Volume 220, number 2, 271-277 FEB 04977 August 1987

Review Letter

Calcium-activated neutral protease and its endogenous inhibitor

Activation at the cell membrane and biological function

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Received 15 May 1987; revised version received 19 June 1987

The structures of calcium-activated neutral protease (CANP) and its endogenous inhibitor elucidated recently have revealed novel features with respect to their structure-function relationship and enzyme activity regulation. The protease is regarded as a proenzyme which can be activated at the cell membrane in the presence of Ca^{2+} and phospholipid, and presumably regulates the functions of proteins, especially membrane-associated proteins, by limited proteolysis. Protein kinase C is hydrolysed and activated by CANP at the cell membrane to a cofactor-independent form. These results are reviewed and the possible involvement of CANP in signal transduction is discussed.

Ca^{2+}-dependent protease; Enzyme inhibitor; Proenzyme activation; Protein kinase C

1. INTRODUCTION

CANP is a typical intracellular cysteine protease and absolutely requires Ca^{2+} for activity (for reviews see [1-6] and references cited therein).

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Abbreviations: CANP, calcium-activated neutral protease. The terms calpain, calcium-dependent protease (CDP), and calcium-activated protease (CAP) are also used. To denote the two isozymes, I and II are sometimes used in place of μ and m, respectively. Calpastatin is another name for the CANP inhibitor

CANP exists ubiquitously in fairly large amounts in various tissues and cells of vertebrates and is presumed to function in various fundamental cellular events mediated by Ca^{2+}, e.g. activation of enzymes like kinases, turnover of myofibrillar proteins, regulation of function of cytoskeletal proteins, receptors for hormones or growth factors, etc. [1-6]. Recently, studies on CANP have been stimulated significantly by the recognition of important roles for Ca^{2+} as a second cellular messenger. This review will summarise mainly recent results (since 1985) on the structure and function of CANP and its endogenous inhibitor, along with the activation of proCANP at the cell membrane, and hypothesise about its biological function in the activation of protein kinase C.

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00145793/87/$3.50

2. GENERAL PROPERTIES OF CANP

At least two isozymes with different calcium sensitivities exist: μ- and mCANPs active at micro- and millimolar Ca^{2+}, respectively [1-6]. Both CANP isozymes have been isolated from various tissues and cells of mammals. The concentration of Ca^{2+} for 50% activity is 2-75 μM for μCANP and 0.2-0.8 mM for mCANP [2]. Their properties, other than calcium sensitivity, are quite similar. CANP is fully active in the neutral pH region only in the presence of reducing reagents such as mercaptoethanol. The activity is inhibited by thiol group-modifying reagents and Ca^{2+}-chelating reagents (e.g. EGTA). Leupeptin, antipain, and E64 (an epoxysuccinyl derivative) are potent inhibitors [7]. It should be noted that the active-site SH group is buried in the molecule and exposed to the surface by a conformational change induced upon binding of Ca^{2+} [2]. The presence of Ca^{2+} is essential for the reaction of CANP with inhibitors as well as substrates.

Limited proteolysis is a typical feature of the action of CANP [3,6]. It hydrolyses protein substrates only to large fragments, not to small peptides or amino acids. It has no strict requirement for the sequence of the cleavage sites, although a relative preference for large hydrophobic residues such as leucine and valine in the P2 site is suggested [8,9]. CANP apparently recognises higher-order structures of protein substrates.

3. STRUCTURE OF CANP

CANP is a heterodimer composed of a large (80 kDa) catalytic and a small (30 kDa) regulatory subunit [1-6]. μ- and mCANPs from the same source have distinct but similar 80 kDa subunits, whereas their 30 kDa subunits are identical [10]. The structures of rabbit [11] and human μ- [12] and mCANPs [6], chicken [13] 80 kDa subunits, and rabbit [14], human [6] and porcine [15] 30 kDa subunits have been established by cDNA cloning. The gene structures of the chicken 80 kDa [16] and human 30 kDa [17] subunits have also been determined.

