The Effect of Colchicine on Human Blood Platelets under Conditions of Short-Term Incubation

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The effects of colchicine on ADP-induced aggregation and on the phosphorylation of tubulin-like protein from human blood platelets were studied. Colchicine at 2 mM concentration completely inhibits ADP-induced aggregation after 8 min incubation. Under the same inhibitory conditions, phosphorylation of tubulin-like materials in intact platelets was also impaired whereas the endogenous kinase activity of tubulin, isolated through polymerization–depolymerization cycles, was not affected. It was also shown that, under conditions of maximal inhibition of both aggregation and tubulin phosphorylation, colchicine does not penetrate into the cells. The results obtained suggest that the effect of colchicine on platelet aggregation might be mainly, although not exclusively, due to a non-specific effect of the alkaloid on the plasma membrane, rather than to a direct action of the drug on the microtubular protein subunits.

In the normal discoid-shaped platelet, microtubules appear as prominent circumferential rings sub-adjacent to the surface membrane. When the cell is activated by a variety of stimuli, a condition that subsequently leads to increased adhesiveness and aggregation, as part of the haemostatic process, the microtubules become disorganized and are often seen extending into the newly formed pseudopodia (Hovig, 1968; Lüscher & Bettex-Galland, 1972; Crawford, 1976). It has been shown that anti-microtubule drugs, such as colchicine and the *Vinca* alkaloids, are capable of modifying the platelet response induced by ADP (Hovig, 1968; Lüscher & Bettex-Galland, 1972; Crawford, 1976). Moreover, morphological changes induced by colchicine on the well-organized microtubular structures of blood platelets have also been reported (White, 1968).

Castle & Crawford (1975) have demonstrated the existence of a kinase activity associated with the tubulin-rich fraction isolated from blood platelet homogenates, and it has been suggested (Crawford, 1976) that phosphorylation/dephosphorylation processes might affect the equilibrium between microtubules and tubulin subunits. However, because of the experimental approach used, it is not yet possible to conclude what the functional role of that activity in intact platelets might be.

The high affinity of the microtubular protein, tubulin, towards colchicine has led to the conclusion

that the effects of the drug on many cellular systems are a consequence of its direct action on the microtubular structures (Olmsted & Borisy, 1973a; Margulis, 1973; Wilson *et al.*, 1974). It has been shown that isolated liver plasma membranes bind colchicine in proportion to the amount of membrane and at a rate compatible with binding to tubulin. However, other properties of the binding were clearly different from those of binding to tubulin (Riordan & Alon, 1977).

In the present paper, we report results on the effect of short-time incubation with colchicine on the ADP-induced aggregation of intact platelets, and the effect of the drug under similar conditions on the degree of phosphorylation associated with the tubulin-rich fraction of intact platelets as compared with its effect on isolated platelet tubulin.

Experimental

Materials

Sephadex and CNBr-activated Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden; DEAE-cellulose filter paper was obtained from W. and R. Balston, Maidstone, Kent, U.K.; ^{32}P as orthophosphate, [γ - ^{32}P]ATP, [*carboxy*- ^{14}C]inulin, [U - ^{14}C]sucrose and [*methoxy*- ^3H]colchicine were from New England Nuclear, Boston, MA, U.S.A. Colchicine, Mes (4-morpholine-ethanesulphonic acid) buffer, EDTA, GTP, ADP, SDS and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A. *NNN*'-Tetramethylethylenediamine (Temed), *NN*'-methylenebisacrylamide, ammonium persulphate, Coomassie Blue and acrylamide were

Abbreviation used: SDS, sodium dodecyl sulphate.

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from Bio-Rad Laboratories, Richmond, CA, U.S.A., of the best electrophoretic grade. K-12 Chinese-hamster fibroblasts were kindly supplied by Dr. Manuel Rieber from the Centro de Biología Celular, IVIC. All other reagents obtained were of the highest purity obtainable.

