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BIOLOGICAL ACTIVITY OF DIAZONIUM COMPOUNDS: STUDIES ON THE MECHANISM OF ACTION OF 4(OR 5)-DIAZOIMIDAZOLE-5(OR 4)-CARBOXAMIDE ON 5-HYDROXYTRYPTAMINE RELEASE FROM RABBIT PLATELETS—I

REQUIREMENT FOR CALCIUM ION

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Abstract—Incubation of 4(or 5)-diazoimidazole-5(or 4)-carboxamide (Diazo-ICA) at 37° with isolated rabbit platelets induces release of 5-hydroxytryptamine (5-HT). This release occurs only in the presence of a trace of calcium ions and the effect is independent of the presence of other plasma components. The release is temperature-dependent, being appreciably lower at room temperature than at 37° and almost negligible at 4°. These results imply that the mechanism of release with Diazo-ICA differs from that with reserpine or bacterial pyrogenic lipopolysaccharide. 4(or 5)-Dialkyltriazenoimidazole-5(or 4)-carboxamides, prepared by coupling Diazo-ICA with corresponding dialkylamines, cause only slight release of 5-HT from platelets, and the release is independent of the presence of calcium ion. In contrast, 4(or 5)-aminoimidazole-5(or 4)-carboxamide (the parent compound of these derivatives), 2-azahypoxanthine (a cyclized product of Diazo-ICA) and S-(5(or 4)-carbamoyl-4(or 5)-imidazolyl azo)cysteine (a coupling product of Diazo-ICA and cysteine) release no 5-HT from platelets. The other diazonium compound tested, diazobenzene sulfonate and diazobenzenesulfonamide, also cause no release.

WE HAVE already reported that 4(or 5)-diazoimidazole-5(or 4)-carboxamide (Diazo-ICA) caused flattening of the EEG, depression of the blood pressure in cats and contraction of isolated intestine and of perfused peripheral blood vessels of the ear in rabbits.[1] A hypothermic effect, prolongation of hexobarbital induced hypnosis and marked antidiuretic activity were also observed after intraperitoneal administration of Diazo-ICA.[2] These multiple pharmacological effects suggested that Diazo-ICA might not only have a direct effect but an indirect effect through release of biogenic amines, especially 5-hydroxytryptamine (5-HT), from storage sites. Accordingly the effect of Diazo-ICA on release of 5-HT from rabbit platelets *in vivo* was studied.[2]

Recently, Iwata *et al.* showed that Diazo-ICA has positive ino- and chronotropic actions on isolated guinea-pig atria and we found that this action of the diazonium salt was in part due to the liberation of catecholamines from their cardiac depots.[3]

In this work on the mechanism of release of 5-HT *in vitro*, we used isolated blood platelets, because Carlson *et al.* found that platelets were a useful experimental tool for studies on the mechanism of amine release.[4]

The present experiments were on the action of Diazo-ICA on isolated rabbit platelets and its dependency upon divalent ion in the medium. To study the influence of the diazonium group in the release, two other diazonium compound, diazobenzene sulfonate and diazobenzene-sulfonamide were also prepared and their effects were tested. The effects were compared with those of bacterial pyrogenic lipopolysaccharide and reserpine, which are also known to release 5-HT from platelets under certain conditions. These studies showed that the release of 5-HT from isolated rabbit platelets by Diazo-ICA was affected by calcium ions and was temperature dependent.

