**INTRODUCTION**

The 2’,3’-cyclic nucleotide 3’-phosphodiesterase (CNP, EC 3.1.4.37) is an enzyme which was originally characterised by its ability to hydrolyse 2’,3’-cyclic nucleotides (but not 3’,5’-cyclic nucleotides) in vitro resulting in the production of 2’-derivatives (Davis and Allen, 1956; Whitfield et al., 1955). Evidence based on subcellular fractionation, developmental studies, neurological mutants, and on histochemical and neurological studies has shown that CNP is found most abundantly in myelin of oligodendrocytes in the CNS and to a lesser extent Schwann cells of the PNS (Sprinkle, 1989; Vogel and Thompson, 1988). The absence of a physiologically relevant substrate in myelinating or any other tissues indicates, however, that this catalytic activity is unlikely to contribute to the role of CNP in vivo and therefore the function of this enzyme is still unknown.

Although the majority of studies have focused exclusively on the expression of CNP in oligodendrocytes and Schwann cells and the involvement of CNP in myelogenesis there is evidence to suggest that this enzyme may be present at lower levels in a variety of other cell types. Some of this evidence is based only on the finding of CNP-like phosphodiesterase activity in preparations derived from a variety of tissues, most notably the spleen, liver, thymus, adrenal and retinal bipolar neurons (Dreiling, 1981; Giulian and Moore, 1980; Jones and Keenan, 1981; Weissbath et al., 1981). More direct evidence comes from immunoblot and northern blot analysis which has shown that CNP is expressed in lymphoid tissues and lymphocytes (Bernier et al., 1987). In addition, mRNA for one isoform of CNP has been shown to have a widespread tissue distribution (Scherer et al., 1994). Relatively little data is available, however, on the expression of CNP protein in non-oligodendrogial cell types. Analysis of purified CNP and immunoblotting has shown that CNP is expressed as two closely migrating polypeptides of around 46 kDa and 48 kDa. These have been designated CNP I and CNP II, respectively (Sprinkle, 1989; Vogel and Thompson, 1988). It appears that mRNA encoding CNP I may be expressed mainly in the CNS whereas mRNA encoding CNP II is expressed in a variety of tissues including non-myelin associated cells (Scherer et al., 1994). It is possible, therefore, that CNP I is a myelin-specific isoform whereas CNP II may have a general function in a variety of non-myelin associated cell types. In addition, CNP has already been shown to have an intracellular distribution in some non-myelin associated cells, particularly neuronal cells. By means of immunofluorescence studies, it was shown that in the neuronal cell line B104, CNP has a discrete and specific intracytoplasmic location, consistent with it being present on an intracellular organelle (Muller et al., 1981). The identity of the organelle was not established.

In an attempt to understand the function of CNP in cells that are not myelin-associated and to examine its expression in non-neuronal cells, the present study further investigated the subcellular localization of CNP using adrenal medullary chromaffin cell cultures. In addition to chromaffin cells, this primary
culture also contains low levels of non-chromaffin cells including adrenal cortical cells and endothelial cells, thus allowing the investigation of a number of different cell types of differing cellular morphologies in the same culture. The results demonstrate that CNP is expressed in all cell types in these cultures. CNP colocalises with mitochondria in the various cell types, present in chromaffin cell culture, which display different mitochondrial morphologies. These results provide insights into possible CNP functions outside of myelin and suggest that it may have a role in mitochondrial function or mitochondrial-cytoskeletal interactions in at least certain cell types.

MATERIALS AND METHODS

Chromaffin cell cultures, rat brain homogenate and immunoblotting with anti-CNP antibody

Bovine chromaffin cell cultures were prepared from the medullas of fresh bovine adrenal glands and maintained as described previously (Burgoyne, 1992). Rat brain homogenate was prepared by homogenisation in 5 mM Tris-HCl, pH 8.0, and solubilisation in SDS-solubilisation buffer at a final protein concentration of 0.5 mg/ml. For immunoblotting, rat brain homogenate and solubilised cell samples were separated on 12.5% or 10% SDS-polyacrylamide gels, blotted onto nitrocellulose by transverse electrophoresis at 90 mA overnight, and probed as described previously (Roth et al., 1994) with anti-CNP mouse monoclonal antibodies 11-5B (at 1:500, Sigma, Dorset, UK) or antibody SMI 91 (at 1:500, produced by Sternberger Monoclonals Inc. and purchased from Affiniti, Maidstone, Kent).