Platelet-rich plasma

This was isolated from human citrated blood of healthy donors at the Blood Bank of the Hospital Universitario de Caracas. The blood was centrifuged in a PR2 International Centrifuge, at 190g for 15 min at 18°C. The supernatant plasma was carefully collected in a transfer bag (Fenwall TA-3M) without anticoagulant. In all cases the residual erythrocytes were removed from the platelet-rich plasma by repeating the centrifugation at 700g for 55 s as previously described (Apitz-Castro *et al.*, 1976). Platelet-poor plasma was obtained by centrifugation of platelet-rich plasma at 3000g for 10 min, followed by 30 min centrifugation of the supernatant plasma at 30000g. The platelet-rich plasma was always kept at room temperature (23–25°C) and used within 2.5 h.

Platelet aggregation

A Gilford 240 spectrophotometer (Gilford Instruments Laboratories, Oberlin, OH, U.S.A.), which was modified to include a magnetic stirrer under the cell compartment, was used to measure the extent of platelet aggregation as described previously (Apitz-Castro *et al.*, 1976). In experiments designed to study the effect of colchicine on the degree of platelet aggregation, a saturation curve for ADP was always established beforehand, in order to select the concentration of ADP giving the maximum and half-maximum effects.

Phosphorylation of tubulin-like material from intact platelets suspended in plasma

Platelet-rich plasma (40 ml) was incubated for 1 h with 150 μ Ci of [32 P]P_i/ml in 0.15 M-NaCl, pH 7.3, at 37°C, as described previously by Apitz-Castro *et al.* (1976). For the experiments designed to study the effect of colchicine on the phosphorylation of tubulin, platelet-rich plasma was divided into two equal samples (after addition of the [32 P]P_i), and colchicine was added to one of them at a final concentration of 2 mM. An equivalent amount of 0.9% NaCl, pH 7.4, was added to the control. Both were incubated at 37°C for 8 min. At the end of the incubation, control and colchicine-treated cells were centrifuged down (1000g for 30 min followed by 5000g for 15 min) in a glycerol gradient (0–40%, v/v), and tubulin-rich material was obtained by affinity chromatography, as described above. The phosphorylated eluates were concentrated and subjected to SDS/polyacrylamide-gel electrophoresis.

Phosphorylation of platelet tubulin-like material in vitro

Depolymerized tubulin-rich material (100 μ g) was incubated with 3.5×10^7 c.p.m. of [γ - 32 P]ATP in phosphate/magnesium buffer (10 mM-phosphate buffer/10 mM-MgCl₂, pH 7.4) at 30°C for 8 min, with and without added colchicine (2 mM). The reaction was stopped by addition of 50 mg of bovine serum albumin/ml, followed by 2 ml of 5% (w/v) trichloroacetic acid. The precipitated proteins were washed as described by Piras & Piras (1974) and dissolved in 0.5 M-NaOH. Samples of these were used for the radioactivity measurements.

Colchicine influx

Platelet-rich plasma (1 ml) was incubated with 200 μ l of [3 H]colchicine (1:500) and 100 μ l of [14 C]inulin (1:100) for 8 min at 37°C. This mixture was then carefully layered on 30 ml of a glycerol gradient (0–40%) and centrifuged, initially at 1000g for 30 min and subsequently for 15 min at 5000g. The supernatant was completely removed by aspiration. The platelet residue was solubilized in 1.1 ml of 1% SDS, from which samples were collected for determination of the $^3\text{H}/^{14}\text{C}$ ratio. The quenching correction factor was determined by comparison between the radioactivity (c.p.m.) from known amounts of [14 C]inulin and [3 H]colchicine in the presence of fixed amounts of solubilized cells and that obtained from the same amounts of both radioisotopes in the presence of an equivalent amount of 1% SDS. The same general procedure was used when [14 C]sucrose was substituted for the inulin. To test the validity of this experimental approach, K-12 Chinese-hamster fibroblasts were used under identical conditions. The cells were grown at 37°C in Dulbecco's medium containing 10% foetal serum, detached from the substrate with 0.5 mM-EDTA in phosphate/saline buffer (50 mM-phosphate/0.9% NaCl), pH 7.4. Cells were then harvested by centrifugation for 10 min at 500g and resuspended in Dulbecco's medium without foetal serum (Rieber & Bacalao, 1974). Samples (1 ml) were incubated and processed as described above for platelet-rich plasma.

