Evidence for the presence of high-M_r microtubule-associated proteins and their Ca^{2+}-dependent proteolysis in synaptosomal cytosol

Robert D. Burgoyne and Richard Cumming

Laboratory of Biological Ultrastructure, National Institute for Medical Research, Mill Hill, London NW7 1AA, England

Received 5 August 1982

Calcium-dependent proteolysis of several polypeptides from rat brain and synaptosomal cytosol was observed including proteolysis of polypeptides of M_r 340 000 and 300 000. These latter polypeptides comigrated with high-M_r microtubule-associated proteins of microtubule preparations from brain or synaptosomal cytosol. Calcium influx into intact synaptosomes due to depolarisation with high potassium or veratridine or treatment with the ionophore A23187 did not result in Ca^{2+}-dependent proteolysis of any polypeptides. This may be due to the low calcium sensitivity of the protease since no proteolysis of the M_r 340 000 and 300 000 polypeptides was seen in synaptosomal cytosol at < 10 μM free Ca^{2+}.

Calcium *Microtubule* *Microtubule-associated protein* *Nerve terminal* *Synaptosome* *Protease*

1. INTRODUCTION

The presynaptic terminal of synaptosomes (pinched-off axon terminals) possesses the ability to assemble an equatorial coil of microtubules on incubation at 37°C [1–3] and using ultrastructural analysis we have recently found that these microtubules are sensitive to calcium influx into the synaptosome and are disassembled as a result of treatment with the depolarising agent veratridine or the calcium ionophore A23187 [4]. A number of proteins known as microtubule-associated proteins (MAPs) copurify with tubulin in assembled microtubules. MAPs promote microtubule assembly and are believed to stabilise microtubules against disassembly [5–8]. High-M_r MAPs are particularly sensitive to a Ca^{2+}-dependent protease present in brain cytosol [9,10] and Ca^{2+}-dependent proteolysis of these MAPs in brain cytosol fractions leads to an irreversible inhibition of microtubule assembly [9].

An immunocytochemical study has indicated that axonal microtubules do not possess high-M_r MAPs and that these MAPs appeared to be absent from presynaptic (axon) terminals [11]. Differences in the amounts of high-M_r MAPs on microtubules from white and gray matter have also been reported [12]. For this reason we examined the question of whether microtubules prepared from synaptosomes possess MAPs.

The results presented here indicate that synaptosomal microtubules have high-M_r MAPs and that MAPs in synaptosomal cytosol are susceptible to an endogenous Ca^{2+}-dependent protease. However, Ca^{2+}-dependent proteolysis was not observed in synaptosomal cytosol following treatment of intact synaptosomes with veratridine, high K^+ or A23187 and is therefore probably not involved in the regulation of the assembly/disassembly of synaptosomal microtubules under physiological conditions.

2. MATERIALS AND METHODS

Brain cytosol was prepared by homogenisation of the forebrain of adult Wistar rats in 5 mM Tris–HCl, 2 mM EGTA (pH 8.0) and centrifugation at 40 000 × *g* for 30 min at 4°C. Synaptosomes were prepared from the forebrain of rats [13] and incubated for 1 h at 37°C in a Krebs–Ringer solution (145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 2 mM $CaCl_2$, 20 mM Hepes (pH 7.5)). Samples of synaptosomes were incubated for a further 5 min in Krebs–

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

Ringer with 55 mM KCl, 75 μM veratridine, 100 μM A23187 or with no additions (control) as in [4]. Synaptosomes were pelleted in an Eppendorf microfuge and lysed by resuspension in 5 mM Tris–HCl, 2 mM EGTA (pH 8.0) and incubation on ice for 15 min. Synaptosomal cytosol and membrane fractions were prepared by centrifugation in an Eppendorf microfuge for 2 min. In experiments in which Ca^{2+}-dependent proteolysis was examined following incubation of lysed synaptosomes, control synaptosomes were lysed as above or in calcium buffers (containing 5 mM EDTA, 5 mM $MgCl_2$ and varying $CaCl_2$ to give free Ca^{2+} of 1 μM–3 mM [14]). Where indicated $CaCl_2$ was added to 4 mM final conc. and samples were incubated at 37°C for either 5 or 30 min. Cytosol and membrane fractions were then prepared as above. Microtubules were prepared from brain and synaptosomal cytosol using cycles of temperature-dependent assembly/disassembly as in [15] except that the concentration of glycerol was reduced to 1%. For SDS–polyacrylamide gel electrophoresis samples were solubilised in 125 mM Tris–HCl (pH 6.8), 1.25% SDS, 1% 2-mercaptoethanol, 10% sucrose, 2 mM EDTA and separated on 8% slab gels [16].