The 80 kDa subunit (~700 residues) comprises 4 domains (I-IV from the N-terminus) [13] and the 30 kDa subunit (~270 residues) is composed of at least two domains (V and IV′ from the N-terminus) [14]. Domain I (residues 1-80 in the chicken 80 kDa subunit) masks the active-site cysteine residue and is processed during activation of the proenzyme. The 80 kDa subunit of μCANP is larger than that of mCANP, a fact that is ascribed to a difference in the size of domain I. Domain II (residues 81-330) is a cysteine protease domain containing the active-site Cys-108 and His-265 residues that are highly conserved among various other cysteine proteases. The protease activity of CANP is ascribed to domain II but the function of domain III (residues 331-560) is not clear. Domains IV (residues 561-705) and IV′ (residues 99-268 of the human 30 kDa subunit), calmodulin-like domains with 4 EF hand structures, are related with 50% sequence homology. These domains bind Ca^{2+} and regulate the activity of domain II. Domain V (residues 1-70) is a glycine-rich hydrophobic domain. The glycine content in this region is about 60% and the amino acids other than glycine are mostly hydrophobic. This domain is essential for the interaction with micelles of phospholipids, potential activators of CANP in vivo.

Studies on the binding of Ca^{2+} to intact CANP are difficult due to rapid autolysis of the enzyme in the presence of Ca^{2+}. Experiments with CANP fragments corresponding to the calmodulin-like domains expressed in *E. coli* have shown that two molecules of Ca^{2+} bind to each of the three calmodulin-like domains in rabbit CANP, and that their apparent average binding constants decrease in the order of the μCANP 80 kDa, mCANP 80 kDa, and 30 kDa subunits [18]. Thus, the calcium sensitivity of CANP is essentially ascribed to the amino acid sequence of the EF hand structure.

Domain II, like other cysteine proteases, should be active without Ca^{2+} when it is isolated from the whole CANP molecule. However, integration of this domain into the whole molecule represses or inhibits the protease activity through interaction with other domains. A conformational change induced by the binding of Ca^{2+} to domains IV and IV′ removes the repression so that the intrinsic protease activity is expressed. To confirm this working hypothesis on the role of Ca^{2+} in the activation of CANP, it is essential to show that domain II itself has proteolytic activity in the absence of Ca^{2+}.

4. ACTIVATION OF THE PROENZYME

CANP, as judged from its calcium sensitivity, is inactive at physiological intracellular Ca^{2+} concentrations. However, CANP undergoes very rapid autolysis in vitro in the presence of Ca^{2+} to generate an autolysed CANP with increased calcium sensitivity that is presumably active in vivo. This autocatalytic activation is observed in CANPs from various sources [19]. Native CANP appears to be an inactive proenzyme that can be converted to an active enzyme by autolysis. In fact, native CANP exhibits a lag phase in the hydrolysis of substrate, whereas the autolysed active form shows linear rates of substrate degradation [20,21]. Moreover, under appropriate conditions, autolysis of CANP precedes hydrolysis of substrates [22]. These results are consistent with the assumption that native CANP is an inactive proenzyme. Treatment with other proteases does not mimic this autocatalytic activation of CANP. The calcium sensitivity of native CANP observed in vitro corresponds to the concentration of Ca^{2+} required for autolysis and not for actual proteolysis. The autolysed CANP requires micromolar Ca^{2+} for activity.

The autocatalytic activation of CANP is not affected by the presence of substrate or the concentration of CANP, indicating that the activation is an intramolecular process [20,21]. During autolysis, the N-terminal regions of both subunits (domains I and V) are modified [19]. In the case of rabbit CANP, about 20 residues are removed from the N-terminus of the 80 kDa subunit, and domain V is completely removed from the 30 kDa subunit. Hybridisation experiments between native and autolysed CANPs have shown that modification of the 80 kDa subunit is obligatory for the change in calcium sensitivity [19]. Modification of the 30 kDa subunit does not alter the enzyme activity, although contradictory results suggesting the importance of the 30 kDa subunit in the Ca^{2+}-dependent regulation of enzyme activity have been obtained, mainly from kinetic studies [20,21].

