THE INFLUENCE OF VARIOUS CATIONS ON THE BINDING OF COLCHICINE BY RAT BRAIN HOMOGENATES. STABILIZATION OF INTACT NEUROTUBULES BY ZINC AND CADMIUM IONS

V.J. NICKOLSON and H. VELDSTRA
Department of Biochemistry, University of Leiden, 64 Wassenaarseweg, Leiden, The Netherlands

Received 2 May 1972

1. Introduction

In 1965 Kane [1] discovered that the mitotic apparatus of sea urchin eggs, containing many microtubules, is stabilized by 1 M hexylene glycol (HG) in phosphate buffer. Kirkpatrick [2–4] showed that neurotubules (NT) in brain homogenates are also stabilized in this medium. If it is assumed that only the neurotubular subunit protein binds colchicine, stabilization of intact NT (for example by HG) is expected to reduce colchicine binding activity:

$$\text{intact tubules} \rightleftarrows \text{tubular protein subunits} \underset{-\text{colchicine}}{\overset{+\text{colchicine}}{\rightleftharpoons}} \begin{pmatrix}\text{subunit}\\ \text{colchicine}\end{pmatrix}\text{complex}$$

Our experiments [5] proved that this is true indeed.

Being interested in factors stabilizing NT *in vivo*, acting at low concentration, we decided to analyze a series of cations as to their capacity in this respect. Based on the assumption that only those cations, which reduce strongly colchicine binding, might be involved in the stabilization of NT we investigated their influence on the colchicine binding capacity of rat brain homogenates.

2. Materials and methods

2.1. *Preparation of homogenates*

Five day old Wistar rats were decapitated and brains were homogenized in 20 vol of 10 mM Tris-HCl buffer, pH 6.5 or 10 mM imidazole-HCl buffer, pH 7.2 Homogenates were diluted with the appropriate buffer to a final conc. of 0.455% (w/v).

2.2. *Determination of colchicine binding activity*

The following technique is partly derived from the method described by Weisenberg et al. [6]. Guanosine-5′-triphosphate (GTP; Serva) and dithiothreitol (DTT, Calbiochem) were added to the samples to final conc. of 0.1 or 1 and 3 mM, respectively, because these substabces stimulate colchicine binding by protecting the colchicine binding protein [5–7]. Samples (1 ml) were then incubated in centrifuge tubes for 45 min at 37° with 1.25×10^{-6} M [^{3}H] colchicine (ring C-methoxyl ^{3}H, 690 mCi/mM, the Radiochemical Centre, Amersham, England). After incubation tubes were transferred to an ice bath. Subsequently 1 ml of 10 mM sodium phosphate, 10 mM magnesium chloride buffer, pH 6.5 (P–Mg buffer), containing 10^{-4} M colchicine (ACF, The Netherlands) and 1 mg per ml of bovine serum albumin (Koch-Light Laboratories, USA) was added, followed by 1 ml of P–Mg buffer containing 100 mg per ml DEAE-cellulose (Serva, W. Germany). Samples were mixed well and after standing 20 min at 0°, spun off at low speed. The resulting pellets, containing protein–colchicine complexes bound to DEAE-cellulose, were washed three times with 4 ml of ice cold P–Mg buffer and suspended in 3.5 ml of a scintillator mixture, consisting of dioxane (1000 ml), naphthalene (100 g), 2,5-diphenyloxazole (PPO, 7 g), 1,4-di-(2-(5-phenyloxazolyl))-benzene (POPOP, 100 mg).

Tubes were rinsed two times with 3.5 ml of the same mixture, washings were combined with the pellet suspension and radioactivity was measured. The values for colchicine binding obtained by this

method were found to be equal to those obtained by gel filtration of the sample, incubated with ^{3}H-colchicine, on a 1 × 15 cm G-100 Sephadex column [6,8].

2.3. *Electron microscopy*

Samples were fixed in 2.5% glutaraldehyde in 10 mM sodium phosphate buffer pH 6.5 and applied to carbon or Formvar coated copper grids. Preparations were negatively stained with 2% uranylacetate (UAc) or 2% phosphotungstic acid (PTA) in water and examined in a Philips EM 200 electron microscope.