MATERIALS AND METHODS

Isolation of platelets

Rabbits of either sex weighing 3·0 to 3·5 kg were fasted for 24 hr. Then blood platelets were isolated as follows: blood from the carotid artery was mixed with $\frac{1}{10}$ volume of 1% EDTA–0·7% NaCl solution, and centrifuged at 53 *g*–69 *g* for 10 min at room temperature to remove blood cells. The resulting supernatant was centrifuged at 870 *g* for 10 min at 4°, and the resulting pellet (containing the platelets) was suspended in saline and recentrifuged at low speed. The resulting suspension of platelets was recentrifuged at 870 *g* for 10 min at 4° and the pellet of platelets so formed suspended in isotonic modified Tyrode's solution. One ml of medium contained twice as many platelets ($1{\cdot}3 \cdot 10^8$) as 1 ml of whole blood. Isotonic modified Tyrode's buffer (pH 7·4) had the following composition per litre: NaCl 7·60 (0·130 M); KCl, 0·42 (0·006 M); EDTA, 0·80 (0·002 M); $NaH_2PO_42H_2O$, 0·14 (0·001 M); $NaHCO_3$, 2·10 (0·003 M); glucose, 2·00 (0·011 M) and saccharose, 4·50 (0·010 M).

Incubation and determination of 5-HT

Samples of 2 ml of platelets suspension in modified Tyrode's buffer with or without 3·0 mM Ca were placed in 15-ml polyethylene tubes, mixed with 0·5 ml of solution of test compound in buffer and 0·5 ml of the buffer, and incubated for 10 to 30 min. The incubation was made at 37°, except in experiments on the effect of temperature. Then the platelets were separated from the incubation medium by centrifugation at 1000 *g* for 10 min at 4°.

The 5-HT of a sample of platelets was determined spectrophotofluorometrically by the method of Weissbuch *et al.*[5] In this determination, the activation and fluorescence wavelengths were 290 mμ and 550 mμ, respectively.

Materials

Lipopolysaccharide from *E. coli* was kindly given by Dr. Kanoh (National Institute of Hygienic Sciences, Osaka). Reserpine phosphate was obtained from CIBA Pharmaceutical Co., Osaka. 4(or 5)-(Dialkyltriazeno)imidazole-5(or 4)-carboxamides were gifts from Prof. K. Hano, Department of Pharmacology, Osaka College of Pharmacy, Takamino-Sato, Matshubara, Osaka-fu. 4(or 5)-Aminoimidazole-5(or 4)-carboxamide hydrochloride was kindly provided by Dr. M. Ohara, Fujisawa Pharmaceutical Co., Ltd., Osaka. Diazonium salts, 2-azahypoxanthine and S-[5(or 4)-carbamoyl-4(or 5)-midazolyl azo]cysteine were prepared in this laboratory.

RESULTS

Effect of Diazo-ICA on 5-HT release from platelets in whole blood or from isolated platelets in modified Tyrode's solution

As shown in Fig. 1, there was little spontaneous release of 5-HT from platelets, in blood or in modified Tyrode's solution, on incubation at 37° for 15 or 30 min. In this experiment, $\frac{1}{10}$ volume of 0·1 % heparin was added to whole blood to prevent clotting in place of EDTA and isolated platelets in the modified Tyrode's solution were prepared as described in the experimental section.

However, incubation of whole blood with Diazo-ICA at concentrations of 0·1 to 0·4 mM resulted in the release of 5-HT from platelets. About 50 per cent of the 5-HT of platelets was released by 0·4 mM Diazo-ICA.

By contrast, addition of Diazo-ICA at relative high concentration (1 to 4 mM) did not increase the release of 5-HT from isolated platelets in isotonic modified Tyrode's solution over the control value. These results suggested that the release of 5-HT from isolated platelets induced by Diazo-ICA involves factors present in the plasma.

