CPK activity found in myocardial cells in rats has been proposed to affect the transfer of high energy phosphates within the myocardial cells and the consequent defect in energy utilization may explain the altered myocardial function present in diabetes mellitus, (Mokhtar, Rouseau, Migneron, Tancrede, & Nadeau, 1992). Prevention of the oxidative deactivation of cardiac CPK during ischemia with myristic acid results in improved CPK activity in rats. The enhanced CPK activity after treatment correlated well with functional benefits to the reperfused myocardium, (Kaplan, Blum, Banerjee, & Whitman, 1993). It is clear that abnormalities in the CPK system of energy transmission are associated with many types of muscle dysfunction

and that the energy consuming sites within the cell rely on the proper functioning of their localized CPK pool. Therefore alteration of CPK activity by proteolysis, and or removal would likely cause functional changes to energy transmission within the cell. These relationships between disturbances in CPK activity and function have been used successfully in numerous clinical studies to assess the presence or absence of tissue damage (see below).

Examples of CPK disturbances

The release of intracellular CPK from diseased muscle is a well known phenomena and well characterized in a number of conditions, (Pennington, 1981). There are many such examples of disturbances in the distribution of CPK and the majority of these examples involve some sort of muscle dysfunction. These include Duchenne muscular dystrophy, As mentioned before, CANP activity is also elevated in muscle from patients with Duchenne muscular dystrophy. MH causes contractures, rigidity, and disturbances of CPK in the muscle. Disturbances in CPK from cardiac muscle after coronary damage has been extensively studied and the plasma levels of CPK are used as an indicator of infarct size, (Roe, 1977 also Jones et al, 1983). CPK disturbance and release has long been used as a marker of muscle damage, (Linjen et al, 1988 also Amelink et al, 1990). CPK has also been shown to become unbound from its intracellular locations in isolated muscles stimulated to fatigue, (Guderley et al, 1989). Although experimentally induced CPK disturbances can lead to loss of muscle function and the clinical evidence is extensive for these disturbances. Little is known about either the chronic changes in disease states or the more transitory alterations in the CPK system as with acute exercise. A number of factors have been suggested to give rise to CPK disturbances and its release from skeletal muscle. Increased membrane permeability due to detergents initiates a quicker release of CPK than is found with contractile activity, suggesting against a simple release of cytoplasmic CPK due to increased membrane permeability in the exercise condition. The time course of CPK release was found to be similar to that of lactate dehydrogenase which has a molecular weight of 140 kDa as

compared to the 81kDa of CPK, also suggesting against only an increased membrane permeability. Acidosis has also been suggested as a cause of CPK release, however muscles stimulated under conditions considered normoxic will release CPK, as do muscles that have been poisoned with iodoacetatic acid and therefore cannot produce lactate. Some of the suggested causes of the CPK release are, influx of extracellular Ca^{2+} and consequent damage to mitochondria, activation of proteases and lipases. Despite much research in this area, the mechanism or mechanisms underlying CPK disturbance and release or reduced CPK activity is not well understood. The modification of CPK activity at its sites within the skeletal muscle cell may cause significant changes in the energy transmission throughout the cell. It is during these times of high energy requirements that the CPK system is used most. These conditions are also where the greatest alterations in the CPK system seem to occur. Therefore any agents that could modify CPK activity may be important during these periods.

A possible mechanism

Elevated intracellular Ca^{2+} concentrations are commonly used to stimulate CPK release from muscle in studies attempting to determine the underlying cause and/or mechanism of CPK release. These include experiments with the calcium ionophore A23187 and caffeine, (Duncan, 1978 also Jones et al, 1983). Incubation of rat skeletal muscle with the calcium ionophore A23187 or with the mitochondrial poison 2.4. Dinitrophenol increases intracellular calcium levels and stimulates CPK disturbances and release from the cell, (Brazeau & Fung, 1990). The same group also found that release of CPK from skeletal muscle exposed to propylene glycol or ethanol was increased when muscles were incubated in a medium containing calcium chloride compared to one containing sodium chloride. The addition of dibucaine, a non specific phospholipase A_2 inhibitor, only modestly reduced the CPK release from the muscle, (Amelink et al, 1990). It was concluded that the cosolvent induced disturbances of the CPK system in skeletal muscle may be caused by an intracellular mechanism rather than by a direct solubilization of the sarcolemma and that this intracellular

mechanism may involve the mobilization of Ca^{2+}. Further support of the role of Ca^{2+} in inducing CPK disturbances comes from exercise or disease studies. The exercise induced release of CPK from rat skeletal muscle is reduced after treatment with dantroline sodium, (Amelink et al, 1990). Dantroline sodium inhibits Ca^{2+} release from the sarcoplasmic reticulum and has been used as an effective treatment for MH. Common to these dysfunctions of skeletal muscle are disturbances in the Ca^{2+} homeostasis within the muscle cell, an example of this is found in prolonged exercise leading to fatigue where a Ca^{2+} imbalance develops. As mentioned previously, CANP is also activated during prolonged running exercise where Ca^{2+} levels have also been reported to be increased with fatigue, (Sembrowitch, Johnson, Wang & Hutchison, 1983 also Belcastro, 1993). This CANP activation may promote a disrupted state within the muscle during running, (Belcastro et al, 1985). Similarly muscles affected by Duchenne muscular dystrophy appear to have increased intracellular Ca^{2+} levels, (Croall & De Martino, 1991). Altered Ca^{2+} levels have been hypothesized to cause MH as a result of a sudden increase in intracellular Ca^{2+} within the muscle. MH muscle has been shown to have a so called "leaky sarcoplasmic reticulum Ca^{2+} release channel" and MH susceptible individuals do demonstrate significantly increased levels of intracellular Ca^{2+}.

It seems clear that elevated Ca^{2+} levels are consistently associated with myopathies and CPK release from the muscle. How this process works is completely unknown. However elevated Ca^{2+} levels may be linked to CPK activity through the Ca^{2+} dependent proteolytic system, CANP/calpastatin.

CANP activity may be elevated during muscle dysfunctions where Ca^{2+} levels are elevated and the CPK system is disturbed. Thus a relationship may exist between increased activity of CANP and these disturbances and or modifications to the CPK system. However no data exists for CPK being a substrate for CANP or of any effect of CANP on CPK activity.

Chapter 3

PROCEDURES

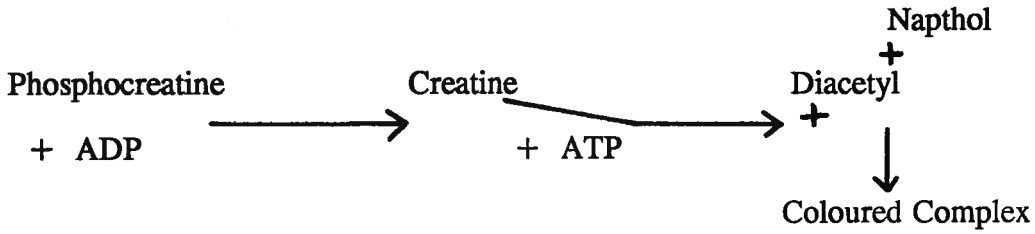
Objective 1: To determine if CPK activity is changed by active CANP in vitro.

CPK activity was measured in vitro, as a standard control and then with 5mM Ca^{2+}, and different amounts and specific activities of active CANP present during the assay.

The MM-CPK purified from rabbit skeletal muscle was obtained from Sigma and its purity was assessed by scanning the control CPK lanes in the SDS-PAGE gels. Purity of the samples ranged from 83% to 98% of the total protein being the 42kDa MM-CPK band, see appendix D (1).

Measuring CPK activity

CPK activity was measured using a colorometric assay based on the method of Hughes ,(1962). The assay relies on the rephosphorylation of ADP at the expense of PCr and the liberation of creatine as shown below:



The basic assay solution contained 6.48mM PCr, 10mM DTT, low salt Tris buffer, pH 7.5, (1M KCl2, 1mM MgCl2, and 1mM DTT), & 25ul of CPK (200ug/ul). The assay contents were incubated at 37^{o}C in a water bath and the CPK reaction was started by adding 50 ul of ADP-Glutathione (final concentration = 13.3uM). The reaction was then stopped after thirty minutes by the addition of 50ul of p-Hydroxymercuribenzoate solution (0.05 mol/L). Then 250ul of alpha-napthol, 250 ul of diacetyl and 1.75ml of water were added to the assay and mixed. The creatine that was liberated during the reaction forms a complex with the alpha-

napthol and diacetyl. This complex is coloured and its formation takes 10 to 15 minutes. The assay contents were then centrifuged in a Hermle Z360K centrifuge with a four place swing out rotor at 3000rpm for five minutes. The amount of coloured complex formed is proportional to the CPK activity and was measured by spectrophotometry using a Shimadzu UV-160 spectrophotometer. The activity of the CPK was found by comparing the absorption at 520 nm to a standard curve using known amounts of creatine treated in the same way as the samples. This was the standard or "control" CPK assay condition. This standard assay was reliable from day to day within each batch of CPK, the descriptive statistics for four assays on different days are shown in Appendices E(1) & E(2). The activity from these four standard assays had a standard deviation of 6.4% of the mean activity.

Because it is necessary to have sufficient Ca^{2+} present to activate the CANP, (1mM free Ca^{2+}). A Ca^{2+} containing assay was used as a "Ca^{2+} control". When modifications are made to the control assay, the results for each set were normalized to the control value for that particular series of experiments and the appropriate blank used to correct for backround. This is because each batch of CPK could vary slightly in activity depending on purity of the CPK. The activity of CPK during the thirty minute incubation was recorded with different amounts and specific activities of CANP present in the assay, (details are provided in the results section, tables and figures).