Isolation of platelet tubulin

The tubulin fraction was prepared from the soluble phase of platelet homogenates, obtained by a combination of the glycerol lysis technique, described by Barber & Jamieson (1970), and a method reported by Shelanski *et al.* (1973). Two methods were used for the purification of tubulin-like material: (i) affinity chromatography on colchicine-Sepharose, as described by Schmitt & Littauer (1974), and (ii) temperature-dependent polymerization, as described by Castle & Crawford (1975, 1977).

Platelet-rich plasma (20 ml) was carefully layered on 30 ml of glycerol gradient (0–40%) and centrifuged,

first at 1000g for 30 min, and then at 5000g for 15 min. The supernatant was completely removed by aspiration. The platelet residue was suspended and homogenized in 2 vol. of phosphate/magnesium buffer, containing 0.3M-sucrose and 1 mM-GTP, for 3 min at 4°C. The homogenate was centrifuged at 100000g for 1 h at 4°C in a Beckman ultracentrifuge type L2-65B. The supernatant (soluble fraction) was diluted to 2 mg of protein/ml with phosphate/magnesium buffer, and used for affinity chromatography on colchicine-Sepharose.

The temperature-dependent polymerization of tubulin-like material was achieved by homogenization of the platelet residue (as described above) with the polymerization buffer used by Crawford & Castle (1976). The soluble phase thus obtained was subjected to polymerization in 4M-glycerol.

In all cases the [^3H]colchicine-binding activity of tubulin was assayed by the procedure of Weisenberg *et al.* (1968).

The tubulin-like material isolated by both methods was either used immediately or stored in phosphate/magnesium buffer containing 0.8M-sucrose and 1 mM-GTP, pH 7.0, at -15°C.

Protein content

This was determined by the Lowry method, as modified by Wang & Smith (1975), and expressed as mg equivalent of bovine serum albumin.

SDS/polyacrylamide-gel electrophoresis

Eluates from colchicine-Sepharose were treated with 2.5% (w/v) SDS in 0.125M-Tris/HCl buffer, pH 8. SDS/polyacrylamide gels (7.5%, w/v) were prepared and run in a buffer system of 25 mM-Tris-base buffer, 192 mM-glycine and 0.1% SDS at pH 8.3. Tubulin-rich fraction isolated by polymerization of the platelet soluble phase was denatured in the same way, but the electrophoresis was performed in 0.4M-boric acid/0.1M-Tris/0.1% SDS at pH 7.0 in 7.5% polyacrylamide gels. All gels were run at 3 mA/gel with Bromophenol Blue as tracking dye, and stained with Coomassie Blue as described by Fairbanks *et al.* (1971). Slicing of the gels for radioactivity counting was accomplished in unfixed gels frozen with solid CO_2 . The slices were incubated overnight in scintillation-counting vials with 0.4 ml of 0.5M-NaOH at 50°C. After cooling, 5 ml of Insta-gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) was added to each vial, which was cooled in the dark for 2 h before the radioactivity-counting procedure.

Results and Discussion

As shown in Fig. 1, there is an inverse linear relationship between time of incubation with colchicine and the degree of platelet aggregation. This relationship is affected by varying the concentration of the aggregating stimulus (ADP), and complete inhibition with 2 mM-colchicine is achieved after 8 min incubation, when the final concentration of ADP is saturating. A lower concentration of colchicine (1 mM) requires more than twice the incubation time to achieve about 70% inhibition, and colcemid produced only 60% of inhibition after 8 min incubation at 4 mM concentration.