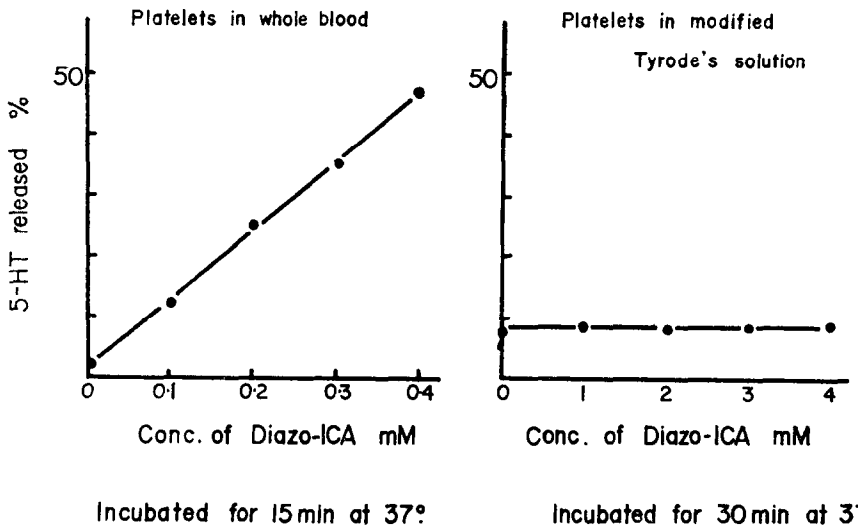

FIG. 1. Effect of Diazo-ICA on 5-HT release from platelets in whole blood or in modified Tyrode's solution.

Diazo-ICA: 4(or 5)-diazoimidazole-5(or 4)-carboxamide.

Ability of various divalent ions to support Diazo-ICA induced release of 5-HT from isolated platelets in modified Tyrode's buffer

The action of Diazo-ICA in liberation of 5-HT from isolated platelets in the presence of divalent ions was investigated in modified Tyrode's solution on 10 min incubation at 37°.

As shown in Fig. 2, with the exception of manganese the divalent ions tested at a concentration of 3 mM had very little effect on the release of 5-HT from the platelets in modified Tyrode's solution. Diazo-ICA (0·5 mM) also had no effect on the release of 5-HT in absence of these metal ions. Addition of calcium ion to the incubation medium with Diazo-ICA stimulated the release of 5-HT from the platelets, while

addition of the other divalent ions, manganese, nickel, strontium, barium, ferrous iron, magnesium, zinc, cobalt and copper with Diazo-ICA did not. These results show that the stimulatory effect of Diazo-ICA on 5-HT release is dependent on the presence of calcium ion in the incubation medium. The additional presence of magnesium ion in the medium did not prevent the release by Diazo-ICA with calcium ion.

Effect of calcium ion on Diazo-ICA induced release of 5-HT from isolated rabbit platelets

The results of Fig. 2 suggested that calcium ion is involved in the process of 5-HT release by Diazo-ICA.

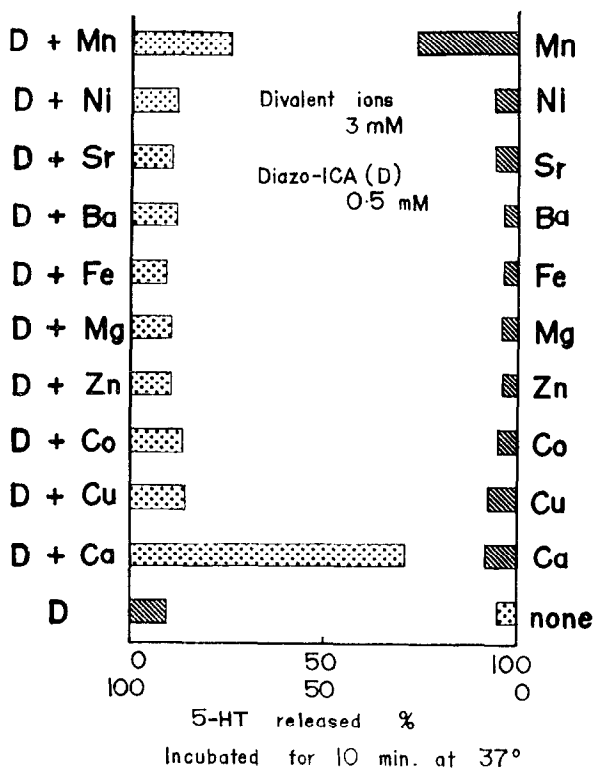

FIG. 2. Ability of various divalent ions to support Diazo-ICA induced release of 5-HT from isolated platelets in modified Tyrode's buffer.

The effect of calcium alone and of Diazo-ICA with different concentrations of calcium on 5-HT release from platelets were therefore examined (Fig. 3).