Measuring CANP activity:

Partially purified (anion exchange chromotography), m-CANP (Sigma), was used in all the assays. Calpain was stored in a buffer containing 50ul of 200mM DTT, 4.9ml of 250mM Tris pH 7.4, and 50 ul of 100mM EGTA. The EGTA is required to chelate any free Ca^{2+} ions and therefore prevent autolytic activation of the calpain during storage. Activity of 0, 10 , and 50ul CANP was standardized to the proteolysis of casein substrate assayed at 37^{o}C in the presence of 2 $mg \cdot ml^{-1}$ casein, 25ul of 200 mM DTT, 75ul of 50mM Ca^{2+} (this gives 1mM free Ca^{2+} due to EGTA) and the total volume for each condition made up to 500ul

with 250mM Tris (pH 7.4). After 30 minutes, caseinolysis was quenched by the addition of 500ul of ice cold 5% TCA. A unit of calpain activity is defined as the amount of TCA soluble product resulting in an increase of 1 unit at 280nm after the calpain digest of casein substrate. Estimates of calpain activity were performed in duplicates.

Measuring Protein Concentration

Protein concentration of the CPK and CANP was measured using the method of Lowry *et al.*, (1951). See appendix A.

Statistical Analysis

To detect any statistically significant alteration in the activity of the CPK with different amounts (0-100ug) of CANP, a repeated measures ANOVA was used. Scheffes post hoc analysis allowed the study of individual differences across conditions, (alpha = 0.05)

Objective 2: To study the enzyme kinetics of the CPK.

To study the enzyme kinetics of CPK it was necessary to study the activity of the enzyme using an end point that lay on the initial linear portion of the plot of enzyme activity against time. Pilot work had shown that with the control assay condition, an end point of 1.5 minutes would ensure that the enzyme kinetics are being studied under a steady state. However when a series of assays with ranges of substrate concentrations were measured under (a) control conditions, (b) control + Ca^{2+}, (c) control + Ca^{2+} + CANP. The small amount of CrP liberated in 90 seconds meant that the variability of the resulting activities were high and made any meaningful enzyme kinetic data analysis unfeasible. See Appendix B; 1 & 2

Objective 3: To determine if CPK would be proteolyzed by active CANP in vitro.

CPK was incubated with different amounts of CANP and proteolytic products visualized.

SDS-PAGE.

The following methods were used.

For the CPK control solution = 8mM Ca^{2+}, 17mM DTT, 14.75mM Tris (pH 7.4) and 10ul of CPK stock.

In the trials where CANP was present, the volume of CANP added was accommodated by reducing the volume of the Tris buffer to keep the total volume constant. The CANP was added to the solution last and the contents incubated at 37°C for 30 minutes and each condition was duplicated. The samples were then mixed with 20ul of digestion buffer (consisiting of 12ul of 200mM DTT, 1ml of 3 X sample buffer and 200ul of 2% bromo-phenyl blue). The samples were then incubated for 10 minutes at 47°C and loaded onto 10% gels. A solution containing known molecular weight markers was loaded into one of the wells on the gel. The gels were then run overnight for approximately 16 hours, then stained in Coomassie brilliant blue stain for 2 hours and destained in graded methanol solutions. After destaining, the gels were dried down in 70% methanol between cellophane sheets.

Densitometry

Quantification of protein bands was carried out using an LKB 2202 Ultrascan Laser Densitometer and an LKB Integrator. The percent total protein of both the CPK, the small subunit of CANP, and any proteolytic products were recorded from these scans.

The molecular weights of the proteolytic fragments were estimated from the gels.

Statistical Analysis: The % total protein of each band visualized on the SDS-PAGE with increasing CANP was compared using a repeated measures ANOVA. The effects of each

increasing amounts of CANP were compared to each other using a Scheffe's post hoc test, (alpha = 0.05)

Chapter 4

RESULTS

Objective 1: Is CPK activity affected by active CANP in vitro ?

CPK activity with Ca^{2+} and CANP

The values for CPK activity of the control assay were typically from 45 to 70 Sigma units (375 to 585 U), the protein concentrations of the CPK used was 200ug/ul. The activity of the control CPK was stated as 100-200 U of activity/mg. The control assay contained 25ul x 200ug = 5mg of CPK. Therefore I could have expected CPK activity of 500 to 1000 units. The assay results were reliable within each CPK batch, see appendix E (1&2). However when new samples of CPK were reconstituted, the activity from one batch to another ranged from 43.7 to 70.3. This was reflected in the SDS-PAGE scans where the CPK batches with higher activity showed higher enzyme purity on the gel, with CPK ranging from 83 to 98% of total protein. The CPK activity data has been represented as percentages of the control assay used for each trial or condition to allow a clearer comparison of the data from different CPK batches.

A preliminary comparison of control CPK activity and CPK with 200ul (200ug) CANP present but no Ca^{2+} allowed any possible effect of the inactive CANP on the activity measured. There was no effect of the inactive CANP on the CPK activity with control activity = 51.36 + 1.83 and with CPK plus inactive CANP = 52.67 + 2.48. Initially the effect of only two different volumes of CANP were measured.

CPK activity was measured as, (1) = standard or "control" assay or, (2) = as control but with 5mM Ca^{2+} added, and also (3) = as in (2), but with 10ul CANP present in the assay, and (4) = as in (2) but with 100ul CANP present in the assay. These two different volumes of CANP resulted in very different CPK activities, see figure 4. In the samples with 5mM Ca^{2+} present, the CPK activity was significantly reduced to 37.64% + 2.6% of the control assay, ($p<0.05$). When 10 ul of CANP was present, the CPK activity was recovered to 97.11 + 1.7% of the control value. The addition of 100ul of CANP to the assay caused

very different results, there was a reduction of CPK activity to 2.08 + 2.56% of control which was significantly different to all three other conditions. See appendix C (5).

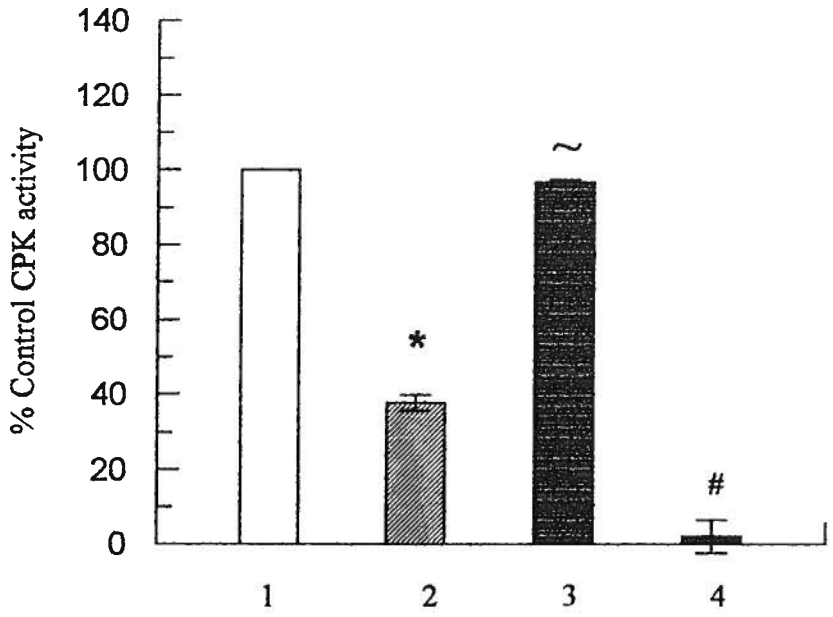


Figure 4: Comparison of two different amounts of CANP on CPK activity. 1 = Control assay, 2 = Control plus 5mM Ca^{2+} (* = significantly different from 1,3 & 4). 3 = As in 2 plus 10ul CANP (~ = Not significantly different from 1). 4 = As in 2 plus 100ul CANP (# = significantly different from 1,2 & 3).

These results suggested that different amounts of CANP will have different effects on CPK activity.

The results of incubating CPK with a range of increasing CANP amounts is shown in figure 5. In this case a further "control" was added for each condition. In addition to the "calcium control", each CANP condition had a duplicate containing an equal volume of the buffer that

the CANP is stored in. This was done to measure any effects of the EGTA in the storage buffer on the inhibition of CPK by Ca^{2+}. The approximately 36% inhibition of CPK activity by the addition of 5mM Ca^{2+} is consistent and this inhibition was not significantly different across conditions, the amount of EGTA added in comparison to the Ca2+ present is so small that there is no significant removal of the Ca2+ inhibition of the CPK, See appendix C (3). This inhibitory effect is overcome by the initial stimulation of CPK activity with 27ul CANP and then CPK activity is lost with increasing amounts of CANP. See figure 5.