In vitro, mCANP requires millimolar Ca^{2+} for autolysis, making autolysis unlikely under physiological conditions. Coolican and Hathaway [23], however, found that phospholipid reduces the Ca^{2+} concentration needed for autolysis from a millimolar to a near micromolar level. Phospholipid has no effect on the autolysis of CANP devoid of domain V, indicating the importance of this domain for interaction with phospholipid liposomes and probably with biological membranes [24]. The following mechanism for the activation of CANP in vivo has been hypothesised (see fig.1) [25,26]. CANP, i.e. proCANP, exists mainly in the cytosol. When the concentration of Ca^{2+} increases to a near micromolar level, the hydrophobic regions of CANP are exposed to the surface by binding of Ca^{2+} and the enzyme translocates to the cell membrane. Autolysis of CANP occurs at the membrane in the presence of Ca^{2+} and phospholipid, and CANP becomes active as a membrane-bound enzyme. Association of CANP with the membrane is inhibited by EDTA or an endogenous CANP inhibitor. For the interaction of CANP with the cell membrane, domain V may also be important [19]. Bound CANP can be dissociated from the membrane by EDTA or endogenous CANP inhibitor. The activation of CANP at the membrane is inhibited by lowering the temperature or by the presence of leupeptin. The activated form of CANP has never been

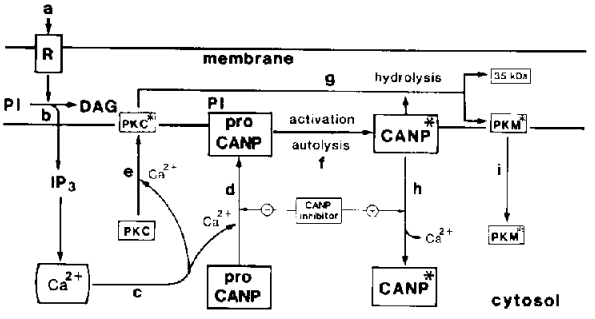

Fig.1. A model for the proteolytic activation of proCANP and protein kinase C (PKC) at the cell membrane. Stimulation of receptor (R) by outer stimuli (a) induces hydrolysis (b) of inositol phospholipids (PI) to generate diacylglycerol (DAG) and inositol phosphate (IP_3), which releases Ca^{2+} from an internal store (c). In the presence of micromolar concentrations of Ca^{2+}, both proCANP and PKC associate with the membrane (d,e). Binding to the membrane results in the autocatalytic activation of proCANP (f), which then hydrolyses PKC into PKM and a 35 kDa fragment (g). CANP and PKM are liberated from the membrane (h,i) and may function in the cytosol. CANP inhibitor inhibits the binding of CANP and promotes the release of CANP from the membrane. Asterisks indicate a catalytically active enzyme species.

isolated from tissues. It may be unstable or only a small portion of proCANP may be activated under ordinary conditions.

5. AN ENDOGENOUS INHIBITOR OF CANP

Like other cellular proteases, an endogenous protease inhibitor that specifically inhibits CANP coexists with CANP in the cytosol [27]. In some cases, this inhibitor is found associated with cellular organelles [28]. The inhibitor can be classified into two molecular species with different molecular masses on SDS gel electrophoresis. The smaller species (70 kDa) exists in erythrocytes while the larger one (110 kDa) exists in liver, heart, and most other tissues [28,29]. Both inhibitors inhibit more than one molecule of CANP, implying the presence of multi-domains for inhibition. The small inhibitor is presumed to be a derivative of the large one, because their properties are indistinguishable except for the stoichiometry of inhibition. We determined the complete structure of the mRNA encoding the rabbit liver CANP inhibitor [30] and analysed the partial structures of the two inhibitors at the protein level [31]. The structures of the liver and erythrocyte inhibitors correspond to sequences from residues 80 and 290, respectively, of the primary translation product composed of 718 residues as shown in fig.2. The two inhibitors are derived from the same precursor by different processing events. Since the CANP inhibitor has no apparent structural homology to other known protease inhibitors, it may represent a new class of protease inhibitors. Thus, the mechanism for inhibition of CANP may be different from that of other inhibitors. The primary translation product of the inhibitor contains four tandemly repeated structures of about 140 residues (fig.2). The erythrocyte and liver inhibitors comprise 3 and 4 repeats, respectively, which correspond to the number of molecules of CANP inhibited by the two inhibitors [31,32]. In addition, each domain expressed in *E. coli* inhibits one molecule of CANP. Therefore, the repeating unit shown in fig.2 is a functional unit of inhibition [30–32]. A peculiar TIPPEYR or a homologous sequence seen in each of the repeats is the presumed reactive site for inhibition. Similar repetitive domains have been reported for the pig inhibitor [34,35].

