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CHARGE EFFECT ON THE COLCHICINE BINDING-SITE OF TUBULIN

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Summary: Poly(L-lysine) was found to enhance colchicine binding activity of brain tubulin to a several folds. Bases of biological interests that were tested and found to be inactive were spermine, spermidine and even L-lysine. Part of this enhance binding is due to the increase in the affinity of colchicine-tubulin interaction in the presence of poly(L-lysine). Moreover, poly(L-lysine) stabilized the colchicine binding site of tubulin against thermal denaturation.

Introduction: The rates of association and dissociation of tubulin and colchicine are slow (1, 2): The activation energy for the binding reaction is approximately 20 kcal/mol (3). Recent reports from our laboratory indicate that these unusual properties of colchicine-tubulin interaction are probably the phenomena of B-ring of colchicine (4, 5). Thus, analogues with smaller (colcemid) or no substituents in the B-ring (desacetamido colchicine) bind tubulin remarkably faster than the colchicine. A compound without the B-ring such as 2-methoxy-5-(2',3',4'-trimethoxyphenyl) tropone binds tubulin even at 4°C and the binding is almost instantaneous at 37°C. However, the rate of binding of colchicine to tubulin could be enhanced significantly by the addition of certain anions

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in the reaction mixture (6). Among the anions tested, sulphate and tartrate are highly effective. Although the rate of binding is influenced by those anions, the affinity constant for colchicine remains uneffected.

We report here that in non-polymerizing conditions poly(L-lysine) enhanced colchicine binding activity to several folds. Part of this enhanced binding is due to the increase in the affinity of the colchicine-tubulin interaction in the presence of poly(L-lysine). Further, poly(L-lysine) stabilized the colchicine binding site of tubulin against thermal denaturation.

Materials and Methods: [ring C, methoxy-^{3}H] Colchicine (specific activity 5 Ci/mmole) was obtained from New England Nuclear Corporation. Podophyllotoxin was a kind gift from Dr. J. Wolff, NIAMDD, NIH, USA. GTP (grade IIs), colchicine and poly(L-lysine) of different molecular weights were the products of Sigma. Goat brain tubulin was purified according to (7) except that DEAE-cellulose (Whatman DE 52) was used instead of DEAE-sephadex A-50, in PMG buffer (10 mM potassium phosphate (pH 7.0), 10 mM $MgCl_2$ and 0.1 mM GTP. The active fractions as judged from [^{3}H]colchicine binding assay, were pooled, concentrated by overnight dialysis against 10 volumes of 8 M glycerol in PMG buffer and stored at -70°C.

Colchicine binding was evaluated by the DE 81 (Whatman) filter disc method of Weisenberg et al. (7). Protein was determined according to Lowry et al. (8) using bovine serum albumin as standard.

Results and Discussion: Fig. 1 shows that addition of poly(L-lysine) to tubulin led to a marked enhancement of colchicine binding activity. At higher poly(L-lysine) concentrations there was a steep inhibitory effect. This results in a rather narrow optimum concentration range for poly(L-lysine). However, the acuteness of this maximum varies with tubulin concentrations, indicating a stoichiometric relations. Preincubation of tubulin with podophyllotoxin, a potent inhibitor of colchicine binding site of tubulin abolished totally the colchicine binding activity of poly(L-lysine) treated and untreated sample. This result indicates that this enhancement in colchicine binding activity

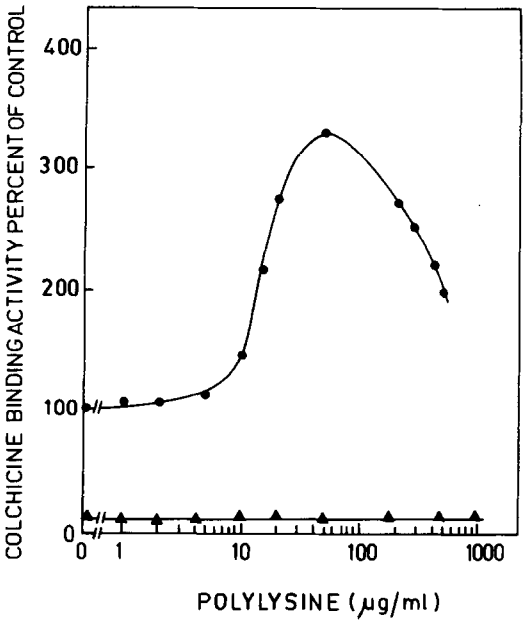

Fig. 1. Effect of poly(L-lysine) on the colchicine binding activity of brain tubulin. Tubulin (0.6 mg/ml) in PMG buffer was incubated with different concentrations of poly(L-lysine) (M.W. 195 K) at 37°C for 10 min. Colchicine binding activity of the samples were determined by further incubating the samples with [^{3}H]colchicine (10^{-6} M) at 37°C for 30 min. Data were expressed as the percent binding activity as compared to a control sample in the absence of poly(L-lysine). (●—●), Samples treated with poly(L-lysine) only; (▲—▲), Samples treated with 1.10^{-5} M podophyllotoxin prior to the colchicine binding assay.

by poly(L-lysine) treatment is not an artifact of binding assay which might arise due to the aggregation and precipitation of protein.

