EFFECT OF COLD TREATMENT AND SOME DRUGS ON THE 5-HYDROXYTRYPTAMINE UPTAKE BY RABBIT BLOOD PLATELETS AND THEIR ULTRASTRUCTURE

Hiroaki NISHIO, Tomio SEGAWA and Hiroshi TAKAGI*

Department of Pharmacology, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima 734 and
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan

Accepted July 30, 1978

Abstract—When utilizing a cold treatment we found that uptake activity of 5-hydroxytryptamine (5HT) by blood platelets of the rabbit was decreased in a non-competitive manner, while endogenous 5HT concentration of the platelets and the platelet counts were not affected. Morphologically, the cold treatment induced a dissociation and disappearance of circumferential bands of microtubules (CB-Mt) and the platelets, protruding pseudopods, lost their discoidal shape. Incubation of platelets with colchicine or vinblastine provided similar morphological alterations of CB-Mt, but there was only a slight inhibition toward the 5HT uptake, a finding which indicated that CB-Mt of platelets do not play an essential role in the 5HT uptake system of platelets. The decreased 5HT uptake by the cold treatment was sharply restored by addition of theophylline to the medium, but not by dibutyryl cyclic AMP. The former also significantly enhanced the 5HT uptake by the intact platelets, and this effect was potentiated by prostaglandin E1 (PG-E1), but not by prostaglandin E2 (PG-E2). The possibility is raised that only endogenous cyclic AMP may regulate the 5HT uptake system of platelets. Pluronic F68, non-ionic detergent, had no effect on 5HT uptake by the intact platelets but stimulated the effect of the cold treatment, probably through facilitating conformational change of membrane induced by the treatment.

It has been indicated that the nature of platelets is altered when such are stored in the cold. Becker et al. (1) demonstrated that the life span of the platelets shortened from the normal eight-ten days to two-four days after less than 24 hr of storage in the cold. Krankenhagen et al. (2) reported that platelets exhibited increasing aggregation with decrease of temperature. Thus clinical application of platelets is limited considerably. Becker et al. (3) showed that when whole blood was stored at 4°C, irreversible clumping of platelets occurred and harvest of platelets was virtually impossible, however, when small amounts of PG-E1 were added to fresh whole blood, the number of harvestable platelets was markedly increased. Behnke (4) and White and Krumwiede (5) presented evidence suggesting that dissociation and disappearance of CB-Mt were related to the cold-induced shape changes of blood platelets.

On the other hand, blood platelets have been used extensively as models for neurological studies, particularly for studies on uptake and binding of monoamines (6, 7). Furthermore, the functional involvement of microtubules and microfilaments in the axonal transport and release mechanism of monoamines in neurons (8) points to morphological and functional
similarities between platelets and neurons.

In an attempt to clarify whether microtubules or microfilaments are involved in an uptake mechanism of 5HT by platelets, we designed a study in which we examined the effects of cold treatment and certain drugs which affect platelet shape and function on the uptake of 5HT by platelets.

MATERIALS AND METHODS

Chemicals and abbreviations used

5-Hydroxy-[side chain-2,14C]tryptamine creatinine sulphate (14C-5HT) and 5-hydroxy-[G-3H]tryptamine creatinine sulphate (3H-5HT) were obtained from the Radiochemical Centre, Amersham, Cytochalasin B from Aldrich, Milwaukee, U.S.A., Dibutyryl cyclic AMP and dibutyryl cyclic GMP from Boehringer, Mannheim, West Germany, and Pluronic F 68 (polyoxyethylene—polyoxypropylene condensate) from Nikko Chemicals Co., Ltd., Tokyo, Japan. The following drugs were kindly donated: Colchicine and cytochalasin D (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan); vinblastine (Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan); prostaglandin E1 (PG-E1) (Ono Pharmaceutical Co., Ltd., Osaka, Japan); prostaglandin E2 (PG-E2) (Japan Upjohn Ltd., Tokyo, Japan). All other chemicals were of analytical grade.