3. Results

Table 1 shows the colchicine binding activities of 0.455% (w/v) homogenates in 10 mM Tris-HCl buffer pH 6.5 containing 0.1 mM GTP, 3 mM DTT and 1 mM of one of the following salts: LiCl, NaCl, KCl, RbCl, CsCl, NH_4Cl, $BeSO_4$, $MgCl_2$, $CaCl_2$, $SrCl_2$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $CoCl_2$, $NiCl_2$. $CuCl_2$, $ZnSO_4$, $CdSO_4$, $AlCl_3$. The activities are expressed as percentage of the colchicine binding of a 0.455% (w/v) homogenate without added salts (28275 cpm ^{3}H-colchicine bound). Table 1A shows that monovalent cations have little or no effect on colchicine binding, whereas the trivalent cations which were tested cause a slight increase (probably due to increased ionic strength (cf. [7]).

Cations of the earth alkali metals, except Be^{2+},

Table 1
Influence of cations (10^{-3} M, A; 10^{-4} M, B) on the colchicine binding of rat brain homogenates.

A						B	
Li^+	104	Be^{2+}	98	Mn^{2+}	91	Zn^{2+}	7
Na^+	101	Mg^{2+}	84	Fe^{2+}	63	Cd^{2+}	7
K^+	101	Ca^{2+}	84	Co^{2+}	79	Hg^{2+}	8
Rb^+	101	Sr^{2+}	83	Ni^{2+}	39	Ag^+	2
Cs^+	101			Cu^{2+}	98	Pd^{2+}	95
NH_4^+	102	Fe^{3+}	106	Zn^{2+}	29		
		Al^{3+}	104	Cd^{2+}	3		

The binding activities are expressed as percentages of the binding of a homogenate without added salts. A. 10 mM Tris-HCl buffer pH 6.5, 3 mM DTT, 0.1 mM GTP. B. 10 mM Imidazole-HCl, pH 7.2, 3 mM DTT, 0.1 mM GTP.

reduce colchicine binding by about 15%. The divalent cations of Mn, Fe, Co, Ni, Cu and Zn, alternately exert relatively weak and strong inhibitory effects on colchicine binding.

The strongest inhibition of colchicine binding activity is obtained by addition of $CdSO_4$. As the UV absorption spectrum of colchicine does not change upon the addition of various concentrations of $CdSO_4$ at various pH values it is not probable that the effect of $CdSO_4$ is caused by the formation of Cd^{2+}–colchicine complexes. The pH optimum for colchicine binding without Cd^{2+}-ions added is about 7.0 If 1 mM $CdSO_4$ is present colchicine binding is strongly decreased, maximal binding being found at pH 6.6. The relation between $CdSO_4$ concentration and colchicine binding at pH 7.3 in a 10 mM imidazole-HCl buffer is shown in fig. 1. The possibility that the Cd^{2+}-effect depends on the formation of a complex of Cd^{2+}-ions and GTP, which protects the neurotubular subunit protein from denaturation, is ruled out by the fact that even 10^{-5} M $CdSO_4$ in the presence of 1 mM GTP causes a decrease of 87% in colchicine binding. Moreover, the relative decrease in colchicine binding caused by the addition of various concentrations of $CdSO_4$ is independent of GTP concentration. When the data for inhibition of colchicine binding by Cd^{2+}-ions are plotted on a Hill plot (according to Loftfield and Eigner [9]) a straight line is obtained with a slope of 0.49 for the concentration range of 10^{-3}–10^{-5} M $CdSO_4$.

This suggests that one Cd^{2+}-ion inhibits the binding of two colchicine molecules. Because one mole of neurotubular subunit protein binds one mole of colchicine (cf. [8,10]), one Cd^{2+}-ion can bind to two protein molecules, inhibiting colchicine binding by both of these molecules. In a separate experiment the effects of the addition of 10^{-4} M $HgCl_2$, $PdCl_2$, $AgNO_3$, $CdCl_2$ and $ZnCl_2$ to a 0.455% (w/v) homogenate in 10 mM imidazole-HCl buffer pH 7.2 containing 0.1 mM GTP and 3 mM DTT were compared. Results, as shown in table 1B, indicate that all of these salts, except $PdCl_2$, exert a strong inhibitory effect on colchicine binding.