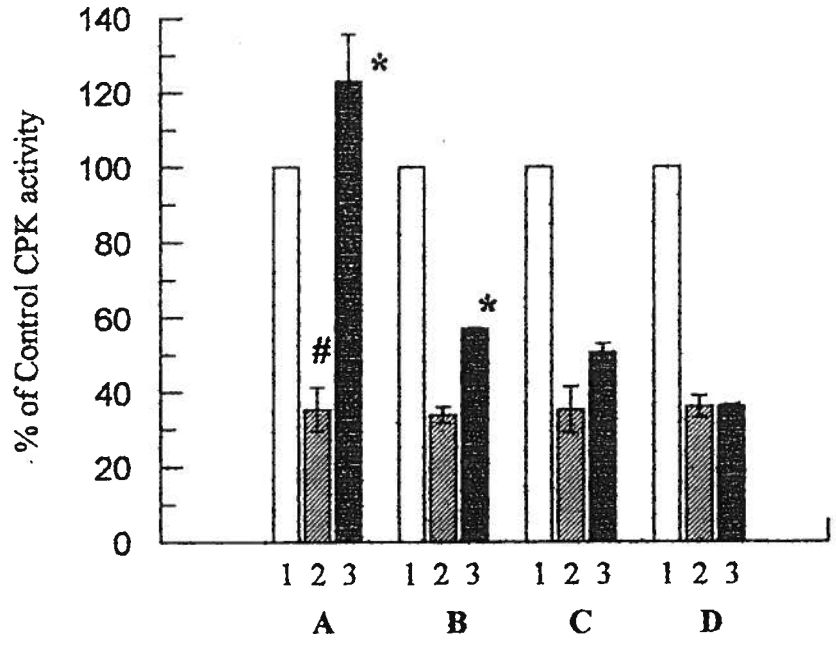


Fig 5: CPK activity with increasing amounts of CANP.
1 = Control assay with CANP buffer. 2 = As in 1 plus 5mM Ca^{2+} also CANP storage buffer. 3 = As in 2 plus CANP. A = 27ul of CANP buffer or CANP. B = 54ul of CANP buffer or CANP. C = 67ul of CANP buffer or CANP. D = 84ul of CANP buffer or CANP. CANP buffer is the buffer that the CANP is reconstituted in.
* = Significantly different from control activity ($p<0.05$).
# = Significantly different from control activity ($p<0.05$).

Each of the conditions from A to D has increasing amounts of CANP in sample number 3. With the presence of 27ul of CANP, (A_3), CPK activity is increased slightly but significantly

to 123.18 + 12.9% of the control value, ($p<0.05$). As the amount of CANP present in the assay is increased to 54ul (B_3), then the CPK activity is recovered to 56.96 + 0.31% of control. With 67ul of CANP (C_3) in the assay the CPK activity was again higher than the "calcium control" but is now only 50.46 + 2.65% of the control activity. When 84ul of CANP (D_3) was present then the CPK activity was 33.06 + 0.5% of the control which is the same as with the 5mM Ca^{2+} control.

When these results are combined with those from Figure 4, we can see the inhibition of CPK activity with 5mMCa^{2+} is removed by the initial stimulation of CPK activity and then CPK activity is lost with increasing amounts of CANP. See figure 6.

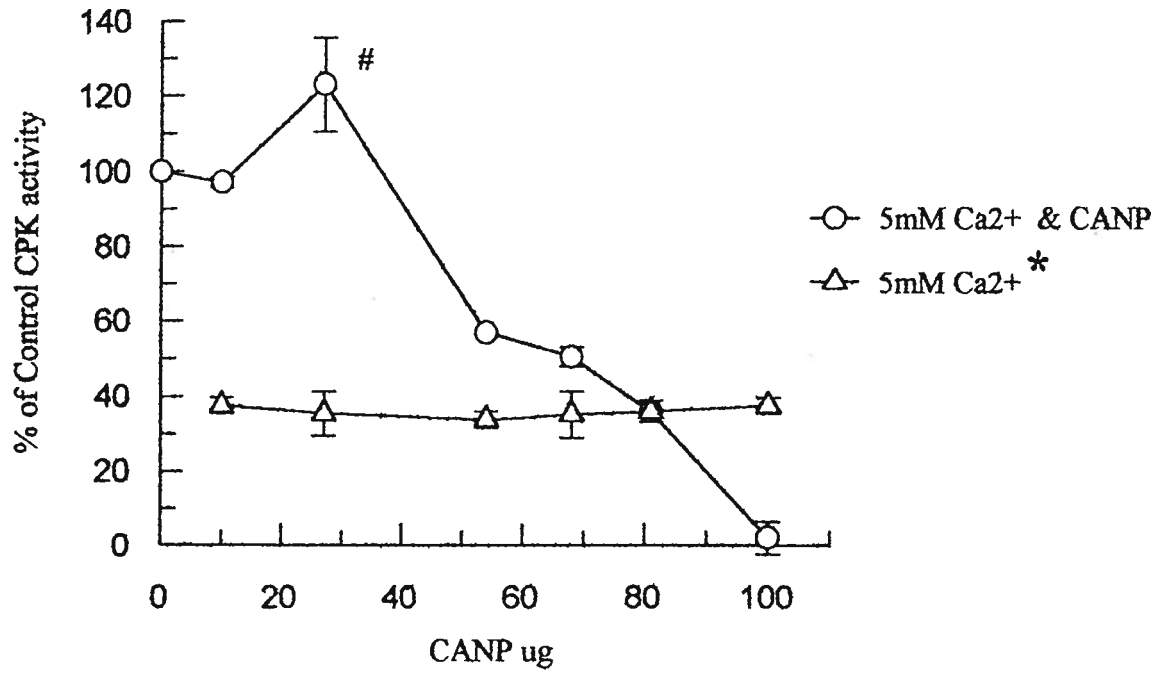


Fig 6: CPK activity as a percentage of control with increasing amounts of CANP. # = significantly different from control ($p<0.05$). * = significantly different from control ($p<0.05$), but not significantly different from each other Ca^{2+} trial.

Objective 3: CPK proteolysis by CANP in vitro

CPK proteolysis and CANP

SDS-PAGE of CPK with active CANP shows that CPK is proteolyzed by CANP. Proteolysis of CPK is shown in figure 7 on a 5-15% gel which has been overloaded with CPK, (1412 ug per lane). CPK proteolysis increases with increasing amounts of CANP. When lanes 1, 2 and 4 are compared with the CPK activity in figure 4, then the degree of proteolysis reflects the resulting CPK activity. The results of the densitometric scans showing significant proteolysis of the CPK, ($p < 0.05$) are shown in appendix D (1).

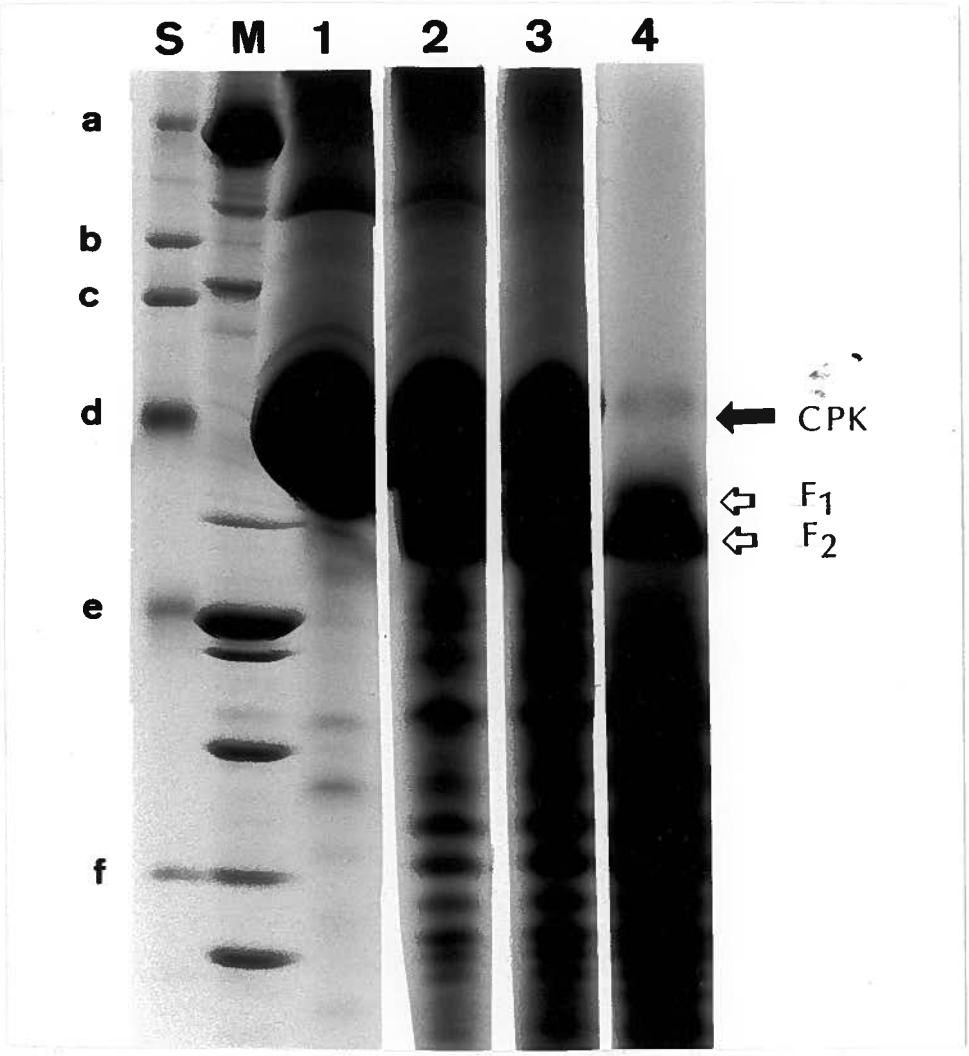


Figure: 7. A 5-15% SDS-PAGE with 1416ug of CPK per lane. Lane 1 = Control CPK, 2 = CPK + 10ul CANP, 3 = CPK + 27ul CANP, 4 = CPK + 100ul CANP. a = 200kDa, b = 97kDa, c = 66kDa, d = 45kDa, e = 31kDa. Position of CPK band is indicated by the solid arrow. F_1 = fragment 1, F_2 = fragment 2.

When 120ug of CPK was loaded in each lane and increasing amounts of CANP were present, the degree of CPK proteolysis was dependent on the amount of CANP present, see figure 8.