Preparation of platelet suspension

Rabbits of both sexes weighing 2.0—2.5 kg were used. Whole blood was collected from the carotid artery, mixed with 1/10 volume of 3.8% sodium citrate and centrifuged at 150 x g for 20 min at room temperature. The 15 ml supernatants were diluted with 35 ml of buffered salt solution (BSS) [NaCl, 134 mM; MgCl2, 3 mM; D-glucose, 5 mM; Tris-HCl buffer pH 7.4, 15 mM], to which heparin solution was added to yield a final concentration of 10 units/ml. The final dilution of platelets rich plasma (dil-PRP) contained 2.37±0.15 x 108 platelets/ml (n=11). The dil-PRP was kept at 37°C until use.

Estimation of 5HT uptake activity

The dil-PRP was separated into 1 ml aliquots, each of which was transferred into a polypropylene test tube containing 0.4 ml of BSS or drug solution to be tested. After aerobic pre-incubation for 30 min at 37°C with gentle shaking (80 strokes/min), 14C-5HT or 3H-5HT (1.27 x 10⁻⁷ M) was added to the samples and the mixtures were further incubated for 3 min. The incubation was terminated by adding 3 ml of ice cold BSS and the mixtures were centrifuged at 1,500 x g for 30 min at 4°C. Platelets thus sedimented were washed twice with ice cold BSS. After draining, platelets were dissolved in 0.1 ml of 1 N NaOH. After neutralization with 1 N HCl the solution was placed in a scintillation vial containing 10 ml of Bray’s scintillant. Radioactivity was determined in a Packard model 3320 Tri-Carb liquid scintillation spectrometer and counts were corrected for d.p.m. by external standardization. Blank values were obtained from samples to which the radioactive material was added after the test tube had been placed in ice water.

Estimation of endogenous 5HT concentration of platelets

Pellets of the platelets obtained by centrifugation at 1,500 x g for 30 min at 4°C, were
extracted and 5-HT content was determined spectrophotofluorometrically by the method of Curzon and Green (9). Endogenous 5-HT concentration of rabbit blood platelets was 2.65 ± 0.21 × 10⁻⁶ g/10⁶ platelets (n = 12), which was referred to as the control value.

Platelet morphology by transmission electron microscopy

The preparation of samples for transmission electron microscopy was done by the method of White (10) with modification. The cell suspension (dil-PRP) was fixed at 37°C for 1 hr after mixing with an equal volume of 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The mixtures were then centrifuged at room temperature to obtain buttons. Supernatant plasma and fixative were discarded and several ml of fresh 2% glutaraldehyde in the phosphate buffer was layered over the buttons of the cells. After hardening overnight at 4°C, the buttons were cut into small pieces and dropped into chilled 1% osmium tetroxide in the phosphate buffer. After 1 hr at 4°C, the samples were dehydrated in a series of graded alcohols and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined under a HITACHI HS-7A electron microscope.

RESULTS

Effect of the cold treatment on ultrastructure of platelets

Under transmission electron microscope, the following findings were noted. The cold treatment of platelets resulted in dissociation and disappearance of CB-Mt as well as enlargement of the canalicular system, and the platelets lost their discoidal form and became irregular with pseudopods (Fig. 1).

Effect of the cold treatment on the 5-HT uptake by platelets

Uptake of 5-HT by the intact platelet was rapid. Thus, as is shown in Fig. 2 [Intact], a rapid 5-HT uptake occurred within 3 min after incubation. On the other hand, cold treated specimens took up much less 5-HT depending on the length of treatment (Figs. 2, 3). The uptake decreased by 90% of control value after 4 hr cold treatment. In contrast, no changes in endogenous 5-HT concentration and platelet counts were observed after the cold treatment (Fig. 3). Fig. 4 demonstrates double reciprocal plots of the 5-HT uptake by the non-treated, and 1 hr and 2 hr cold treated platelets. The apparent non-competitive fashion of the figure may indicate that the cold treatment denatures the plasma membrane non-selectively. A conformational change might be an explanation.