RT-PCR

Total RNA was extracted from whole brain of Wistar rats and cultured chromaffin cells using an RNeasy isolation kit (Qiagen, Surrey, UK), cDNA synthesised with a reverse transcription system and cDNA encoding CNP amplified in PCR reactions using an Omne-E dryblock thermocycler (Hybaid, Middlesex, UK). The sense and antisense primers used were (5’-CCAGGATCCAGAAGCAACGCTTGGCCG-3’) and (5’-GCGTGAATTCTCAGATGAGGTGCAAGAT-3’), respectively. These were based on the nucleotide sequence of rat CNP II (Gravel et al., 1994; GenBank accession number L16532) and incorporated BamHI and EcoRI restriction sites (underlined) to facilitate cloning of the product. PCR was carried out using Taq polymerase (Promega, Southampton, UK) according to the suppliers protocol. PCR consisted of an initial denaturation cycle (95°C for 5 minutes), 30 cycles of annealing (61°C for 1 minute), elongation (72°C for 2 minutes) and denaturation (95°C for 1 minute) followed by a final elongation cycle (72°C for 10 minutes) and cooling (30°C for 1 minute).

Cell permeabilisation and assay of protein leakage

Bovine chromaffin cells, prepared and maintained in culture were permeabilised in a buffer consisting of 139 mM potassium glutamate, 5 mM EGTA, 2 mM ATP, 2 mM MgCl2 and 20 μM digitonin, pH 6.5 (300 μl/106 cells). After various times, the buffer from the cells was removed and spun at 13,000 g to remove any detached cells. The leaked proteins contained within the buffer were precipitated with an equal volume of methanol at –20°C and resuspended in 300 μl of SDS dissociation buffer/3x106 cells. Following removal of the buffer, the permeabilised cells were also solubilised in SDS buffer (300 μl/106 cells). The leaked and cell retained proteins were then separated on 12.5% SDS-polyacrylamide gel and immunoblotted with anti-CNP antibody.

Mitochondrial autofluorescence and CNP and mitochondrial immunofluorescence

Chromaffin cells were grown in culture (Burgoyne, 1992) on glass coverslips for 3 days before fixation in 4% formaldehyde in PBS. In some experiments, mitochondria were visualised by inducing specific mitochondrial autofluorescence as described previously (Cambray-Deakin et al., 1988; Willingham and Pastan, 1985). Prior to immunofluorescence labelling, cells were fixed for 30 minutes in 0.4% glutaraldehyde in PBS. The cells were then incubated in 100 mM glycine in PBS, pH 10, for 45 minutes. Immunofluorescence labelling was performed as follows. The cells were washed twice in PBS and incubated for 30 minutes in PBT(0.1% Triton X-100 and 0.3% bovine serum albumin in PBS). Following this, the cells were incubated with anti-CNP antibody (1:40) or MAB1273 monoclonal antibody against human mitochondria (1:40; Sigma, Dorset, UK) in PBT for 1 hour and washed three times in PBT. The cells were then incubated in anti-mouse IgG biotinylated (1:100) for 1 hour, washed three times in PBT followed by a further incubation in Streptavidin-fluorescein (1:50) for 30 minutes. The cells were again washed three times in PBT, and the coverslip was blotted and allowed to air dry. The coverslip was mounted on anti-fade glycerol (glycerol:PBS (9:1) containing 0.25% 1,4-diazabicyclo[2.2.2]octane and 0.002% p-phenyldiamine). All photomicroscopy was then performed using a Zeiss Universal microscope fitted with appropriate filter sets.

RESULTS

These studies used two mouse monoclonal anti-CNP antibodies. One (11-5B) has been raised against purified human CNP and was reported to recognise both CNPI and CNPII isoforms (Sprinkle et al., 1987) in brain and peripheral nerve extracts from a range of species. 11-5B recognised a major band in rat brain and slightly faster migrating band in chromaffin cells when analysed on 12.5% gels (Fig. 1A). In

![Image](https://via.placeholder.com/150)