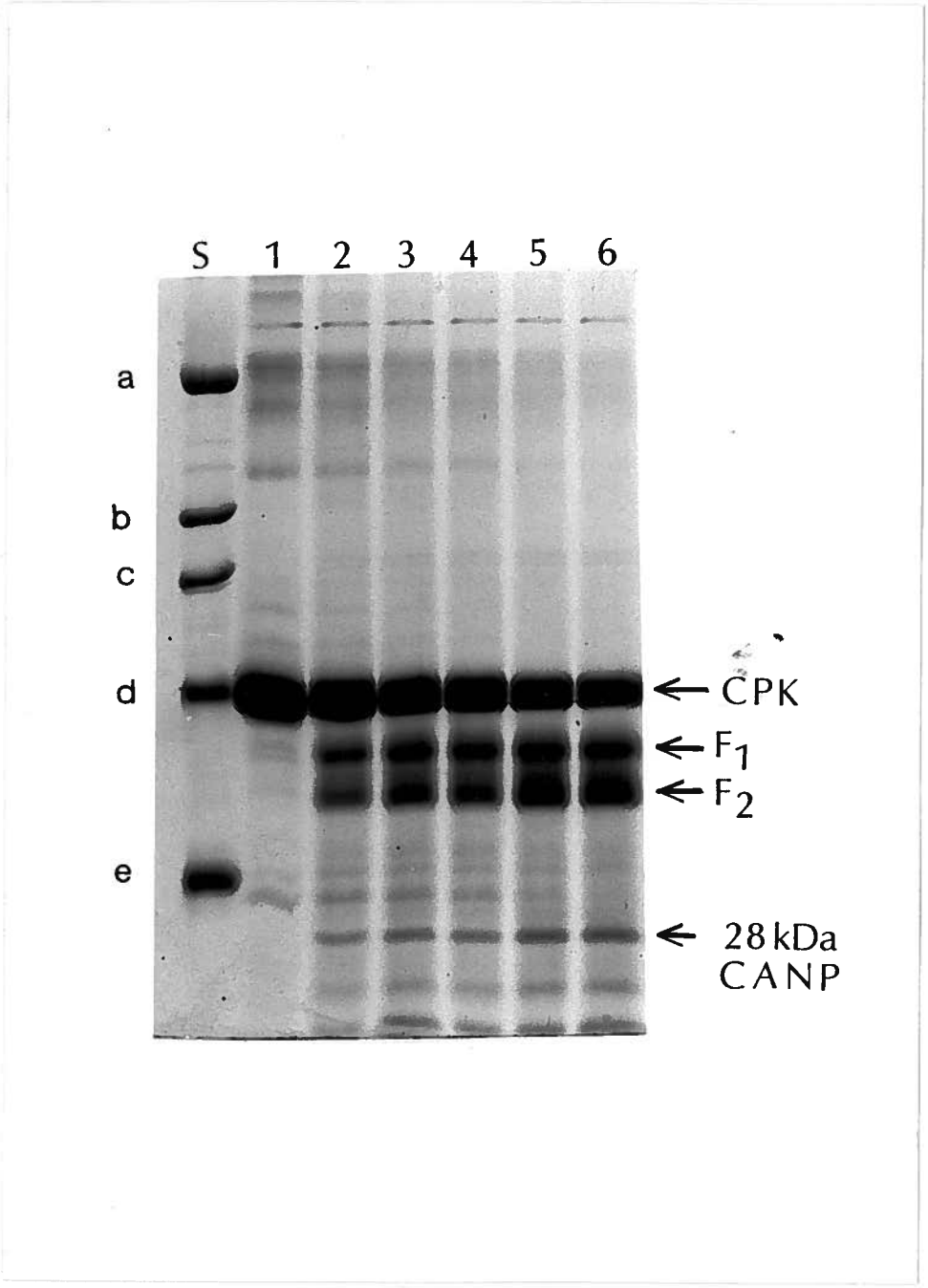


Figure 8: Gel showing proteolysis of 120ug CPK with increasing amounts of CANP. Lane 1 = Control CPK, 2 = CPK + 10ul CANP, 3 = CPK + 15ul CANP, 4 = CPK + 20ul CANP, 5 = CPK + 25ul CANP, and 6 = CPK + 30ul CANP. S = Molecular weight standards. a = 200kDa, b = 97kDa, c = 66kDa, d = 45kDa, e = 31kDa. The CPK and small CANP subunit bands are labelled. F_1 = fragment 1, F_2 = fragment 2

The data from densitometric scans of each lane from five SDS-PAGE trials are plotted in figure 9(A). Data was reported for each protein band as a percentage of total protein on the lane being scanned, See appendix D (1).

The increased CPK proteolysis is shown in figure 8. The corresponding data from this gel is shown in figure 9(B). In the control lane the CPK represents 83.2% of the total protein loaded in that lane. Lane 1 has 10ul of CANP added and this constitutes 2.79% of the total protein, the small 28kDa subunit of CANP is visible on the gel. Lane 2 has 15ul of CANP which was 4.93% of total protein, the resulting decrease of CPK to 61.23% total protein for CPK shows the effect of the increasing CANP. The loss of the CPK band continues with increasing CANP, 20ul CANP left 59.95% as CPK, 25ul CANP left 43.79%, and 30ul CANP left 36.38%. These results demonstrate the dose dependency of the CPK proteolysis on the amount of CANP actually present.

Fragment analysis

The proteolysis of the CPK, as shown by the loss of the CPK band in figures 7 & 8, results in the formation of two main fragment products. These fragments are a few kDa smaller than the original CPK. The significant formation of these fragments with increasing amounts of CANP is shown in figure 10.

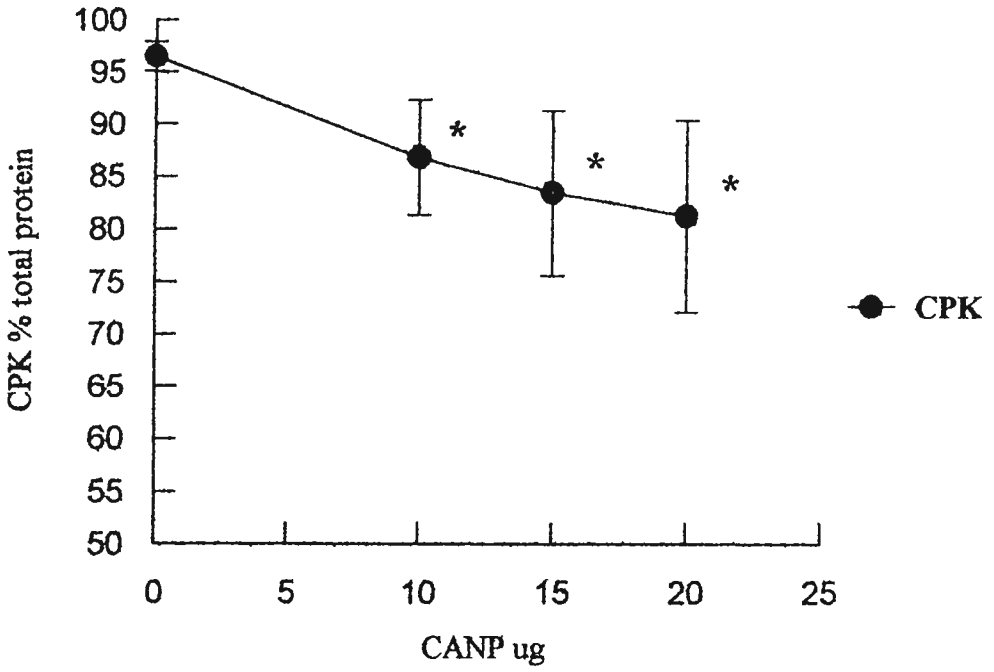


Figure 9(A): CPK proteolysis with CANP. Data is the mean for five SDS-PAGE trials. * = significantly different from control ($p < 0.05$).

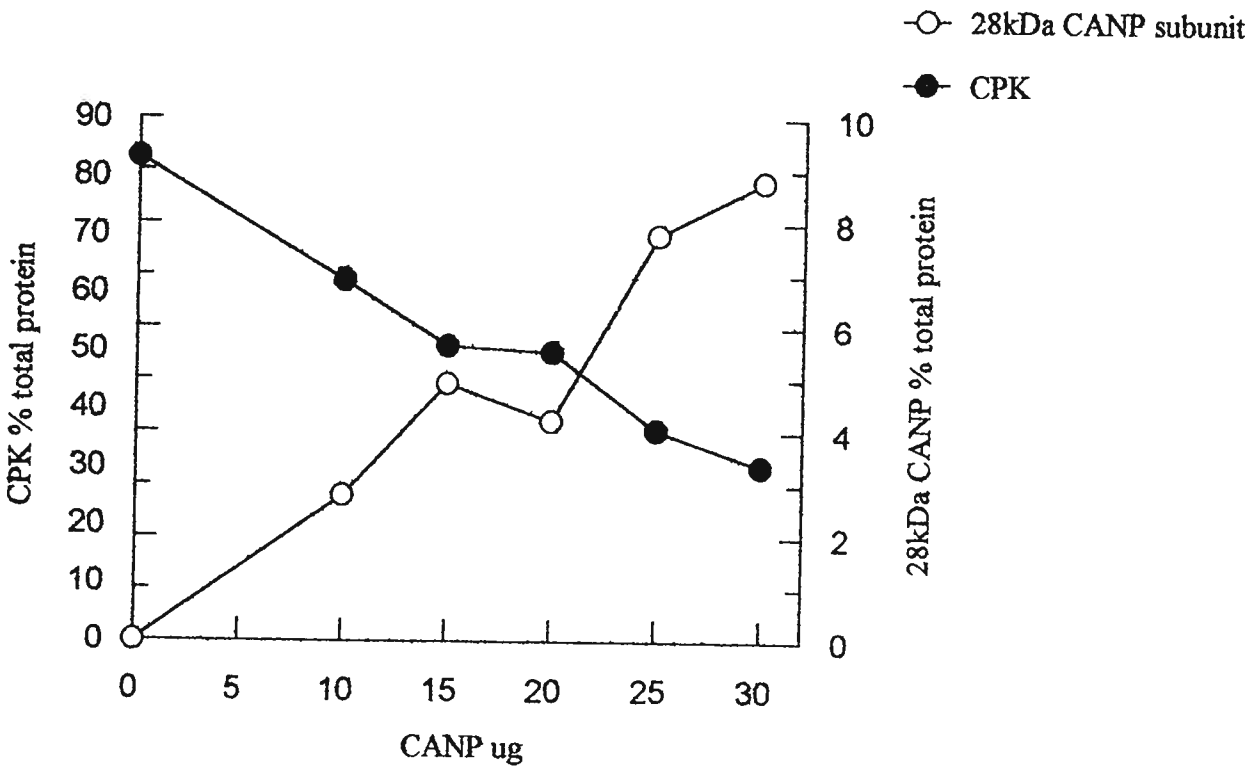


Figure 9(B): Data from densitometric scans of 10% SDS-PAGE from figure 8. The % total protein of CPK and the 28 kDa CANP subunit are shown. Each lane was loaded with 120ug CPK.