3. RESULTS

Incubation of brain cytosol at 37°C for 5 min in the presence of 2 mM Ca^{2+} (excess over EGTA) resulted in the almost complete proteolysis of M_r

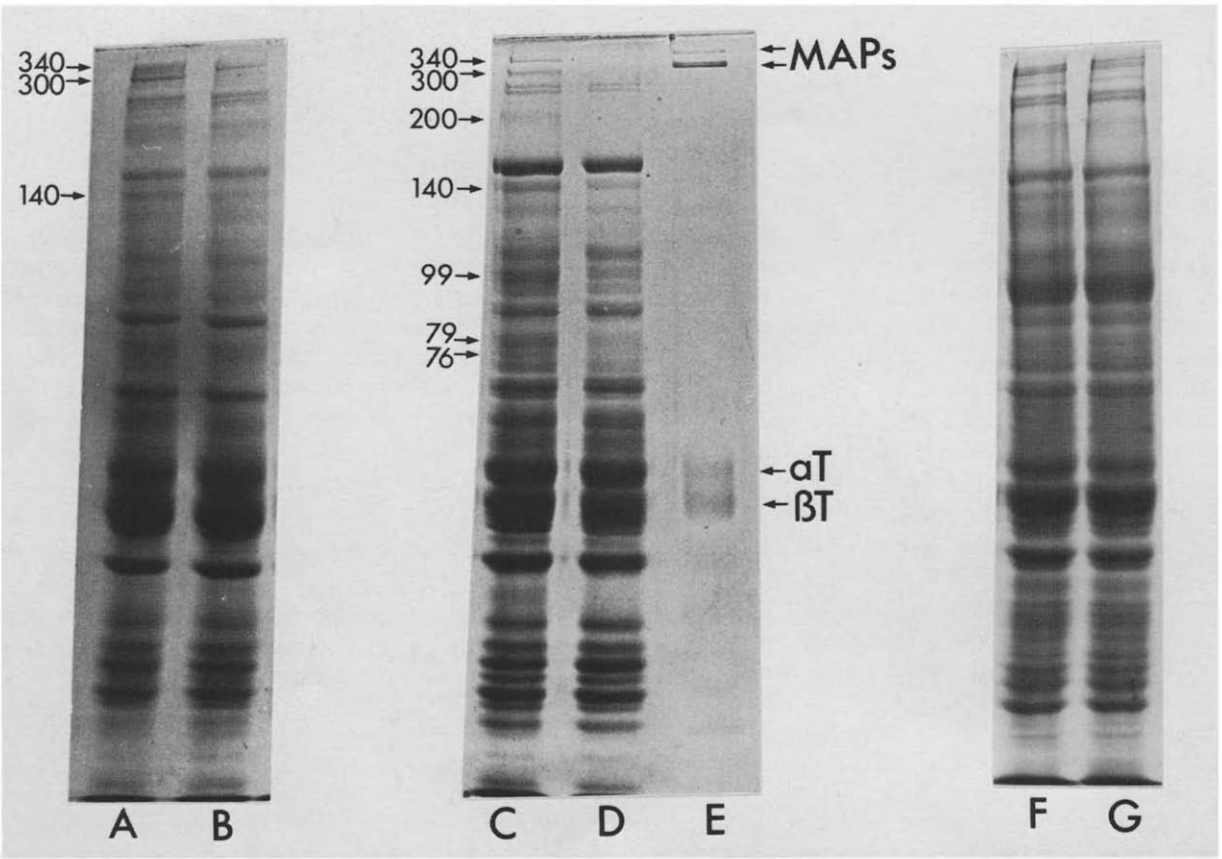

Fig.1. Coomassie blue-stained polyacrylamide gel showing whole brain cytosol (A,B), synaptosomal cytosol (C,D) and synaptosomal membranes (F,G) following incubation for 5 min at 37°C either with 2 mM EGTA (A,C,F) or with 2 mM EGTA + 4 mM $CaCl_2$ (B,D,G). A microtubule preparation purified from brain cytosol through 2 cycles of assembly/disassembly is shown in (E). Those polypeptides showing Ca^{2+}-dependent proteolysis are indicated by $M_r \times 10^{-3}$. aT,α-tubulin; βT, β-tubulin; MAPs, high-M_r microtubule-associated proteins.