**Fig. 1.** Detection of CNP proteins by immunoblotting with anti-CNP monoclonal antibodies in rat brain and bovine chromaffin cell cultures. Aliquots of solubilised rat brain homogenate and bovine chromaffin cell cultures were separated by SDS-polyacrylamide gel electrophoresis on 12.5% (A) or 10% gels (B) and probed by immunoblotting using two anti-CNP monoclonal antibodies as indicated. (A) The lower bands indicated by an arrow represent CNP monomer whereas the upper bands indicated by a spot represent a higher molecular mass cross-reacting polypeptide. (B) Antibody SMI 91 detected a CNP doublet in rat brain and chromaffin cells (arrows).
addition to this major band, 11-5B also recognises a band in both tissues with a molecular mass of around 80-90 kDa. In the original study using this monoclonal antibody a similar band was detected in brain and it was claimed that it represented a CNP dimer (Sprinkle et al., 1987). In contrast, a second monoclonal antibody directed against CNP (SMI 91) failed to recognise the larger polypeptide in either rat brain or chromaffin cells suggesting that its presence in 11-5B immunoblots may be due to cross-reactivity with another protein rather than due to the presence of a CNP dimer. On 10% SDS-PAGE, both 11-5B (not shown) and SMI 91 recognised a closely migrating doublet of polypeptides of around 45-50 kDa and thus likely CNPI and II in rat brain (Fig. 1B). In chromaffin cells the anti-CNP antibodies recognised a doublet on some blots but the upper of the doublet polypeptides was present at only low levels. This may suggest the presence of both CNPI and II in chromaffin cultures. Data from both anti-CNP monoclonals suggested that CNP in the bovine chromaffin cell cultures migrated ahead of that in rat brain (Fig. 1). Such a species difference between rat and bovine CNP has previously been reported from studies using 11-5B (Sprinkle et al., 1987) and also an unrelated anti-CNP monoclonal (Reiser et al., 1994) and is thus expected.

In addition to detection by immunoblotting, CNP expression was also found in chromaffin cell culture using reverse transcription and PCR amplification (RT-PCR). Using primers corresponding to the 5' and 3' ends of the coding region of CNP, co-migrating PCR products of the correct size (around 1.2 kb) were amplified from cDNA prepared from both chromaffin cell cultures and from rat brain (Fig. 2). The primers used would be expected to amplify from both CNPI and CNPII sequences. No PCR products were detected using mock reverse transcribed samples with inactivated reverse transcriptase and other primers amplified distinct PCR products. The bovine CNP sequence (Vogel and Thomson, 1987; Genbank accession number Y00405) differs in 3 positions for each primer but would still be predicted to be amplified by the primers used which were based on the rat sequence. These data are, therefore, in support of the findings on protein expression from immunoblotting.

CNP associates with membranes as a result of isoprenylation and carboxyl methylation of a cysteine residue at the C-terminal CXXX sequence and is not found as a soluble protein in brain (Braun et al., 1991; Cox et al., 1994; De Angelis and Braun, 1994). Soluble proteins leak, in a time dependent manner from digitonin-permeabilised chromaffin cells (Chamberlain et al., 1996; Sarafian et al., 1987). No leakage of CNP was detected from such permeabilised cells in two separate experiments using either the 11-5B or SMI 91 monoclonals. As a control for soluble protein leakage, the appearance of the γ-14-3-3 protein was demonstrated in the same samples used for the SMI 91 immunoblot (Fig. 3). Partial leakage of this soluble protein from permeabilised chromaffin cells has previously been demonstrated (Roth et al., 1994). In addition, no CNP was detected in cytosol fractions from adrenal medulla (not shown). These studies confirmed that CNP is membrane-associated in chromaffin cells.

In order to determine with which organelles CNP was associated, immunofluorescence studies were carried out using both CNP monoclonal antibodies. When chromaffin cells (the predominant cells in culture) were examined by immunofluorescent labelling with either anti-CNP antibody, a discrete,
punctate staining pattern was observed, consistent with CNP being present on intracellular organelles (Fig. 4a and d). No staining was observed in the nucleus or on the plasma membrane. No staining was detected (data not shown) in the absence of primary antibody and a distinct staining pattern was observed with control mouse monoclonal antibodies (HPC1 monoclonal which is directed against the plasma membrane protein syntaxin and an anti-rab3A monoclonal which stained secretory granules). Since the staining pattern differed from that of ER or Golgi markers (not shown) but was reminiscent of mitochondria, we used an anti-mitochondrial monoclonal antibody (Fig. 4g). This gave a similar staining pattern to that seen with the anti-CNP monoclonals 11-5B and SMI 91. The small size of the chromaffin cells makes analysis of distribution of proteins on intracellular organelles difficult to interpret and, therefore, we also examined the staining patterns of the larger, flat, non-chromaffin cells in the culture. Two morphologies of intracellular staining were observed with the anti-CNP monoclonals 11-5B and SMI 91 with either small elongated structures (Fig. 4b,e) or larger round structures (Fig. 4c,f). Cells with essentially the same intracellular staining patterns were also observed using the anti-mitochondrial monoclonal antibody.