The CANP inhibitor inhibits μ- and mCANPs equally regardless of their origins by forming a complex in the presence of Ca^{2+} [27,33]. This inhibition is reversible and both active inhibitor and CANP can be recovered by dissociation of the complex [33]. CANP is not inhibited by cystatins, typical cysteine protease inhibitors. Conversely, the CANP inhibitor does not inhibit other cysteine

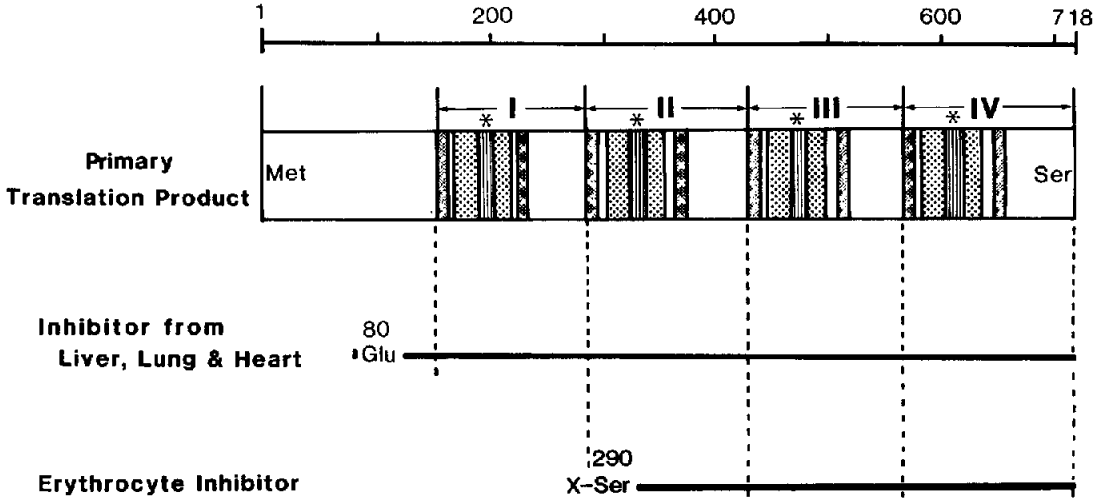

Fig.2. The structure of the primary translation product of the CANP inhibitor. Four internal repeats (I–IV) are shown. Corresponding regions are denoted by the same form of shading, the borders of domains are tentative. Asterisks show the position of a TIPPEYR or a homologous sequence.

proteases. Kininogen is exceptional among cysteine protease inhibitors and inhibits both CANP and other cysteine proteases [36].

6. BIOLOGICAL FUNCTION

As discussed, CANP is considered to function in various Ca^{2+}-mediated cellular processes as a processing protease. Among the many protein substrates hydrolysed in vitro, protein kinases are the most intriguing. The activities of various kinases increase rather than decrease following hydrolysis by CANP [1-6]. These include protein kinase C (C kinase), phosphorylase kinase, pyruvate kinase, cAMP-dependent kinase, protease-activated kinase, etc.

Protein kinase C (PKC) is a key enzyme in transmembrane signalling [37,38]. Various results indicate that PKC exists in an inactive, soluble form and translocates from the cytosol to the membrane in response to Ca^{2+} mobilisation. PKC is activated at the membrane by forming a quaternary complex with Ca^{2+}, membrane-associated phospholipid and diacylglycerol or its analog (phorbol ester).