300 000 and 340 000 polypeptides (fig.1). A reduction in the amount of a 140 000 M_r polypeptide was also seen. When a synaptosomal lysate was incubated under similar conditions prior to separation of cytosol and membrane fractions Ca^{2+}-dependent proteolysis was observed in the cytosol but not in the membrane fraction (fig.1). Ca^{2+}-dependent proteolysis of M_r 340 000, 300 000, 200 000, 140 000, 99 000, 79 000 and 76 000 polypeptides occurred in the cytosol fraction. Proteolysis was essentially complete within 5 min at 37°C.

The 340 000 and 300 000 M_r polypeptides comigrated with high-M_r MAPs of a 2 cycle brain microtubule preparation (fig.1, track E). In order to confirm the presence of high-M_r MAPs in synaptosomal cytosol we prepared a microtubule preparation by assembly/disassembly cycles from synaptosomal cytosol. It was evident that a set of high-M_r MAPs similar to those found in microtubule preparations from whole brain cytosol were present in microtubules polymerised from synaptosomal cytosol (fig.2). Components of these MAPs in the synaptosomal microtubule preparation comigrated with the 340 000 and 300 000 M_r polypeptides from the synaptosomal cytosol which were susceptible to the Ca^{2+}-dependent protease (fig.2).

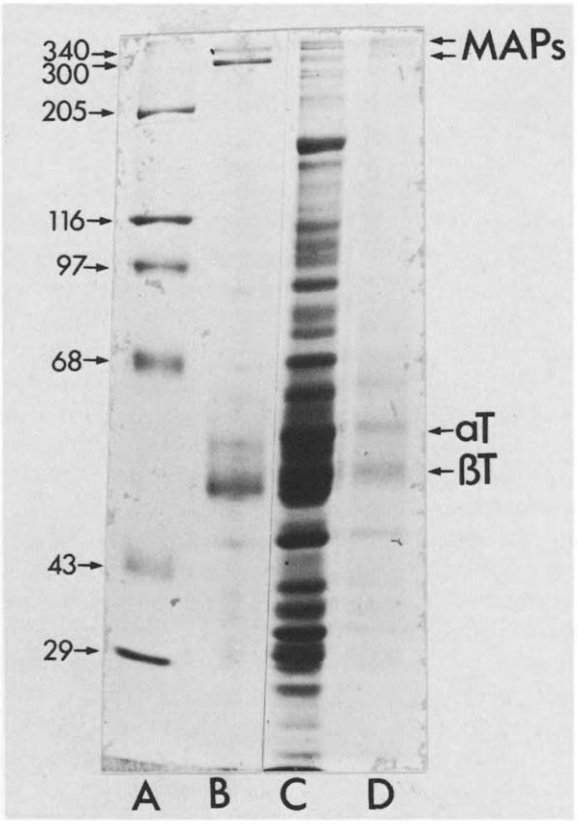

Fig.2. Polyacrylamide gel electrophoresis of: (A) M_r-standards ($\times 10^{-3}$), myosin (205), β-galactoside (116), phosphorylase B (97), albumin (68), ovalbumin (43), carbonic anhydrase (29); (B) a microtubule preparation from whole brain cytosol; (C) synaptosomal cytosol, (D) a microtubule preparation from synaptosomal cytosol.