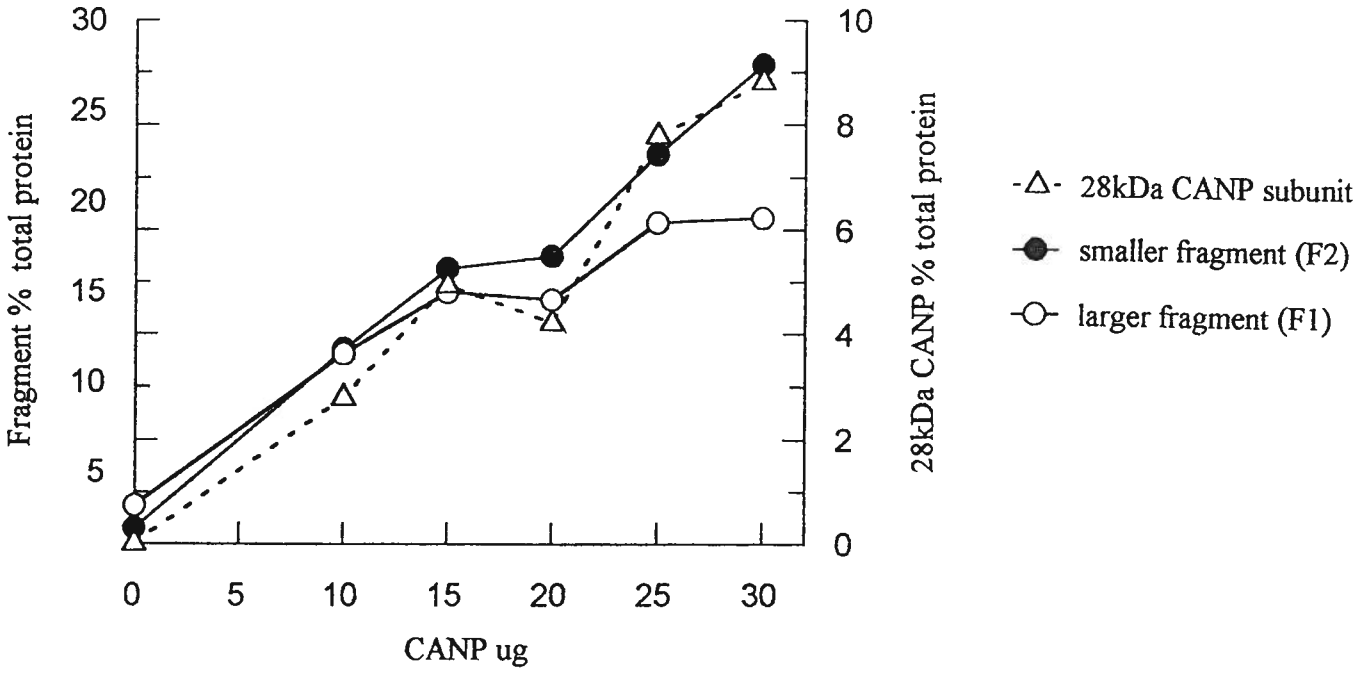


Figure 10: Data from densitometric scans of 10% SDS-PAGE with 120ug CPK per lane incubated with increasing volumes of CANP.

An approximation of the molecular weights of these proteolytic products was made by creating a linear regression equation using the log Molecular weight of the known standards plotted against their relative mobilities from the gel.

Chapter 5

DISCUSSION

From the results of this study, two of the three proposed hypotheses can be accepted.

The increase in CPK activity and then its loss as the ratio of CANP to CPK is increased confirms the first hypothesis. That is: "CANP will change CPK activity in vitro".

The data from the SDS-PAGE of CANP and CPK showing significant yet restricted proteolysis of CPK by CANP also leads to the confirmation of the third hypothesis, which is "CANP will cause proteolysis of CPK in vitro".

The inhibition of CPK with 5mM Ca^{2+} was consistent across trials and 10 to 20% CPK inhibition with 4mM ATP and Ca^{2+} has been reported before (Walliman & Eppenberger, 1985). Free Ca^{2+} resulting from the 5mM Ca^{2+} added to the control assay will likely be reduced upon the addition of the CANP buffer which contains 0.1mM EGTA. Therefore it may have been expected that the addition of increasing volumes of CANP storage buffer may have just increased the chelation of Ca^{2+} and would have caused removal of the inhibition on the CPK activity. The fact that the effect on CPK activity of the Ca^{2+} conditions with increasing volumes of CANP buffer were not significantly different from each other demonstrates that this effect did not occur. Any possible recovery of CPK activity with removal of Ca^{2+} by the presence of the CANP alone is unlikely because if this was the case it is unlikely that the CPK activity could have been increased to a level greater than the control value. Indeed any removal of this inhibitory effect would have caused recovery of CPK activity with increasing amounts of CANP and not the opposite, as observed.

This is the first demonstration that MM-CPK is a substrate for CANP in vitro. The activity of CPK was increased above control values when 27ug of protease was incubated with 1412ug of substrate, this is a protease to substrate ratio of 1:185. Other examples in the literature demonstrating CANP mediated proteolysis of enzyme substrates have used protease to substrate ratios ranging from 1:500 with tyrosine hydroxylase, (Kirsch, Kirsch, Titani, Fugita, Suzuki & Nayatsu, 1991),; 1:3, 1:16.67 or 1:33.33 with PKC, (Kishimoto et al,

1989),and 1:25 with calcineurin, (Wang et al, 1989a). The CANP content of skeletal muscle is approximately 2mg/g wet weight and the CPK content is around 5mg/g wet weight, (Walliman & Eppenberger, 1985). This gives a ratio of 1:2.5 which is obviously a gross estimate and has many confounding factors, including the various different locations of both CANP and CPK. Conclusions and assumptions made regarding the ratio of protease to substrate at this time is at best a rough indicator of the actual intracellular ratios.

The modification of CPK activity can be loosely correlated to the changes in CPK structure visualized on the SDS-PAGE. The SDS-PAGE gel in figure 7 shows the effect of 10ul CANP. There is definite proteolysis of the CPK at this ratio of protease to substrate and in the activity assay this is reflected as a recovery of the CPK activity back to control values. The 27ug of CANP shows increased proteolysis of the CPK and activity is increased above the control level. In these cases the ratio of protease to substrate on the gel is 1:148 and in the assay is 1:185. Similar activation of substrate enzymes after proteolysis by CANP has been documented with, for example: tyrosine hydroxylase, (Kirsch et al, 1991),; PKC, (Kishimoto et al, 1989), and phosphorylase-b-kinase, (Meyer et al, 1964). The loss of CPK activity with 100ug of CANP correlates to the almost complete loss of the original 41kDa CPK band on the SDS-PAGE.

The fact that two large fragments are formed rather than an absolute degradation of the enzyme suggests some kind of regulatory role of CANP with CPK and the other enzyme substrates that are not degraded but are slightly modified, such as PKC, TH, and calcineurin as mentioned previously. The sites of CANP cleavage in these enzymes have been shown to be near to the PEST regions on their amino acid sequences. The fact that CPK contains two weak PEST sequences of -0.21 and -0.4 adds to the evidence that these sequences are likely to be involved in some kind of substrate recognition process before CANP proteolysis occurs. The PEST sequences on CPK were found to be at the amino end of the protein and if the CANP cleavages were to occur close to these sequences, then the approximate molecular weight of the fragments produced would be 39 and 35 kDa. The approximate

molecular weights of the fragments as determined under the conditions in this study were 38 and 35kDa. Production of large fragments after cleavage of substrates close to their PEST sequences has been shown previously, (Chin et al, 1984 also Liscum et al, 1985). PEST localized CANP cleavage was shown by Liscum et al with the sites of CANP proteolysis on HMG-CoA reductase being 5 and 2kDa from the two PEST sequences on the enzyme. It seems that on CPK, like the other enzyme substrates, there are sites that CANP can recognize and proteolysis then occurs at or near these PEST sequences, (Wang et al, 1989b). Proteolysis of CPK has been shown before with proteinase K, in this case the fragment produced was 37kDa this proteolysis resulted in a loss of CPK activity. The inactivation was thought to be due to a small change in the tertiary structure of the enzyme which prevented the formation of a transition state analogue, (Price, Murray, & Milner-White, 1981). CANP is known to activate some of its kinase substrates, just how the proteolysis of these enzymes by CANP results in their activation is unclear.