Effect of colchicine, vinblastine and cytochalasin B on ultrastructure of platelets

Incubation of platelets with colchicine (10⁻³ M) for 30 min induced a dissociation and disappearance of CB-Mt as was observed in the cold treated platelets. However, here the platelets became spherical (Fig. 5-A). Similar morphological changes were observed in platelets treated with vinblastine (2 × 10⁻³ M). Furthermore, a typical crystalline material was occasionally present in these platelets (Fig. 5-B). On the other hand, cytochalasin B (10⁻¹ M) induced no morphological changes.

Effects of colchicine, vinblastine and cytochalasin on the 5-HT uptake by platelets

Colchicine (10⁻³ M) inhibited the 5-HT uptake by 26.6%, and vinblastine (2 × 10⁻³ M)
Fig. 1. Transmission electronmicrograph of rabbit blood platelets. (A) Intact platelets: Following structures of platelets could be defined. CM; cell membrane. EC; exterior coat. CB-Mt; circumferential bands of microtubules. α-G; α-granule. Mt; mitochondoria. GG; glycogen granule. DB; dense body. (B) Cold treated platelets: Formation of pseudopods, loss of CB-Mt and enlargement of canalicular system are evident. Scale: 1.0 μm.

Fig. 2. Time course of 5HT uptake by intact and cold treated platelets. Platelets were incubated in plasma-BSS medium containing 1.27×10⁻⁷ M ¹³C-5HT as described in Methods. Values were obtained from the samples incubated for 1, 3 and 10 min. ( —●—) : intact platelets. ( — ■ —): 1 hr cold treated platelets. ( — ▲— ) : 4 hr cold treated platelets. Ordinate: ratio of [¹³C-5HT in platelets] to [¹³C-5HT in medium]. Vertical bars show S.E. mean from 3 experiments.
FIG. 3. Effect of cold treatment of rabbit blood platelets on $^{14}$C-5HT uptake activity (●), endogenous 5HT concentration (▲) and platelet counts (■). Platelets had been subjected to cold treatment for 30 min-4 hr. Number of experiments is given in parentheses. Vertical bars show S.E. mean. Significantly different from control, *$P\leq0.005$ **$P\leq0.001$.

FIG. 4. Double reciprocal plots of 5HT uptake by intact and cold treated platelets. The 5HT concentrations range from $1.27\text{ to }5.08\times10^{-7}\text{ M}$. Velocity is expressed as n moles/10$^9$ platelets/min.

by 89.3%. The latter induced release of the endogenous 5HT by 37.0%, and the marked apparent inhibition of the uptake may be attributed, at least in part, to the 5HT releasing activity (Table 1). Cytochalasin B ($10^{-4}\text{ M}$) slightly inhibited the 5HT uptake, but the same concentration of cytochalasin D had no effect. Neither cytochalasin affected the endogenous concentration of 5HT (Table 1).
Effects of theophylline, cyclic AMP, cyclic GMP and Pluronic F68 on the 5HT uptake by platelets

To clarify whether or not cyclic AMP is involved in an uptake process of 5HT, the effects of theophylline, cyclic AMP and cyclic GMP were studied. Theophylline, at $10^{-3}$ and $5 \times 10^{-3}$ M, significantly augmented the uptake of 5HT by the intact platelets, and this effect of theophylline was potentiated by $2 \times 10^{-6}$ M PG-E₁, but not by this same concentration of PG-E₂. On the other hand, cyclic AMP ($10^{-3}$ M) and cyclic GMP ($10^{-3}$ M) had no effect on the 5HT uptake (Table 2).