The absence of inhibition by Pd^{2+}-ions can be explained by assuming that Pd^{2+}-ions form stable 6-coordinated complexes with imidazole, whereas Hg^{2+}, Ag^+, Cd^{2+} and Zn^{2+} form more or less unstable complexes with this compound [11]. The most intriguing

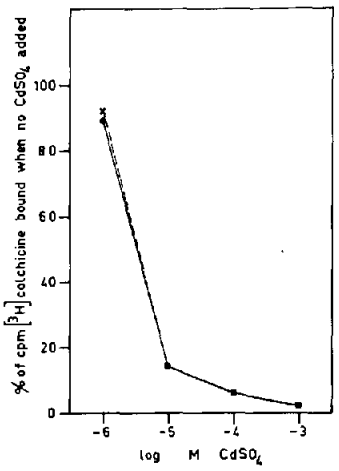

Fig. 1. Inhibition by $CdSO_4$ of colchicine binding by 0.455% (w/v) rat brain homogenate in 10 mM imidazole HCl buffer pH 7.2, containing 0.1 mM GTP (•—•—•) and 1.0 mM GTP (x--x--x). Values for colchicine binding are expressed as percentage of the value obtained when no $CdSO_4$ is added (35,000 and 35,000 cpm [^{3}H]-colchicine bound in the presence of 0.1 and 1.0 mM GTP, respectively).

question was whether inhibition of colchicine binding is directly related to stabilization of intact neurotubules as in the case of HG.

To investigate this problem, homogenates made in 10 mM imidazole-HCl buffer pH 7.2 containing 1 mM $CdCl_2$, $ZnCl_2$ or $AgNO_3$ were examined by electron microscopy.

Silver ions appeared to have no stabilizing effect because intact neurotubules were not found. In the homogenates containing cadmium ions structures were frequently found resembling neurotubules but lacking a clearly visible inner space as is seen in neurotubules in intact cells and in neurotubules stabilized by HG (fig. 2, cf. fig. 3). In the homogenate containing zinc ions many intact neurotubules were present, which were morphologically undistinguishable from neurotubules stabilized by HG (fig. 4, cf. fig. 3). Later experiments showed that even 10^{-4} M $ZnCl_2$ in 10 mM imidazole-HCl buffer pH 7.2 stabilizes neurotubules in rat brain homogenates.

4. Discussion

In rat brain homogenates no intact neurotubules are found unless stabilizing agents are added. Neurotubules apparently depolymerize very fast when the cells are disrupted. As a result of depolymerization microtubular subunit protein is liberated, which is supposed to bind colchicine [6–8, 10]. If under stabilizing conditions equilibrium exists between protein subunits and intact neurotubules (cf. scheme under Introduction) colchicine binding activity changes with different degrees of stabilization. Determination of the influence of a particular compound on colchicine binding activity therefore may yield information about the effect of this compound on

Fig. 2. Electron micrograph of a 5% (w/v) rat brain homogenate in 10 mM imidazole-HCl buffer pH 7.2 containing 1 mM $CdCl_2$; negatively stained with uranyl acetate. Note tubular structure without visible inner space. 56,400 ×.

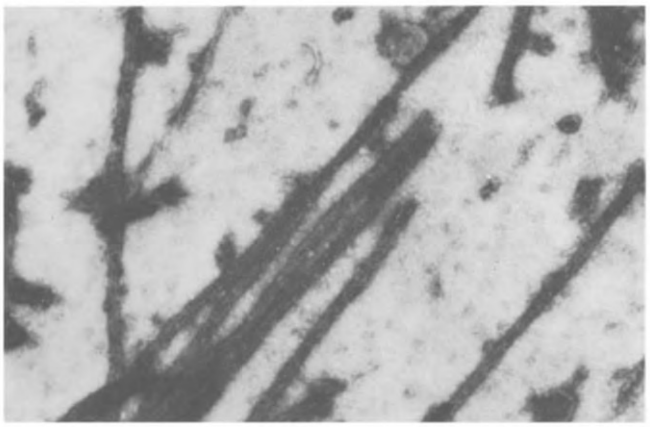

Fig. 3. Electron micrograph of a 5% (w/v) rat brain homogenate in 10 mM sodium phosphate buffer pH 6.5 containing 1 M hexylene glycol; negatively stained with uranylacetate. Neurotubules with a clearly visible inner space. 56,400 ×.