An explanation of the bi-phasic response of CPK activity to increasing CANP that is demonstrated in this work could be explained in terms of quaternary structure changes with specific CPK cleavage. The quaternary structure of CPK is as yet unknown. Research to date has shown that there are certain amino acid residues that are close to Cys-278 at the active site. These residues are; Cys-69, Lys-191 and Asp-335, (James, Wyss, Lutsenk, Walliman, & Carafoli, 1990). The close proximity of these residues makes it likely that the CPK molecule is complex and folded. The presence of Cys-69 near the active site means that CPK's two pest sequences could be quite close to the active site where the catalytic complex is formed. An initial cleavage at or near the first and stronger pest sequence could remove the first 30 or so amino acids. This could act to remove Ca^{2+} ions that could bind to the pest sequence and possibly interfere with the formation of the active complex. With increasing amounts of CANP present then the second and weaker pest sequence would become a target for CANP proteolysis. Cleavage at this site on the enzyme may act to remove essential residues involved in active site complex formation. These explanations are

at best speculative but do fit the available data. The fact that the fragments formed were the size that cleavage at or near the PEST sequences would predict, makes it likely that some kind of limited proteolysis is occurring. Then with increased proteolysis of the CPK residues essential for activity could be lost. How the activity is related to proteolysis in vivo could be affected by a range of conditions not found in the in vitro experiments. These include binding of the protein to the M-line or to plasma membranes which may mask sites of proteolysis etc.

Studies of CPK's reactive cysteine side chain suggests that the environment of this side chain, which is believed to be close to the active site is somewhat modified in the proteolyzed enzyme. It has been proposed on the basis of structure prediction methods that this side chain is at the beginning of a B turn separating two portions of a B sheet, (Maggio, Kenyon, Markham, & Reed, 1977). If this is the case then this side chain may play a role in mediating conformational changes associated with the formation of the catalytically active complex. In support of this is the fact that small modifications of this side chain led to only small decreases in activity but larger modifications resulted in more significant activity losses.

The effect of CANP on this side chain could be to allow easier formation of the catalytic complex. More severe proteolysis of the CPK may then act to prohibit the formation of the active complex. An example of increased CPK activity has been shown during natural development, myofibrillar CPK Vmax is increased with no change in the Km of the enzyme. It is suggested that the alteration of CPK Vmax occurs via an interacting subunit domain effect on the conformation of the enzyme which in turn influences enzyme activity, (Dowell & Fu, 1992). However the detailed structure of the enzyme is not known and therefore the exact role that this reactive cysteine side chain may play in inhibition or activation is unclear. Comparison of the MB-CPK and MM-CPK isozymes suggested that the succeptability of the different CPK isozymes to proteolysis was not the same but that the MM (muscle) isozyme was a more compact, less flexible protein than the brain (BB) isozyme which is thought to be

a "looser" protein, (Grossman, Akinade, & Garcia-Rubio, 1990). The conformational flexibility of the CPK may be linked to its catalytic cooperative & regulation. Therefore it would be interesting to compare the CANP to CPK ratio required for activation and/or inactivation for both BB & MM CPK isozymes.

The degradation rate of a protein might depend on its intracellular location and its association with intracellular structures such as membranes or myofilaments. The localization of CPK within the cell may increase its chances of proteolysis by CANP. CPK is commonly located at cellular membranes close to sites of energy consumption. These include the Ca^{2+} ATP'ase on the sarcoplasmic reticulum and at the plasma membrane Na/K ATP'ase dependent pump, (Levitsky et al, 1977 and Sharov et al, 1977). Considering the support given to the membrane activation hypothesis of CANP, it is likely that this position on the membrane could locate CPK close to the membrane lipids that can reduce the Ca^{2+} required for CANP activation. CPK has also been shown to be localized with the enzymes of glycolysis at the I band in rat skeletal muscle, (Walliman & Eppenberger, 1985). Immunogold labelling and EM also showed uCANP to be located predominantly at the I band, (Kumamoto et al, 1992). The location of a protein can also confer added resistance to proteolytic agents in the cell, for example; neuron specific enolase and CPK both associate with structural proteins during axonal transport and are remarkably stable, (Rechsteiner et al, 1987). MM CPK is localized within the M line of skeletal muscle and has both an enzymatic and structural role. These different locations of CPK pools within the cell may help to determine their succeptability to CANP proteolysis. Therefore the susceptibility of the different CPK isozymes to CANP proteolysis within a cell could vary due to their location and conformation of the actual isozymes.

CANP may act on CPK at its specific intracellular locations within the cell depending on the factors previously mentioned, for example; protein to protein; or protein to membrane interactions; and local environmental factors such as hydrophobicity, phosphorylation of CPK

etc. CPK is subject to phosphorylation and dephosphorylation, (Hemmer, Skarli, Perriard & Walliman, 1993).

Conclusions regarding the role of CANP proteolysis of its in vitro substrates must therefore consider the many possible regulatory factors involved such as: substrate availability; intracellular location of substrate; the substrates local environment; endogenous inhibitors of the protease eg: calpain to calpastatin ratio. The physiological role of the CANP/calpastatin system is not clear despite various suggestions for its role in many different cell types. Whether CANP proteolysis of CPK plays a physiological role within the muscle cell remains to be determined. A protease that can cause limited but specific proteolysis of CPK would be well suited to influence processes such as energy transmission, ion transport, Ca^{2+} transport, and protein synthesis in the muscle cell. As it stands, the conclusions from this study should provide a basis from which to investigate the CANP/calpastatin system and its effects on CPK during periods of acute and chronic muscle dysfunction.

Chapter 6

CONCLUSIONS

The muscle isoform of CPK is a substrate for CANP in vitro. Proteolysis of the CPK results in a stimulation of CPK activity with lower amounts of CANP and activity of CPK is lost as the ratio of protease to CPK is increased.

CANP induced activation of CPK can overcome the inhibitory effect of Ca^{2+}.

CANP proteolysis of CPK results in the production of two large fragments. The size of these fragments suggest that the CPK is cleaved at or near one of the two "weak" PEST regions near the N-terminal end of its primary structure.

Suggested further research

The effect of CANP on muscle myofibrills shown to rely on CPK for the re-synthesis of ATP at the M-line would be a suitable place to start work on a more physiological model.

The succeptability of the different isozymes of CPK to CANP proteolysis would give information on the specificity of CANP towards CPK.

Development of specific antibodies towards the fragments produced by CANP proteolysis of CPK would allow these fragments to be visualized in muscle tissue. This would allow for an in vivo measure of CPK proteolysis during various types of muscle dysfunction where CANP has already shown to be active and disturbances in CPK occur.

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Appendix A:

Lowry assay procedure: Determination with Folin-Ciocalteau reagent.
Stock reagents:
A. 0.5% w/v copper sulphate ($CuS0_4.5H_20$)
B. 1% w/v sodium potassium tartrate
C. 0.1M sodium hydroxide & 2% w/v sodium carbonate, pH 12.5.
D. Folin-Ciocalteau Reagent.
E. 0.3M potassium hydroxide.
F. 5mg/ml Bovine serum albumin (BSA), in distilled water.

Method:
1. Preperation of standards.

Protein Stock (ml)	Buffer (ml)	Concentration (Final)
0	0.5	0
0.1	0.4	1 mg/ml
0.2	0.3	2
0.3	0.2	3
0.4	0.1	4
0.5	0	5

2. Dilute 0.1 ml from all standards and samples with 0.2 ml of reagent E, mix and let stand at room temperature for 10 minutes.