Cold treatment (4 C, 1 hr) resulted in a decrease of the 5HT uptake by approx. 50%, (Fig. 3). The decreased uptake was significantly restored by theophylline ($10^{-3}$ M) but not by the same concentration of cyclic AMP and cyclic GMP (Table 3). Pluronic F68, a non-
TABLE 1. Effect of colchicine, vinblastine and cytochalasins on 5HT uptake by blood platelets

| Drug        | Concentration (M) | 5HT uptake (%) | Endogenous 5HT concentration (%) |
|-------------|-------------------|----------------|----------------------------------|
|             |                   |                |                                  |
|             |                   |                |                                  |
| Colchicine  | $10^{-5}$         | 99.0±2.2       | (3)                              |
|            | $10^{-4}$         | 95.5±4.2       | (3)                              |
|            | $10^{-3}$         | 73.4±4.9*      | (3)                              |
|            | $2 \times 10^{-5}$| 93.3±7.5       | (3)                              |
| Vinblastine | $2 \times 10^{-5}$| 81.5±11.5      | (6)                              |
|            | $2 \times 10^{-4}$| 10.7±7.6**     | (9)                              |
| Cytochalasin B | $10^{-4}$   | 80.3±7.7*      | (6)                              |
| Cytochalasin D  | $10^{-4}$   | 98.8±0.9       | (4)                              |

The control values are referred to 100%. Values are the mean±S.E. Number of experiments is given in parentheses. Significantly different from the control, *P<0.05, **P<0.001.

TABLE 2. Effect of theophylline, prostaglandins, cyclic AMP and cyclic GMP on 5HT uptake by blood platelets

| Drug                  | Concentration (M) | 5HT uptake (%) | Endogenous 5HT concentration (%) |
|-----------------------|-------------------|----------------|----------------------------------|
| Theophylline           | $10^{-4}$         | 104.2±2.3      | (6)                              |
|                       | $10^{-3}$         | 106.5±2.4*     | (8)                              |
|                       | $5 \times 10^{-3}$| 112.1±3.8*     | (6)                              |
| Prostaglandin E₁      | $2 \times 10^{-6}$| 121.6±7.5**    | (6)                              |
| Theophylline +        | $10^{-3}$         | 97.2±5.1       | (9)                              |
| Prostaglandin E₂      | $2 \times 10^{-6}$| 95.7±1.6       | (3)                              |
| Db-c-AMP              | $10^{-4}$         | 95.9±6.2       | (8)                              |
|                       | $10^{-3}$         | 93.0±5.5       | (5)                              |
| Db-c-GMP              | $10^{-3}$         | 98.8±2.5       | (3)                              |
| Theophylline +        | $2 \times 10^{-6}$| 97.2±5.1       | (9)                              |
| Prostaglandin E₂      | $2 \times 10^{-6}$| 95.7±1.6       | (3)                              |
| Db-c-AMP              | $10^{-3}$         | 95.9±6.2       | (8)                              |
|                       | $10^{-3}$         | 93.0±5.5       | (5)                              |
| Db-c-GMP              | $10^{-3}$         | 98.8±2.5       | (3)                              |

The control values are referred to 100%. Values are the mean±S.E. Number of experiments is given in parentheses. Significantly different from the control, *P<0.05, **P<0.02.

TABLE 3. Effect of theophylline, cyclic AMP, cyclic GMP and Pluronic F68 on 5HT uptake by cold treated platelets

| Drug         | Concentration (M) | 5HT uptake (%) |  |
|--------------|-------------------|----------------|---|
| Theophylline | $10^{-3}$ M       | 155.0±11.7**   | (6) |
| Db-c-AMP     | $10^{-3}$ M       | 80.1±13.8      | (6) |
| Db-c-GMP     | $10^{-3}$ M       | 95.5±12.7      | (3) |
| Pluronic F68 | $5 \times 10^{-3}$ g/ml | 64.9±7.5*    | (3) |

Rabbit platelets were treated with cold (4°C) for 1hr. 5HT uptake in absence of the drugs is referred to as 100%. Values are the mean±S.E. Number of experiments is given in parentheses. Significantly different from the control, *P<0.05, **P<0.01.
ionized surfactant, which was expected to stabilize protein like other surfactants (11, 12) and which is known to influence platelet function by induction of conformational changes of the cell membrane (13), decreased the uptake by the cold treated platelets (Table 3).