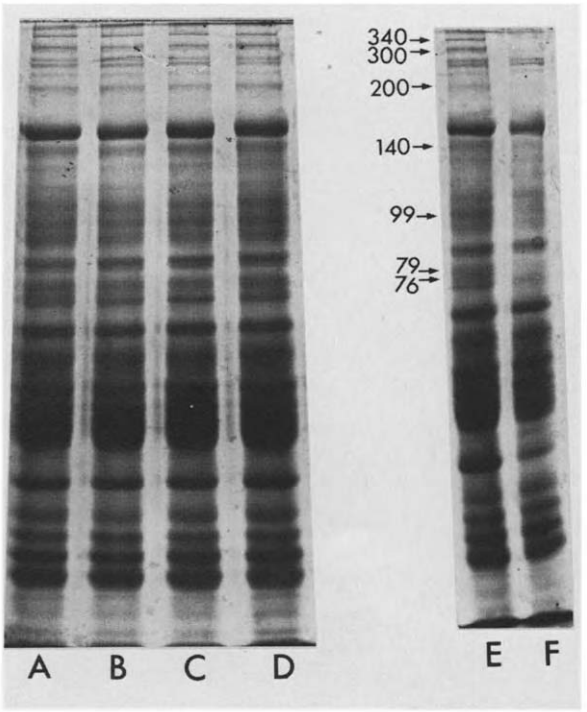

Fig.3. Polyacrylamide gel electrophoresis of synaptosomal cytosol from (A) control, (B) synaptosomes treated with 75 μM veratridine, (C) synaptosomes treated with 100 μM A23187, (D) synaptosomes treated with 55 mM K^+. Cytosol from controls was further incubated for 5 min at 37°C with 2 mM EGTA (E) or 2 mM EGTA + 4 mM $CaCl_2$ (F).

Further support for the identification of the 340 000 and 300 000 M_r polypeptides in synaptosomal cytosol as MAPs came from the observation that after pelleting of microtubules from synaptosomal cytosol, during the first cycle of assembly, the 340 000 and 300 000 M_r polypeptides co-sedi-

mented with the microtubules and the supernatant was almost completely devoid of these polypeptides (not shown). Furthermore, using conditions in which MAP 2 has been shown to be heat stable [17] we found that boiling of synaptosomal cytosol for 5 min led to the precipitation of the majority of the polypeptides while the 300 000 M_r polypeptide was heat stable (not shown). Therefore this polypeptide is probably MAP 2.

To determine whether the Ca^{2+}-dependent protease can be activated under physiological conditions we treated synaptosomes by depolarisation with high K^+ or veratridine or with the ionophore A23187. None of these treatments resulted in the proteolysis of any polypeptide in either the synaptosomal cytosol (fig.3) or membrane fractions. However, cytosol prepared from these synaptosomes in the presence of 2 mM EGTA showed marked proteolysis of a number of proteins following addition of 4 mM Ca^{2+} and incubation for 5 min at 37°C.

The absence of Ca^{2+}-dependent proteolysis in intact synaptosomes treated with veratridine, high K^+ or A23187 could be due to the low calcium sensitivity of the protease [9]. In experiments in which synaptosomal cytosol was prepared from synaptosomes lysed in calcium buffers with a range of free Ca^{2+} from 1 μM–3 mM we were unable to detect any Ca^{2+}-dependent proteolysis at or < 10 μM free Ca^{2+} (fig.4).

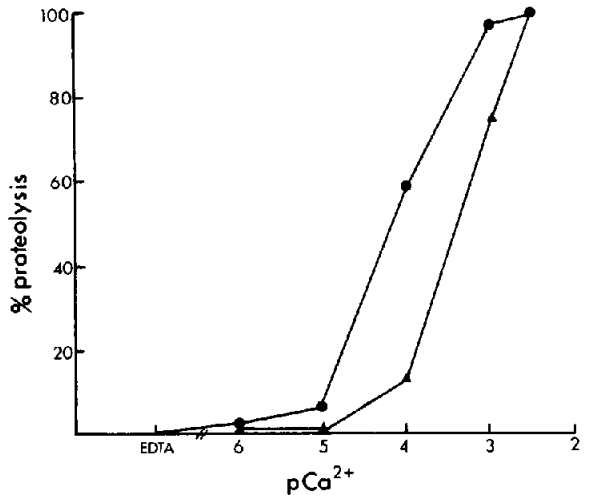

Fig.4. Ca^{2+}-dependency of proteolysis of the 340 000 (•—•) and 300 000 (▲—▲) M_r polypeptides in synaptosomal cytosol. Synaptosomes were lysed in calcium buffers containing various levels of free Ca^{2+} and incubated at 37°C for 5 min. Cytosol fractions were prepared, analysed by SDS–polyacrylamide gel electrophoresis and the level of proteolysis of polypeptides determined using quantitative densitometry.