3. To 48ml of reagent C add 1ml of reagents A and B. This copper alkaline solution was prepared fresh when required.

4. Dilute reagent D (F-C reagent 1v:1v with distilled water).

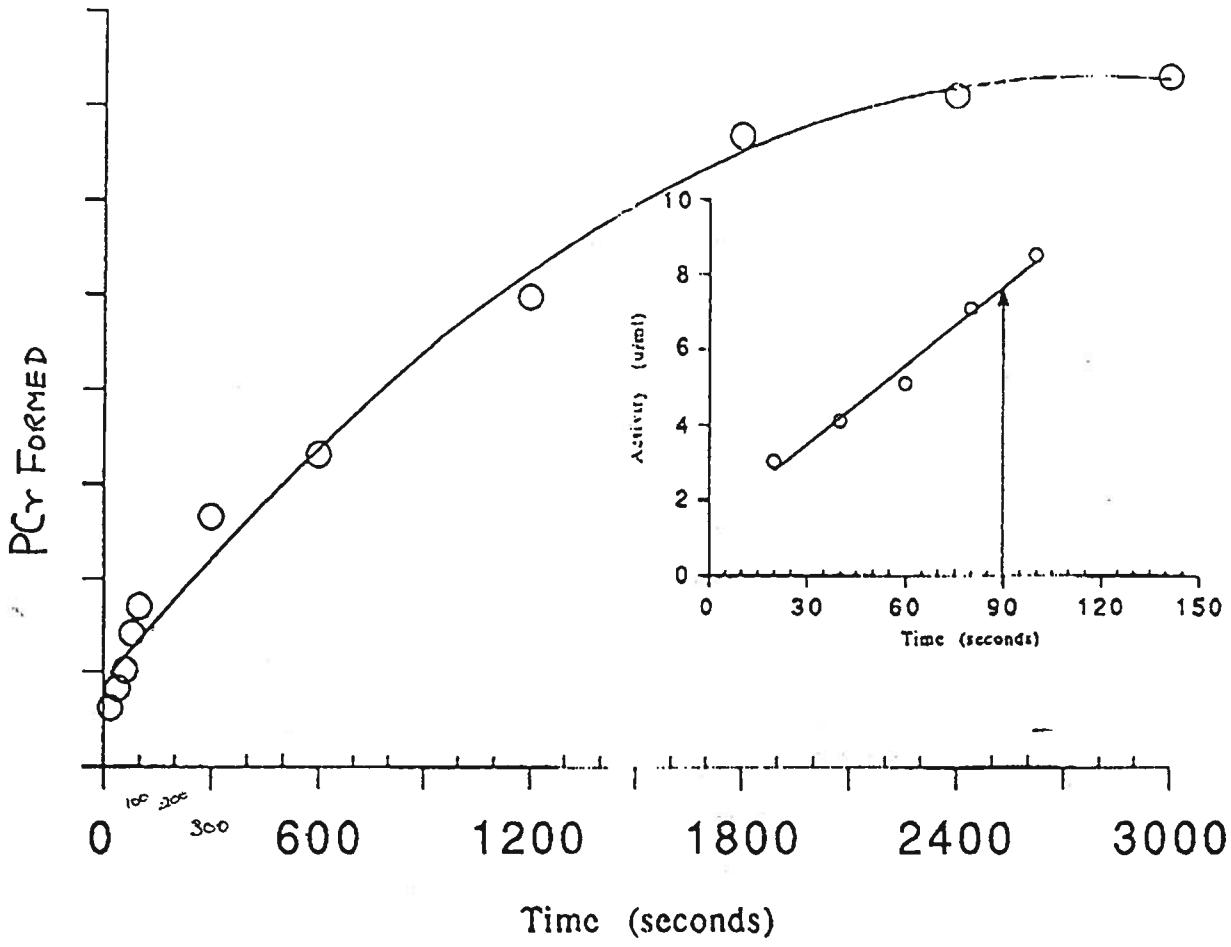
5. Take 0.1ml of all standards and samples and add 2.5 ml of copper alkaline solution (from step 3), mix and let stand at room temperature for 10 minutes.

6. Add 0.25ml of diluted F-C reagent (from step 4) to each tube while mixing with the vortex. Let stand for 30 minutes.

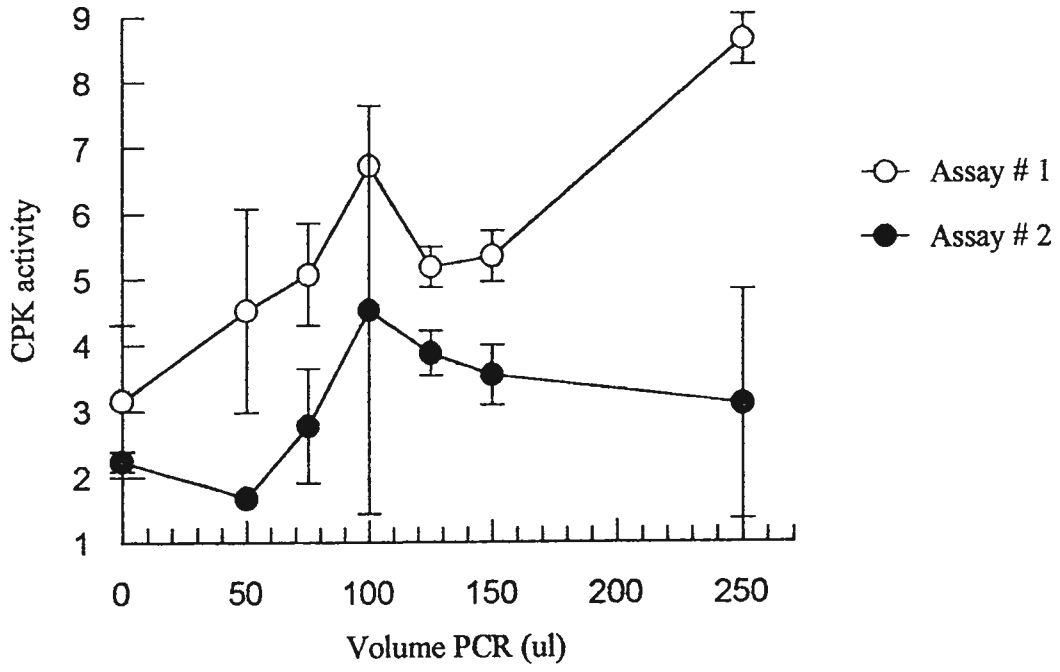
7. Measure absorbances at 750nm against the 0 (blank tube) prepared as the standards and samples.

8. Prepare a standard curve of OD750 vs protein concentration (mg/ml) to determine unknown concentrations.

Appendix B(1):
Time course of CPK activity showing the creatine produced by CPK over increasing durations of incubation under the control assay conditions, the fitted curve has R = 0.987. The smaller inset graph shows the initial linear section of the time course and the arrow at 90 seconds indicates that using this time as the end point will ensure that the measurements of CPK activity are on the linear portion of the curve.



Appendix B(2):
Data from two independent phosphocreatine curves are shown, the small degree of PCR hydrolysis allowed in 90 seconds resulted in low values for activity and high degrees of variation. Therefore I decided it would not be valid to make any comparisons from this data.



Appendix C (1):
Data for CPK activity (Sigma Units), each trial having a control assay; assay plus 5mM Ca^{2+}; and assay plus 5mM Ca^{2+} plus CANP.

	CPK activity				
	Control	Ca2+	10ug CANP	100ug CANP	
	43.7	15.61	42.3	2.8	
	43.9	16.155	41.7	-1.062	
	43.08	17.425	42.3	0.976	
			42.1	1.62	
Mean	43.56	16.4	42.1	0.9	
Std Dev	0.43	0.93	0.35	1.93	
	Control	Ca2+	27ug CANP		
	46.282	19.059	51.086		
	43.151	13.614	62.723		
	49.165	16.347	56.921		
Mean	46.20	16.34	56.91		
Std Dev	3.01	2.72	5.82		
	Control	Ca2+	54ug CANP		
	70.303	22.728	39.343		
	68.595	25.251	39.77		
	69.449	22.489	39.57		
Mean	69.45	23.49	39.56		
Std Dev	0.85	1.53	0.21		
	Control	Ca2+	67ug CANP		
	57.919	18.312	28.667		
	61.22	17.458	26.959		
	50.553	23.863	29.948		
Mean	56.56	19.88	28.52		
Std Dev	5.46	3.48	1.50		
	Control	Ca2+	84ug CANP		
	54.929	19.1	19.1		
	51.086	20.66	20.66		
	53.015	17.564	17.564		
Mean	53.01	19.11	19.11		
Std Dev	1.92	1.55	1.55		

Appendix C (2):
Data for CPK activity assays with 5mM Ca^{2+}. The data is normalized and represented as a percentage of the control assay.

	CPK activity			CPK activity	
	Control %			67 Ca2+	
	100.00			32.39	
	100.00			30.88	
	100.00			42.21	
Mean	100.00		Mean	35.16	
Std Dev	0.00		Std Dev	6.15	
	10 Ca2+			84 Ca2+	
	35.84			36.05	
	37.09			38.97	
	40.00			33.13	
Mean	37.64		Mean	36.05	
Std Dev	2.14		Std Dev	2.92	
	27 Ca2+			100 Ca2+	
	29.42			35.84	
	41.25			37.09	
	29.47			40.00	
Mean	33.38		Mean	37.64	
Std Dev	6.82		Std Dev	2.14	
	54 Ca2+				
	32.73				
	36.36				
	32.38				
Mean	33.82				
Std Dev	2.20				

Appendix C (3):
Repeated measures ANOVA of control CPK activity and CPK activity with 5mM Ca^{2+} added. Again the data is normalized and represented as a percentage of control.

Source	df	Sum of Squares	Mean Square	F-test	P value
Between Subjects		13.09	6.55	0.01	0.9891
Within Subjects		10760.47	597.8		
Treatment		10583.4	1763.9	119.54	0.0001
Residual		177.07	14.76		
Total		10773.57			
Group	Count	Mean	Std Dev	Std Error	
Control (100%)	3	100	0	0	
10 Ca2+	3	37.64	2.14	1.23	
27 Ca2+	3	35.37	5.89	3.4	
54 Ca2+	3	33.82	2.2	1.27	
67 Ca2+	3	35.16	6.15	3.55	
84 Ca2+	3	36.05	2.92	1.69	
100 Ca2+	3	37.64	2.14	1.23	
Comparison		Mean Diff	Scheffe F-test		
Control v 10 Ca2+		62.36	65.88*		
Control v 27 Ca2+		64.63	70.77*		
Control v 54 Ca2+		66.18	74.2*		
Control v 67 Ca2+		64.84	71.22*		
Control v 84 Ca2+		63.95	69.28*		
Control v 100 Ca2+		62.36	65.88*		
10 Ca2+ v 27 Ca2+		2.27	0.09		
10 Ca2+ v 54 Ca2+		3.82	0.25		
10 Ca2+ v 67 Ca2+		2.48	0.1		
10 Ca2+ v 84 Ca2+		1.59	0.04		
10 Ca2+ v 100 Ca2+		0	0		
27 Ca2+ v 54 Ca2+		1.55	0.04		
27 Ca2+ v 67 Ca2+		0.21	0.07		
27 Ca2+ v 84 Ca2+		-0.68	0.01		
27Ca2+ v 100 Ca2+		-2.27	0.09		
54 Ca2+ v 67 Ca2+		-1.34	0.03		
54 Ca2+ v 84 Ca2+		-2.23	0.08		
54 Ca2+ v 100 Ca2+		-3.82	0.25		
67 Ca2+ v 84 Ca2+		-0.89	0.01		
67 Ca2+ v 100 Ca2+		-2.48	0.1		
84 Ca2+ v 100 Ca2+		-1.59	0.04		
		* =significant at 95%			

Appendix C (4):
Data for CPK activity assays with 5mM Ca^{2+} and CANP present. Data is normalized with the activity for each CANP condition represented as a percentage of the control activity.