DISCUSSION

Kattlove and Alexander (14) reported that low temperature induced platelet aggregation and suggested that the mechanism of this phenomenon may be similar to ADP-induced aggregation. This is not due to the extrusion of ADP from platelets during storage, but rather to an increase in the conversion of adenine to metabolites (15). Dissociation and disappearance of CB-Mt had been observed by Behnke (4) and White and Krumwiede (5). Okuma et al. (16) found a reduction of lipid content in platelets as well as decreased incorporation of free fatty acid into platelets following cold treatment.

Our morphological results with rabbit blood platelets were similar to those with human platelets, except that no evident aggregation was observed. Depending on the time of storage, the cold treatment resulted in a decrease in 5HT uptake. This observation is different from that of Kattlove et al. (17) who reported that 5HT uptake by human platelets was unaffected by prior chilling. The difference could be explained by the difference of animal species and/or experimental conditions. In our experiments, platelets were incubated only for 3 min, while Kattlove et al. (17) reported an incubation time of 30 min. In such a long time incubation, contribution of non-specific mechanisms other than the specific active transport into platelets probably should be investigated. Cold treatment may have an inhibitory influence only on the specific active transport of 5HT, therefore, special care should be taken when 5HT uptake is determined in platelets isolated at a low temperature.

Recently, there has been considerable evidence suggesting the involvement of microtubules and microfilaments in transport mechanism of monoamines in cells (8). However, colchicine and vinblastine were found to affect the 5HT uptake only slightly. Furthermore, there was a certain difference in ultrastructural changes between cold treated and anti-mitotic drug treated platelets. Those results suggest that the inhibition of 5HT uptake by the cold treatment is not due to a dissociation of microtubules. This is further supported by the fact that microtubules disappeared from the majority of platelets after exposure to 4°C for only 5 min (4), while almost complete inhibition of 5HT uptake was observed after 4 hr exposure (Fig. 3).

Uptake of 5HT by platelets was slightly inhibited by cytochalasin B, but not by cytochalasin D. Since the latter drug is thought to be more specific in interfering with the functions of microfilaments (18), there may be no involvement of microfilaments in the mechanism of 5HT uptake. The slight inhibitory action of cytochalasin B can be explained by its action on the platelet membrane, since cytochalasin B was found to interact with plasma membrane.

Salzman and Weisenberger (19) observed that epinephrine, collagen and thrombin which induced or augmented platelet aggregation reduced the content of cyclic AMP, while PG-E1, caffeine and theophylline which antagonized platelet aggregation increased the content of
this nucleotide. A certain relationship of cyclic AMP to platelet function in terms of 5HT uptake is suggested from our observation that theophylline increased uptake of 5HT and that this effect was potentiated by PG-E1. On the other hand, the fact that cyclic AMP had no effect on 5HT uptake does not support the notion that this nucleotide is a mediator of the 5HT uptake potentiating activity of PG-E1 and theophylline. There was also a dissociation of the effect of theophylline and cyclic AMP or cyclic GMP on the cold treatment of platelets. Thus, the decreased uptake of 5HT by the cold treatment was restored by theophylline but not by the two nucleotides. Although the precise mode of the theophylline effect remains to be determined, one explanation may be that only intracellularly synthesized cyclic AMP is effective on the 5HT uptake.