PKC (80 kDa) can also be irreversibly activated in vitro by digestion with CANP to produce a 50 kDa fragment (PKM) which is active in the absence of Ca^{2+} and phospholipid [37]. A micromolar level of Ca^{2+} induces the translocation of both CANP and PKC to isolated neutrophil membranes [39]. Association with membranes results in the activation of CANP, which then catalyses the proteolytic conversion of PKC to PKM as shown in fig.1. This proteolytic activation of PKC has been observed in other systems in response to the tumor-promoting phorbol ester TPA [40], phorbol myristic acid [41], TPA plus a Ca^{2+} ionophore [42], etc. Proteolytic activation of PKC appears to be essential for exocytosis of granule enzymes in neutrophils, while the presence of leupeptin, a potent inhibitor of CANP, enhances the production of oxygen radicals and release of a serine protease from neutrophils stimulated by a low concentration of TPA. These results reflect the essential role of native membrane-bound PKC [43].

Although the behaviour of PKC varies among cells and tissues, and depending on the nature of stimuli, PKM can be detected in most cases in which PKM accumulates predominantly in the cytosol [41]. It is, therefore, clear that CANP degrades PKC. The critical point, however, is whether the proteolysis of PKC is physiologically important, or whether it is simply a degradation of active PKC [44].

We have recently analysed the structure of rabbit PKC [45]. The results show for the first time that PKC, which has been considered to be unique, is a mixture of at least four molecular species [46-48], and that the expression of these species varies significantly among cells and tissues. These molecular species may have different substrate specificities and affinities for cofactors, such as phospholipid, Ca^{2+}, and diacylglycerol. Therefore, the extent of translocation to the cell membrane, the mode of association with membranes, and thus the activation might be different among molecular species of PKC. These differences will affect the susceptibility to CANP. PKC shows diverse responses among cells and tissues to stimulation by external stimuli [37,38]. For example, translocation and down-regulation of PKC induced by TPA vary with the concentration of TPA, time, and cell type. Diverse responses of PKC can be explained, at least partly, in terms of the molecular diversity of PKC, which suggests the existence of multiple signalling pathways where distinct types of PKC are involved. Determination of the levels of PKC molecular species and the ratio of each in cytosolic and membrane fractions in various tissues and cells, which vary with the physiological state, are needed to clarify this point.

Structural studies [45-48] indicate that PKM corresponds to the C-terminal catalytic domain of PKC which is highly homologous to the catalytic domain of other protein kinases. The other fragment (~35 kDa) [49], produced concomitantly with PKM, is the N-terminal regulatory domain containing a binding site for diacylglycerol and possibly those for Ca^{2+} and phospholipids. CANP hydrolyses the region connecting the two domains in PKC. The activity of the kinase domain is negatively controlled by the regulatory domain, and cofactors repress the negative control. This situation is similar to that predicted for the control of the activity of the protease domain in CANP.

PKM species derived from different PKC molecular species, though their activities are cofactor-independent, may have different substrate specificities, and when released from the membrane, can phosphorylate a number of intracellular protein

substrates other than membrane-bound proteins. The 35 kDa fragment remains in the membrane [49]. Since no extreme hydrophobic regions are found in the fragment or PKC, a membrane-binding domain(s) may be induced upon binding of cofactors. This fragment may play some role in signal transduction, because it binds phospholipids, diacylglycerol and Ca^{2+}. It is worth mentioning that this fragment has cysteine-rich tandem repeats [46–49]. Similar cysteine-rich repeats are found in receptors and DNA-binding proteins. It is tempting to speculate that this fragment or PKC binds DNA and modulates its expression. It is likely that both PKM and the 35 kDa fragment may have biological functions. If so, the degradation of PKC by CANP is a physiological process.

7. CONCLUDING REMARKS

CANP first attracted attention as a protease that catalyses the degradation of muscle proteins. It is true that CANP is responsible for muscle atrophy in muscular dystrophy [2,3]. There is now increasing evidence that indicates its importance in a number of cellular functions. Although significant progress has been achieved recently in studies on the structure-function relationship of CANP and its inhibitor, its biological function has remained obscure. Unfortunately, CANP has not been found in yeast or bacteria. Nevertheless, since cDNA, genomic DNA and antibodies for CANP and its inhibitor are available, a variety of approaches for analysing the biological function of CANP are now feasible. It is hoped that the physiological function will be clarified in the near future.

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