4. DISCUSSION

We have identified substrates for endogenous Ca^{2+}-dependent protease activity in brain cytosol similar to those in [10] and also in synaptosomal cytosol. On the basis of comigration, heat-stability and co-sedimentation with microtubules two of these polypeptides in synaptosomal cytosol (M_r 340 000 and 300 000) appear to be MAPs probably corresponding to MAP 1 and MAP 2, respectively [5]. High-M_r MAPs have been shown to be particularly susceptible to a Ca^{2+}-dependent protease in whole brain cytosol [9]. The physiological significance of the Ca^{2+}-dependent proteolysis of MAPs and other polypeptides in synaptosomes is unclear since we could not demonstrate proteolysis in intact synaptosomes with conditions known to stimulate Ca^{2+} influx [18]. The absence of proteolysis can be explained on the basis of the low calcium sensitivity of the protease. No activity could be detected at free Ca^{2+} ≤ 10 μM. Stimulation of synaptosomes by depolarisation or with A23187 would be unlikely to raise the concentration of free Ca^{2+} in synaptosomes by more than 1–10 μM [19,20]. By contrast, A23187 has been shown to stimulate proteolysis of high-M_r MAPs in nucleated erythrocytes [21].

Previously, it was uncertain whether or not high-M_r MAPs are present in presynaptic nerve terminals [11]. Proteins reach the nerve terminal by axonal transport and conflicting reports exist concerning the axonal transport of high-M_r MAPs in peripheral axons [22,23]. Our demonstration that microtubules prepared from synaptosomal cytosol do possess high-M_r MAPs would appear to resolve the question of the existence of high-M_r MAPs in central nerve terminals. However, the amount of MAPs in the nerve terminal may be below the level detectable using immunocytochemistry. Alternatively, the susceptibility of the MAPs to the Ca^{2+}-dependent protease could explain the apparent absence of high-M_r MAPs from nerve terminals using immunocytochemical techniques [11].

Thus, the fixation conditions used for immunocytochemistry could permeabilise the membrane allowing influx of high concentrations of Ca^{2+} and rapid proteolysis of nerve terminal MAPs prior to immunocytochemistry. Such an explanation would require there to be a differential distribution of the protease as MAPs were detected on dendritic microtubules [11]. As yet the distribution and physiological role of the Ca^{2+}-dependent protease are unknown.

REFERENCES

[1] Chan, K.Y. and Bunt, A.H. (1978) J. Neurocytol. 7, 137–143.
[2] Hajos, F., Csillag, A. and Kalman, M. (1979) Exp. Brain Res. 35, 382–393.
[3] Gordon-Weeks, P.R., Burgoyne, R.D. and Gray, E.G. (1982) Neuroscience 7, 739–749.
[4] Cumming, R., Burgoyne, R.D. and Gray, E.G. (1982) Biochem. Soc. Trans. in press.
[5] Dentler, W.L., Granett, A. and Rosenbaum, J.L. (1975) J. Cell Biol. 65, 237–241.
[6] Keates, R.A.B. and Hall, R.H. (1975) Nature 257, 418–420.
[7] Murphy, D.B. and Borisy, G.G. (1975) Proc. Natl. Acad. Sci. USA 72, 2696–2700.
[8] Weingarten, M.D., Lockwood, A.H., Hwo, S.-Y. and Kirschner, M.W. (1975) Proc. Natl. Acad. Sci. USA 72, 1858–1862.
[9] Sandoval, I.V. and Weber, K. (1978) Eur. J. Biochem. 92, 463–470.
[10] Malik, M.N., Meyers, L.A., Iqbal, K., Sheik, A.M., Scotto, L. and Wisnieski, H.M. (1981) Life Sci. 29, 792–802.
[11] Matus, A., Bernhardt, R. and Hugh-Jones, T. (1981) Proc. Natl. Acad. Sci. USA 78, 3010–3014.
[12] Vallee, R.B. (1982) J. Cell Biol. 92, 435–442.
[13] Cotman, C.W. and Matthews, D.A. (1971) Biochim. Biophys. Acta 380–394.
[14] Burgoyne, R.D. (1981) FEBS Lett. 127, 144–148.
[15] Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Francon, J., Fellows, A., Lennon, A.M. and Nunez, J. (1978) Eur. J. Biochem. 85, 43–53.
[18] Blaustein, M.P. (1975) J. Physiol. 247, 617–655.
[19] Akerman, K.E.O. and Nicholls, D.G. (1981) Eur. J. Biochem. 115, 67–73.
[20] Nicholls, D.G. and Akerman, K.E.O. (1981) Phil. Trans. R. Soc. Lond. B. 296, 115–122.
[21] Allan, D. (1982) personal communication.
[22] Takenaka, T. and Inomata, K. (1981) J. Neurobiol. 12, 479–486.
[23] Tytell, M., Brady, S.T. and Lasek, R.J. (1980) Soc. Neurosci. abst. 6, 501.