Fig. 1. Time-dependence of the inhibition of platelet aggregation induced by colchicine

Each point represents the mean value obtained from three different donors. The temperature was 37°C. ○, Saturating ADP concentration; △, half-saturating ADP concentration.

White (1968) has reported that, to eliminate microtubules from over 90% of the platelets, the alkaloid concentrations needed were of the order of 1–2 mM. Unfortunately, time-dependent experiments were not performed and all the data reported came from experiments in which platelets were incubated for 30 min. It should be noted that, in contrast with many other cellular systems (White, 1968; Olmsted & Borisy, 1973a,b; Wilson *et al.*, 1975), in the case of

blood platelets relatively high concentrations of the alkaloids are needed to (a) inhibit ADP-induced aggregation, or (b) produce morphological alterations of the microtubular structures. To explain this behaviour, two alternatives seem attractive: (1) that colchicine is bound to some macromolecular components present in platelet-rich plasma and, in consequence, the effective alkaloid concentration would be lower, and (2) that in this particular case colchicine is not being taken up by the platelet, or the uptake rate is very low.

The first alternative can be dismissed, since it has been shown (Donigian & Owellsen, 1973) that, although plasma proteins are capable of binding colchicine, this binding represents less than 10% of total drug. Our own experiments, using a molecular-sieve approach (not discussed here), confirm these results.

In order to study the second possibility, platelet-rich plasma was incubated with a mixture of [^3H]-colchicine and [^{14}C]inulin or [^{14}C]sucrose, as described in the Experimental section. Table 1 shows that the $^3\text{H}/^{14}\text{C}$ ratio, when inulin is used as the extracellular marker, is slightly decreased, whereas when sucrose is used no change in the $^3\text{H}/^{14}\text{C}$ ratio is observed. These results are in contrast with those obtained when platelets were replaced by Chinese-hamster fibroblasts. In this case, as shown in Table 1, a 23-fold increase in the $^3\text{H}/^{14}\text{C}$ ratio after 8 min incubation with the drug was obtained. The increased $^3\text{H}/^{14}\text{C}$ ratio clearly demonstrates that colchicine has been taken up by these cells.

These findings suggest that, under conditions of maximal inhibition of the platelet response to ADP-induced aggregation, colchicine does not penetrate the cells. They also suggest that the binding of colchicine to the platelet plasma membrane must be very weak, and in consequence the drug is easily washed out during the cell passage through the glycerol gradient.

Table 1. [^3H]Colchicine influx into platelets suspended in plasma compared with its influx into Chinese-hamster fibroblasts

Results are means of three duplicate experiments $\pm$ s.d. For Chinese-hamster fibroblasts, only [^{14}C]-sucrose was used as external marker (see the text for details).

	$^3\text{H}/^{14}\text{C}$ ratio	
	[^{14}C]Inulin	[^{14}C]Sucrose
Incubation medium (platelet-rich plasma)	1.01 ± 0.03	1.10 ± 0.05
Platelets (1% SDS)	0.65 ± 0.08	1.03 ± 0.07
Chinese-hamster fibroblasts	—	23.30 ± 0.25

Fig. 2. ^{32}P labelling of the tubulin-rich fraction from intact platelets suspended in plasma

Radioactivity distribution of 150 μg of proteins eluted from colchicine-Sepharose and subjected to SDS/polyacrylamide-gel electrophoresis. S, beginning of running gel (7.5% acrylamide); T, position of tracking dye at the end of the run. Gels obtained with Coomassie Blue were monitored at 650 nm as described by Fairbanks *et al.* (1971). Fractions were of 2 mm. Continuous trace, A_{650} ; histogram, radioactivity.

The decrease in $^3\text{H}/^{14}\text{C}$ ratio with inulin might indicate that inulin is partially absorbed on the platelet surface, a condition that should be kept in mind when this compound is used as extracellular marker, at least for platelet studies.