Scource	df	Sum of Squares	Mean Square	F-test	P value
Between Subjects	2	8.95	4.48	0.00248	0.9975
Within subjects	18	32458.2	1803.23		0.0001
Treatment	6	32092.07	5348.68	175.31	
Residual	12	366.13	30.51		
Total	20	32467.15			
Group	Count	Mean	Std Dev	Std Error	
Control (100%)	3	100	0	0	
10ug CANP	3	97.11	1.38	0.8	
27ug CANP	3	123.18	12.59	7.27	
54ug CANP	3	56.96	0.31	0.18	
67ug CANP	3	50.46	2.65	1.53	
84ug CANP	3	36.06	0.5	0.29	
100ug CANP	3	2.08	4.44	2.56	
Comparison		Mean Diff	Scheffe F-test		
Control v 10ug CANP		2.89	0.07		
Control v 27ug CANP		-23.18	4.4*		
Control v 54ug CANP		43.04	15.18*		
Control v 67ug CANP		49.54	20.11*		
Control v 84ug CANP		63.94	33.5*		
Control v 100ug CANP		97.92	78.57*		
10ug CANP v 27ug CANP		26.08	5.57*		
10ug CANP v 54ug CANP		40.14	13.21*		
10ug CANP v 67ug CANP		46.65	17.83*		
10ug CANP v 84ug CANP		61.05	30.54*		
10ug CANP v 100ug CANP		95.03	74*		
27ug CANP v 54ug CANP		66.22	35.93*		
27ug CANP v 67ug CANP		72.72	43.33*		
27ug CANP v 84ug CANP		87.13	62.2*		
27ug CANP v 100ug CANP		121.11	120.18*		
54ug CANP v 67ug CANP		6.5	0.35		
54ug CANP v 84ug CANP		20.91	3.58*		
54ug CANP v 100ug CANP		54.89	24.68*		
67ug CANP v 84ug CANP		14.4	1.7		
67ug CANP v 100ug CANP		48.38	19.17*		
84ug CANP v 100ug CANP		33.98	9.46*		
			*=significant at 95%		

Appendix C (5):
Repeated measures ANOVA of CPK activity (shown in figure 2). Control assay; assay with 5mM Ca^{2+} added ; and assay with 5mM Ca^{2+} added and either 10 or 100ug of CANP added. Activity expressed as Sigma units of activity.

Source	df	Sum of Squares	Mean Square	F-test	P value
Between subjects	2	1.98	0.99	0.0023	0.977
Within subjects	9	3875.74	430.64		
Treatments	3	3867.91	1289.3	988.42	0.0001
Residual	6	7.83	1.3		
Total	11	3877.72			
Group	Count	Mean	Std Dev	Std Error	
Control	3	43.56	0.43	0.25	
Ca2+	3	16.4	0.93	0.54	
10ug CANP	3	42.1	0.35	0.2	
100ug CANP	3	0.9	1.93	1.12	
Comparison		Mean Diff	Scheffe F-test		
Control v Ca2+		27.61	282.83*		
Control v 10ug CANP		1.46	0.82		
Control v 100ug CANP		42.66	697.44*		
Ca2+ v 10ug CANP		-25.7	253.24*		
Ca2+ v 100ug CANP		15.49	92*		
10ug CANP v 100ug CANP		41.2	650.52*		
			*=significant at 95%		

Appendix D (1):
Data from densitometric scans of 10% SDS-PAGE of CPK and increasing amounts of CANP, as shown in figure 7. Values for each protein band are reported as percent of total protein of each lane. F1 = fragment 1, F2 = fragment 2.

GEL #1	CPK	F2	F1	28kDa CANP	
Control	83.151	1.887	3.143	0	
10ug CANP	62.164	11.764	11.506	2.793	
15ug CANP	50.917	16.238	14.907	4.93	
20ug CANP	49.852	16.929	14.502	4.21	
25ug CANP	36.413	22.563	18.797	7.78	
30ug CANP	30.249	27.518	19.053	8.805	
GEL #2					
Control	97.55	0.494		0	
10ug CANP	77.454	5.178	10.738	2.177	
15ug CANP	70.631	8.137	13.355	2.592	
20ug CANP	65.04	9.445	15.463	2.908	
25ug CANP	62.07	11.679	17.43	2.646	
30ug CANP	62.105	12.189	17.46	2.425	
GEL #3					
Control	97.975		0.504	0	
10ug CANP	88.601		3.265	0.223	
15ug CANP	84.802		4.931	2.223	
20ug CANP	85.697		4.345	0.89	
25ug CANP	84.803		5.995	0.458	
GEL #4					
Control	94.825		0.969	0	
10ug CANP	90.781		2.654	0.282	
15ug CANP	87.954		4.379	539	
20ug CANP	84.33		5.313	1.159	
25ug CANP	84.752		4.204	0.627	
30ug CANP	83.123				
GEL #5					
Control	96.911		0.468	0	
10ug CANP	87.881		3.887	1.18	
15ug CANP	88.453		3.923	0.464	
20ug CANP	84.251		4.435	0.937	
25ug CANP					
GEL #6					
Control	95.259		0.9	0	
10ug CANP	86.723		4.754	0.173	
15ug CANP	82.804		4.876	1.862	
20ug CANP	84.515		4.568	0.6	
25ug CANP	91.485		3.479	0.413	

Appendix D (2):
Repeated measures ANOVA of the percent total protein of the small CANP subunit band. Control and increasing amounts of CANP added to the gels. Data from gels 2-6 were used.

Source	df	Sum of Squares	Mean Square	F-test	P value
Between Subjects	2	0.15	0.07	0.15	0.8664
Within Subjects	12	6.06	0.51		
Treatment	4	4.43	1.11	5.45	0.0204
Residual	8	1.63	0.2		
Total	14	6.21			
Group	Count	Mean	Std Dev	Std Error	
Control	3	0	0	0	
10ug CANP	3	0.23	0.05	0.03	
15ug CANP	3	1.54	0.89	0.51	
20ug CANP	3	0.88	0.28	0.16	
25ug CANP		0.5	0.11	0.07	
Comparison		Mean Diff	Scheffe F-test		
Control v 10ug CANP		-0.23	0.09		
Control v 15ug CANP		-1.54	4.4*		
Control v 20ug CANP		-0.88	1.44		
Control v 25ug CANP		-0.5	0.46		
10ug CANP v 15ug CANP		-1.32	3.21		
10ug CANP v 20ug CANP		-0.66	0.8		
10ug CANP v 25ug CANP		-0.27	0.14		
15ug CANP v 20ug CANP		0.66	0.81		
15ug CANP v 25ug CANP		1.05	2.01		
20ug CANP v 25ug CANP		0.38	0.27		
			*=significant at 95%		

Appendix D (3):
Repeated measures ANOVA of the percent total protein of fragment 1 produced with increasing amounts of CANP.

Source	df	Sum of Squares	Mean Square	F-test	P value
Between Subjects	3	0.85	0.28	0.08	0.9707
Within Subjects	12	43.68	3.64		
Treatment	3	40.65	13.55	40.33	0.0001
Residual	9	3.02	0.34		
Total	15	44.53			
Group	Count	Mean	Std Dev	Std Error	
Control	4	0.71	0.26	0.13	
10ug CANP	4	3.64	0.9	0.45	
15ug CANP	4	4.53	0.47	0.24	
20ug CANP	4	4.67	0.44	0.22	
Comparison		Mean Diff	Scheffe F-test		
Control v 10ug CANP		-2.93	17.03*		
Control v 15ug CANP		-3.82	28.91*		
Control v 20ug CANP		-3.95	31.04*		
10ug CANP v 15ug CANP		-0.89	1.56		
10ug CANP v 20ug CANP		-1.03	2.09		
15ug CANP v 20ug CANP		-1.4	0.4		
			*=significant at 95%		

Appendix E (1):
Data from four independent trials of control CPK activity (Sigma units) from the same batch of CPK.

	Trial 1	Trial 2	Trial 3	Trial 4	
	CPK activity	" "	" "	" "	
	40.944	40.411	39.944	40.838	
	41.709	38.279	36.712	38.43	
	39.865	37.01	38.73	39.421	
	41.037	40.62	38.631	41.65	
Mean	40.89	39.08	38.50	40.08	
Std Dev	0.76	1.74	1.34	1.44	

Appendix E (2):
Repeated measures ANOVA of the four independent trials of CPK activity (Sigma units) shown in appendix E (1).

Source	df	Sum of Squares	Mean Square	F-test	P value
Between Subjects	3	13.443	4.481	2.404	0.1183
Within Subjects	12	22.364	1.864		
Treatment	3	12.115	4.038	3.546	0.061
Residual	9	10.249	1.139		
Total	15	35.807			
Group	Count	Mean	Std Dev	Std Error	
Trial 1	4	40.534	0.456	0.228	
Trial 2	4	38.783	2.1	1.05	
Trial 3	4	38.756	1.254	0.627	
Trial 4	4	40.485	1.306	0.653	
Comparison		Mean Diff	Scheffe F-test		
Trial 1 v Trial 2		1.752	1.796		
Trial 1 v Trial 3		1.778	1.85		
Trial 1 v Trial 4		0.05	0.001		
Trial 2 v Trial 3		0.026	3.96E-04		
Trial 2 v Trial 4		-1.702	1.696		
Trial 3 v Trial 4		-1.728	1.748		