**Fig. 4.** Immunofluorescence staining of three cell types in chromaffin cell cultures with anti-CNP or anti-mitochondrial monoclonal antibodies. Cultured chromaffin cells were immunostained with the anti-CNP monoclonal antibodies 11-5B (a,b,c) or SMI 91 (d,e,f) or anti-mitochondrial antibodies (g,h,i). Each vertical row shows cell types with similar cell and mitochondrial morphologies immunostained with each antibody. (a,d and g) Chromaffin cells, the most predominant cells in the culture. (b,e,h and c,f,i) Two types of non-chromaffin cell. The staining patterns with each monoclonal antibody in each cell type were closely similar. Bar, 10 μm.
The two control monoclonal antibodies (HPC1 and anti-rab3A) did not give any staining of these cell types.

The data shown in Fig. 4, show that both anti-CNP monoclonal antibodies give similar staining patterns to each other and to anti-mitochondrial monoclonal antibody, strongly suggesting that CNP was localised on mitochondria in the various cell types in chromaffin cell cultures. To confirm this point, double-labelling is required. Since all of the antibodies available were mouse monoclonals we made use of a fluorescence technique based on induced autofluorescence of mitochondria. Organelles labelled by this autofluorescence method have previously been shown to be indistinguishable from mitochondria labelled with antibodies to the mitochondrial F1-ATPase (Cambray-Deakin et al., 1988; Willingham and Pastan, 1985). Mitochondrial autofluorescence revealed a staining pattern which substantially overlapped with that observed with anti-CNP antibody immunolabelling (Fig. 5). Mitochondrial autofluorescence was still observed in the absence of primary antibody but was not observed in cells untreated with glycine. The small size and rounded morphology of chromaffin cells made detailed comparison of immunofluorescence and mitochondrial autofluorescence difficult but clear overlap of fluorescence was seen in the non-chromaffin cell types even though cell types with distinct mitochondrial morphologies were evident (Fig. 5). It seems, therefore, that CNP is likely to be present on the mitochondria of at least three different cell types present in adrenal medullary chromaffin cell culture which display differing cellular and mitochondrial morphologies.

**DISCUSSION**

CNP has usually been regarded as a specific marker of oligodendrocytes and Schwann cells and it is clear that the majority of CNP protein in the developing and adult brain is present in myelin. Reports of CNP activity (Giulian and Moore, 1980; Weissbarth et al., 1981) or protein (Muller et al., 1981) in non-myelin associated cell types have received relatively little attention despite the fact that these would suggest a more general role for CNP than merely one concerned with the specialised function of myelin. The exact role of CNP has been mysterious since the well-characterised enzymatic activity which has been assayed is based on hydrolysis of a synthetic substrate that is not found in nature and thus it is uncertain whether this activity has any relationship to its function in vivo. CNP has been suggested to function in the organisation of myelin (Vogel and Thompson, 1988) but its presence in other cell types must indicate an additional and more general cellular function. Recent work has suggested an interaction of CNP with the actin-based cytoskeleton (De Angelis and Braun, 1996).

![Fig. 5. Immunofluorescence staining of non-chromaffin cells with anti-CNP antibody and mitochondrial autofluorescence.](image) Non-chromaffin cells also present in adrenal medullary cultures were pre-treated to induce mitochondrial autofluorescence after which CNP was immunostained. (a and c) Immunostaining of CNP (11-5B), (b and d) mitochondrial autofluorescence showing cell types with distinct mitochondrial morphologies. Staining with anti-CNP monoclonal antibody revealed a punctate pattern, consistent with CNP being located on discrete intracellular organelles. Mitochondrial autofluorescence produced a staining pattern essentially identical to that produced by anti-CNP antibody, suggesting that CNP is located on the mitochondria of these cell types. Bar, 10 μm.
It was the aim of the present study to gain an understanding of the possible function of CNP in non-myelin associated cells by examining its subcellular localisation and in particular to determine whether CNP is associated with any specific organelle.