Other bases of biological interest that were tested and found to be inactive were spermine and spermidine. Similarly, L-lysine itself was inactive at 1.10^{-3} M (data not shown). However, large molecular weight poly(L-lysine) were increasingly more potent on a molar basis although the degree of enhancement of colchicine binding activity did not increase significantly. Thus, poly(L-lysine) of average molecular weight 350 K showed maximum enhancement of colchicine binding activity at 1.10^{-7} M, whereas this value shifted to 1.10^{-5} M for poly(L-lysine) of molecular weight 4 K. These results suggest that only monomer

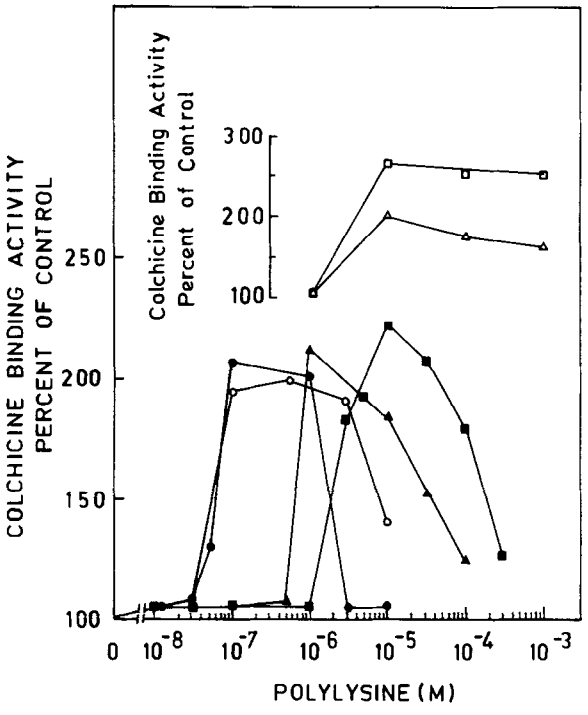

Fig. 2. Effect of poly(L-lysine) of different molecular weights on the colchicine binding activity of brain tubulin. Tubulin (0.45 mg/ml) in PMG buffer was incubated with different concentrations of poly(L-lysine) at 37°C for 10 min. Colchicine binding activity of the samples were determined by further incubating the samples with [^{3}H]colchicine (10^{-6} M) at 37°C for 30 min. Data were expressed as the percent binding activity as compared to a control sample in the absence of poly(L-lysine). Molecular weights of different poly(L-lysine) are ●—● 350 K; ○—○ 195 K; ▲—▲ 40 K; ■—■ 20 K; △—△ 13 K; □—□ 4 K.

is incapable and it required a minimal number of poly(L-lysine) residues for the stimulation of colchicine binding activity. A progressive improvement in potency occurs with large L-lysine polymers (Fig. 2).

To investigate the possibility that charge-charge interactions play a role in the effects of these bases, effect of high ionic strength on the enhancement of colchicine binding activity was investigated. As shown in Table 1, the stimulatory effect of poly(L-lysine) of molecular weight 195 K was progressively eliminated at increasing KCl concentrations. Whereas, under identical KCl concentrations the colchicine binding activity of control tubes remain unaffected. It seems clear from the KCl data

Table 1
Effect of salt concentrations on the poly(L-lysine) induced stimulation of colchicine binding activity of Tubulin.

KCl (M)	Percent stimulation of colchicine binding activity
0	110
0.1	112
0.3	125
0.5	139
0.6	95
0.7	51
0.8	36
0.9	21

Aliquots of tubulin (0.5 mg/ml) in PMG buffer were incubated with poly(L-lysine) of M.W. 195 K (50 ug/ml) in the presence of different concentrations of KCl at 37°C for 10 min. Colchicine binding activity of the samples were subsequently determined by further incubating the samples with [^{3}H]colchicine (1.10^{-6} M) at 37°C for 30 min. Data were expressed as the percent stimulation of the binding activity as compared to a control sample in the absence of poly(L-lysine).

that the charge-charge interactions play a dominant role on the vicinity of the colchicine binding-site of tubulin.