However, in spite of the apparent impermeability of platelets towards colchicine, the pattern of phosphorylation of tubulin-like material in intact platelets is strongly affected by the presence of inhibitory concentrations of the alkaloid. Fig. 2 shows the electrophoretic pattern of the protein fraction eluted from colchicine bound to Sepharose, as described in the Experimental section, after incubation of platelet-rich plasma with [^{32}P]P_i for 1 h at 37°C. The ^{32}P -labelled material, which appears at 3.7 cm from the origin, coincides in electrophoretic mobility with

purified tubulin. Although we do not have any direct evidence, the radioactivity band at 4.9cm from the origin might be due to phosphorylated actin, a serious contaminant of most tubulin preparations. The high-molecular-weight contaminants, described by many authors as dynein-type proteins, are also able to incorporate the label. Crawford & Castle (1976) have suggested that these high-molecular-weight proteins are important components of cytoplasmic microtubules, and possibly represent the short filamentous projections often seen on them. Samples obtained as before, derived from colchicine-treated (2mM), platelets and subjected to SDS/polyacrylamide-gel electrophoresis, show (Fig. 3) a pattern of radioisotope distribution qualitatively similar to the above described for control cells. However, quantitatively a striking difference in the degree of labelling of the tubulin and actin-like materials can be observed, whereas no significant

Table 2. *Phosphorylation in vitro of tubulin isolated by polymerization of the platelet-soluble phase*
Results are means of three replicate experiments $\pm$ S.D.

Addition	^{32}P incorporation (c.p.m./100 μg of protein)
None	12900 $\pm$ 100
Colchicine (10 μM)	10600 $\pm$ 90
Colchicine (2mM)	13500 $\pm$ 100

difference is observed in the high-molecular-weight region of the gel.

The differences in the degree of labelling might be ascribed to a differential uptake of the labelled compound by the cell; however, experiments performed to check this possibility consistently showed a $20 \pm 3\%$ ($n = 5$) increase in total radioisotope incorporation in the colchicine-treated cells.

Hence the observed decrease in the degree of phosphorylation in colchicine-treated platelets of at least two of the intraplatelet contractile systems described by Crawford & Castle (1976) does not seem to be due to an impaired uptake of the labelled compound.

Piras & Piras (1974) have described the phosphorylation of chick-embryo muscle and HeLa-cell tubulin, with both intact cells and partially purified preparations. These authors suggest that the phosphorylation of the microtubular fractions, isolated from chick-embryo muscle, is catalysed by a kinase activity apparently associated with that fraction. Colchicine (10 μM) does not seem to affect the endogenous phosphorylation of their tubulin preparation; however, it does affect the exogenous kinase activity (casein and ATP as substrates). As shown in Table 2, in platelets, colchicine, even at concentrations of 2 mM, does not affect the phosphorylation *in vitro* of the tubulin-rich fraction isolated by the polymerization method (Castle & Crawford, 1975). These results confirm those of Piras & Piras (1974), but contrast with the colchicine-induced inhibition of phosphorylation that we have found with intact platelets suspended in plasma.

At the present, we do not have a clear explanation for this behaviour; however, taken together, the results discussed seem to suggest that the inhibitory effect of colchicine on platelet aggregation, under the conditions described, might mainly be related to a non-specific effect of the alkaloid on the plasma membrane rather than to a direct action of the drug on the cytoplasmic microtubular protein subunit.

A relationship between plasma-membrane proteins and intracellular contractile elements has been proposed in several cell systems (Riordan & Alon, 1977; Crawford, 1976). The possibility arises that, in our

Fig. 3. *Effect of colchicine on the ^{32}P incorporation in the tubulin-rich fraction from intact platelets suspended in plasma*

All other conditions were as in Fig. 2. $\square$, Control; $\blacksquare$, 2mM-colchicine for 8 min. Fractions were of 2mm.

experiments, changes induced in the plasma membrane by colchicine can disrupt its functional relationship with subadjacent contractile proteins, impairing in that way the response of the latter to stimuli.

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