Pluronic F68 is a non-ionized surfactant (11, 12), and this property, as suggested by Benner and Brunner (13), might serve to explain the action of Pluronic F68 on platelets in such a way that the hydrophobic units (polyoxypropylene) could penetrate into the membrane protein while the hydrophilic tails (polyoxyethylene) projected from the protein. The observations that cold treatment non-competitively inhibited 5HT uptake and that Pluronic F68 promoted the influence of cold treatment indicate that conformational changes in platelet membrane induced by cold treatment are facilitated by Pluronic F68.

REFERENCES
1) BECKER, G.A., TUCCELLI, M., KUNICKI, T., CHALOS, M.K. AND ASTER, R.H.: Studies of platelet concentrates stored at 22°C and 4°C. Transfusion 13, 61–68 (1973)
2) KRANKENHAGEN, B., MANN, H. AND HIRSCH, H.: In vitro-Thrombocyten-aggregation durch Kälte. Pflügers Arch. 334, 62–73 (1972)
3) BECKER, G.A., KUNICKI, T. AND ASTER, R.H.: Effect of prostaglandin E1 on harvesting of platelets from refrigerated whole blood. J. Lab. clin. Med. 83, 304–309 (1974)
4) BEHNKE, O.: Some possible practical implications of the lability of blood platelet microtubules. Vox Sang. 13, 502–507 (1967)
5) WHITT, J.G. AND KRUMWIDE, M.: Influence of cytochalasin B on the shape change induced in platelets by cold. Blood 41, 823–832 (1973)
6) PLETSCHER, A.: Metabolism, transfer and storage of 5-hydroxytryptamine in blood platelets. Brit. J. Pharmacol. 32, 1–16 (1968)
7) TUOMISTO, J.: A new modification for studying 5HT uptake by blood platelets; a re-evaluation of tricyclic antidepressants as uptake inhibitors. J. Pharm. Pharmacol. 26, 92–100 (1974)
8) ALLISON, A.C. AND DAVIES, P.: Advances in Cytopharmacology, Edited by CECCARELLI, B., CLEMENTI, F. AND MELDOLESI, J., Vol. 2, p. 237–248, Raven Press, New York (1973)
9) CURZON, G. AND GREEN, A.R.: Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. Brit. J. Pharmacol. 39, 653–655 (1970)
10) WHITE, J.G.: Tubular elements in platelet granules. Blood 32, 148–156 (1968)
11) DOWBEN, R.M. AND KOEHLER, W.R.: The interaction of a nonionic detergent with protein. I. Physical properties of the protein detergent complex. Archs Biochem. Biophys. 93, 496–500 (1961)
12) JIRGENSONS, B.: Effect of detergents on the conformation of proteins. I. An abnormal increase of the optical rotatory dispersion constant. Archs Biochem. Biophys. 94, 59–67 (1961)
13) BENNER, K.U. AND BRUNNER, R.: Cold-induced platelet aggregation in vivo and its inhibition by a nonionic surface active substance. Thrombos Res. 2, 331–342 (1973)
14) KATTLOVE, H.E. AND ALEXANDER, B.: The effect of cold on platelets. I. Cold-induced
15) KATTLOVE, H.E.: The effect of cold on platelets. III. Adenine nucleotide metabolism after brief storage at cold temperature. Blood 42, 557-564 (1973)

16) OKUMA, M., STEINER, M. AND BALDINI, M.: Lipids of human platelets: The effect of storage at 4°C. Blood 38, 27-38 (1971)

17) KATTLOVE, H.E., ALEXANDER, B. AND WHITE, F.: The effect of cold on platelets. II. Platelet function after short-term storage at cold temperatures. Blood 40, 688-696 (1972)

18) PUSZKIN, E., PUSZKIN, S., LO, L.W. AND TANENBAUM, S.W.: Binding of cytochalasin D to platelet and muscle myosin. J. biol. Chem. 248, 7754-7761 (1973)

19) SALZMAN, E.W. AND WEISENBERGER, H.: Advances in Cyclic Nucleotide Research, Edited by GREENGARD, P. AND ROBISON, G.A., Vol. 1, p. 231-247, Raven Press, New York (1972)