Scatchard plot of colchicine binding to tubulin was done in the presence of poly(L-lysine) with an object to see that whether the enhanced binding was due to a change in the number of binding sites or in the apparent affinity constant. As shown in the Fig. 3 there was no change in the number of binding sites in the presence of poly(L-lysine), however, there are substantial increase in the apparent affinity of colchicine tubulin interaction. Thus, the K_A for the colchicine binding in the presence and the absence of poly(L-lysine) (MW 195 K) were 6.5×10^{6} M^{-1} and 4.1×10^{5} M^{-1} respectively.

It is also possible that part of the enhancement of the colchicine binding by poly(L-lysine) might be due to the protection of the binding site against denaturation. Thus, to see if the poly(L-lysine) effect was due to a stabilization of the binding

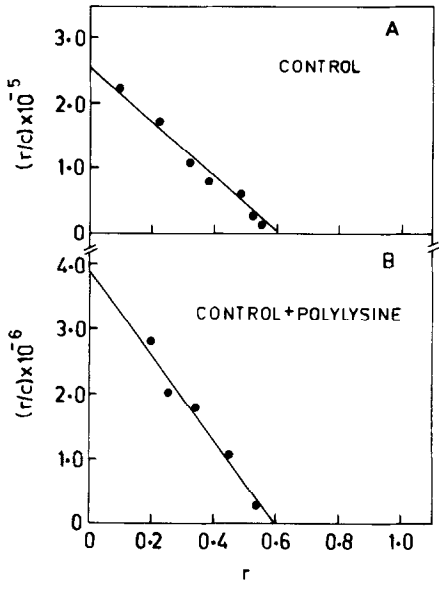

Fig. 3. Scatchard analysis of colchicine binding to tubulin in the presence and absence of poly(L-lysine). Aliquots of tubulin (0.4 mg/ml) in PMG buffer was incubated either alone (Panel A) or with 50 ug/ml poly(L-lysine) of molecular weight 195 K (Panel B) at 37°C for 10 min. Samples were further incubated with various concentrations of [^{3}H]colchicine at 37°C for 3 h and subsequently assayed for the bound [^{3}H]colchicine. Free colchicine concentrations were determined from the difference between total and bound ligand concentrations.

site, we measured the decay of the colchicine binding site of tubulin with and without poly(L-lysine). It is apparent from the Fig. 4 that there are in fact protection of the colchicine binding-site of tubulin against thermal decay. This protection of the colchicine binding-site against thermal decay is very similar to that obtained in the presence of vinblastine (9) and MAP(s) (10). However, vinblastine has been found to effect rate of the colchicine tubulin interaction without changing the apparent K_A of the reaction. In this context it is to be mentioned that most of the ionic stabilizers of the colchicine binding-site of tubulin such as vinblastine and MAP(s) are cationic in nature. On the other hand, non-ionic stabilizers like glycerol and sucrose did not

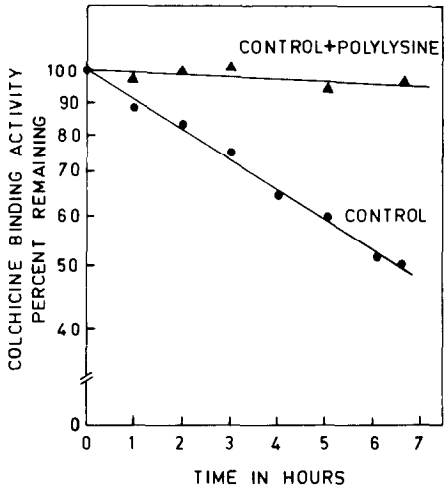

Fig. 4. Decay of colchicine binding activity of tubulin in the absence and in the presence of poly(L-lysine). Aliquots of tubulin (0.36 mg/ml) in PMG buffer were incubated either alone (●—●) or in the presence of poly(L-lysine) of molecular weight 195 K (▲—▲) at 37°C for 10 min. Samples were further incubated at 37°C for various periods. Colchicine binding activity of the samples were determined after each incubation period by further incubating the samples with [^{3}H]colchicine (1.10^{-6} M) at 37°C for 30 min. Data were expressed as the percent binding activity remained as compared to a control for which the second incubation at 37°C was omitted.

increase the apparent K_A despite a substantial reduction in the rate of denaturation of the uncomplexed tubulin.

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