We used two mouse monoclonal antibodies generated against CNP. Both monoclonals recognised polypeptides corresponding to CNP in rat brain and the more rapidly migrating bovine CNP in chromaffin cell cultures. There was an indication of a doublet detected by the anti-CNP in chromaffin cell cultures consistent with expression of both CNPI and II but the upper polypeptide was present at low levels and difficult to resolve in some gels. One monoclonal (11-5B) also detected a larger polypeptide, previously described and claimed to be a dimer form of CNP (Sprinkle et al., 1987). This polypeptide was, however, not detected by the second monoclonal (SMI 91) indicating that it is unlikely to be a CNP dimer but instead to be a cross-reacting polypeptide. One possibility is a mammalian homologue of the goldfish gRICH protein which is of this size and is closely related to CNP in sequence and activity (Ballestero et al., 1997). Despite the additional polypeptide recognised by 11-5B both antibodies gave very similar immunofluorescence staining patterns suggesting that the major immunoreactivity detected in immunofluorescence was due to CNP.

We were able to show, by means of immunofluorescence, that CNP is found in all of the cell types present in adrenal medullary chromaffin cell culture and that this protein is apparently located on the mitochondria of these cells. These observations are consistent with previous immunofluorescence studies on the B104 neuronal cell line employing a distinct polyclonal anti-CNP antibody (Muller et al., 1981). In common with the present study, staining revealed a discrete and specific intracelluar location for CNP. Although the authors suggested that CNP might be present on the endoplasmic reticulum (Muller et al., 1981), this was not demonstrated and, in fact, the staining pattern observed was very similar to previously observed mitochondrial staining and quite distinct from characteristic ER localization patterns (Willingham and Pastan, 1985). Also in agreement with the present findings is an early report of CNP enzymic activity associated with mitochondrial membranes isolated from liver (Dreiling, 1981), though it is not certain that this activity was due to the same CNP protein characterised here.

Despite the finding of CNP associated with mitochondria in the present study, it is clear that in brain its major site of localisation is with myelin. Electron microscopy with immunoperoxidase staining has shown immunoreactivity throughout the cytoplasm of oligodendrocytes (Braun et al., 1988) but immunogold labelling studies have demonstrated its apparent specific localisation to myelin (e.g. Trapp et al., 1988). Immunogold labelling of mitochondria in brain has not been reported but this could be a consequence of the high concentration of CNP on myelin and antibody concentrations sufficient to detect lower levels of mitochondria-associated CNP. CNPs are expressed in non-myelin associated cell types (Bernier et al., 1987; Muller et al., 1981; Scherer et al., 1994) indicating that CNP must have a non-myelin function. The immunofluorescence localisation of CNP reported in the present study was based on two distinct monoclonal antibodies against CNP arguing against a peculiar cross-reaction due to one antibody. In addition, it was specific as mitochondrial staining was not detected with control incubations omitting the primary antibody or with other mouse monoclonal antibodies. It seems possible, therefore, that CNP could have a functional role on mitochondria in addition to the role that it has a myelin protein.

Although the exact role of CNP still remains unclear, a number of observations have been made which may give clues to its function. There is evidence to suggest that CNP associates with membranes and that this interaction is dependent upon post-translational modifications (Braun et al., 1991; Cox et al., 1994; De Angelis and Braun, 1994). In addition, it also appears that CNP can interact with the actin-based cytoskeleton (Braun et al., 1990; De Angelis and Braun, 1996; Dyer and Benjamins, 1989; Gillespie et al., 1989; Pererya et al., 1988; Wilson and Brophy, 1989). These observations recently prompted De Angelis and Braun (1996) to propose a model in which the role of CNP is to link membranes to the actin-based cytoskeleton. Recent studies have suggested that mitochondria are associated with the actin-based cytoskeleton (Morris and Hollenbeck, 1995; Simon et al., 1995) and, in addition an ATP-dependent motor activity has been located on the outer surface of the mitochondrial membrane (Simon et al., 1995) that may be responsible for mitochondrial motility and distribution within the cell. Although no CNP-associated ATPase activity has yet been demonstrated, it is interesting to note that this protein does have a number of motifs that together, in other proteins, are capable of binding and hydrolysing ATP (Gravel et al., 1994). In light of the findings of the present study, showing a clear localisation of CNP to mitochondria in cells in culture, it is therefore tempting to speculate that CNP has a role in the attachment of the mitochondria to the cytoskeleton or in mitochondrial motility on the cytoskeleton in addition to its role in myelin. Further work will be required to test this possibility but this does provide a framework for the investigation of CNP function as well as identifying for the first time a particular intracellular organelle with which this protein is associated.

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