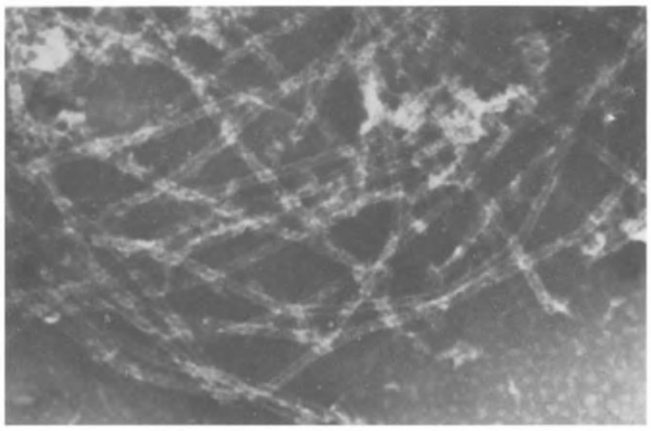

Fig. 4. Electron micrograph of a 5% (w/v) rat brain homogenate in 10 mM imidazole HCl buffer 7.2 containing 1 mM $ZnCl_2$. Negatively stained with phosphotungstic acid. Neurotubules with a clearly visible inner space. 56,400 ×.

stabilization of intact neurotubules.

The stimulating effect of DTT on the binding of colchicine by rat brain homogenates suggests that sulfhydryl residues are involved in the binding of colchicine.

It is well known [12] that Zn^{2+}, Cd^{2+}, Ag^{+} and Hg^{2+}-ions form complexes with sulfur containing ligands. In the case of neurotubular protein, binding of metal ions to the sulfhydryl groups, probably involved in the binding of colchicine, is expected to inhibit the latter process. This has been observed indeed.

The fact that one cadmium ion inhibits the binding of two colchicine molecules, suggests that Cd^{2+} binds to two protein molecules, inhibiting colchicine binding by both of them. The binding of one cadmium ion to more than one protein subunit might account for the stabilizing action of cadmium (and zinc ions) on neurotubules.

The capacity of Ag^{+}-ions to form complexes with only two ligands, whereas cadmium and zinc ions form 4-coordinated complexes, may explain why Ag^{+}-ions fail to stabilize neurotubules.

The morphological difference of neurotubules, stabilized by cadmium and zinc ions, can be explained

by the fact that complexes of cadmium and zinc, although resembling each other, are not quite equal. The observation that neurotubules stabilized by zinc ions are morphologically similar to those found in intact cells suggests that zinc ions act more physiological than cadmium ions.

The question arises whether neurotubules and other microtubules are also stabilized *in vivo* by zinc ions. Suggestive in this sense is the fact that in leucocytes, and in the spermatogenic epithelium, both containing many microtubules, a high amount of zinc is found [13], as well as in the microtubules isolated from sea urchin spermatozoa [14].

Acknowledgement

We thank Dr. J. Reedijk for helpful discussions.

References

[1] R.E. Kane, J. Cell Biol. 25 (1965) 137.
[2] J.B. Kirkpatrick, J. Cell Biol. 42 (1969) 600.
[3] J.B. Kirkpatrick, Science 163 (1969) 187.
[4] J.B. Kirkpatrick, L. Hyams, V.L. Thomas and P.M. Howley, J. Cell Biol. 47 (1970) 384.
[5] V.J. Nickolson, Thesis, Leiden (1971).
[6] R.C. Weisenberg, G.G. Borisy and E.W. Taylor, Biochemistry 7 (1968) 4466.
[7] L. Wilson, Biochemistry 9 (1970) 4999.
[8] M.L. Shelanski and E.W. Taylor, J. Cell Biol. 34 (1967) 549.
[9] R.B. Loftfield and E.A. Eigner, Science 164 (1969) 305.
[10] M.L. Shelanski and E.W. Taylor, J. Cell Biol. 38 (1968) 304.
[11] J. Reedijk, Rec. Trav. Chim. 88 (1969) 1451.
[12] O.K. Jörgensen, in: Inorganic Complexes (Acad. Press, London, 1963) chapter 7.
[13] G.H. Bell, J.N. Davidson and H. Scarborough, in: Textbook of Physiology and Biochemistry (E and S Livingstone Ltd, Edinburgh and London, 1965).
[14] M. Morisawa and H. Mohri, Exp. Cell Res. 70 (1972) 311.