Concentrations of calcium ion of up to 4·6 mM in the presence of 2·0 mM EDTA did not cause liberation of 5-HT. Moreover, in the presence of 2·0 mM EDTA contained in Tyrode's buffer, Diazo-ICA caused no release of 5-HT.

However, incubation of platelets with Diazo-ICA (0·5 mM) and calcium ion resulted in marked 5-HT release. Approximately 85 per cent of the 5-HT in platelets was released by Diazo-ICA into surrounding medium in the presence of 2·8 mM of

calcium. Further increase in the concentration of calcium ion did not result in further release with Diazo-ICA. Thus a minimum concentration of calcium ion is required for Diazo-ICA induced 5-HT liberation from platelets.

5-HT release at various Diazo-ICA concentrations in the presence of calcium ion

Platelets were extremely sensitive to Diazo-ICA in the presence of calcium ion and there was a definite release of 5-HT as little as 0·1 mM Diazo-ICA on incubation

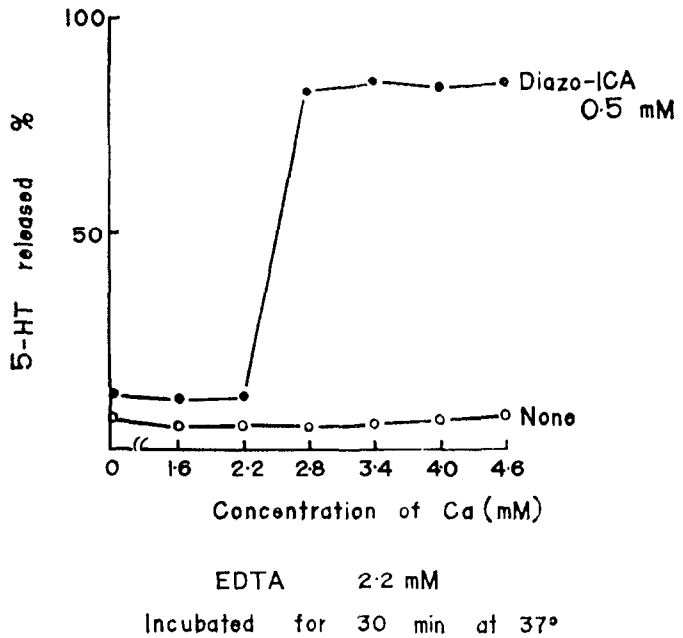

FIG. 3. Effect of calcium ion on Diazo-ICA induced release of 5-HT from isolated rabbit platelets.

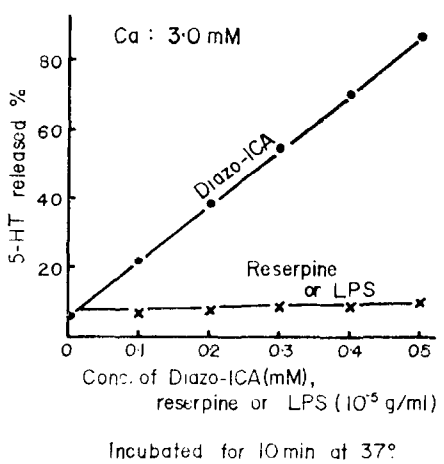

FIG. 4. 5-HT release at various Diazo-ICA concentrations in the presence of calcium ion. LPS: lipopolysaccharide from *E. coli*.

for 10 min at 37°. In the presence of calcium (3·0 mM) at 37°, the liberation of 5-HT from the platelets into the medium increased with the concentration of Diazo-ICA. Typical results are shown in Fig. 4. In the presence of calcium and lipopolysaccharide or reserpine (5×10^{-5} g/ml, respectively) but absence of the plasma component, there was no 5-HT release.

Effect of Diazo-ICA and related compounds on 5-HT release from isolated platelets in presence and absence of calcium ion

Platelet suspensions were incubated at 37° for 30 min with several compounds related to Diazo-ICA and their effects on 5-HT release in the presence and absence of calcium ion (3·0 mM) were measured. The results are shown in Fig. 5.

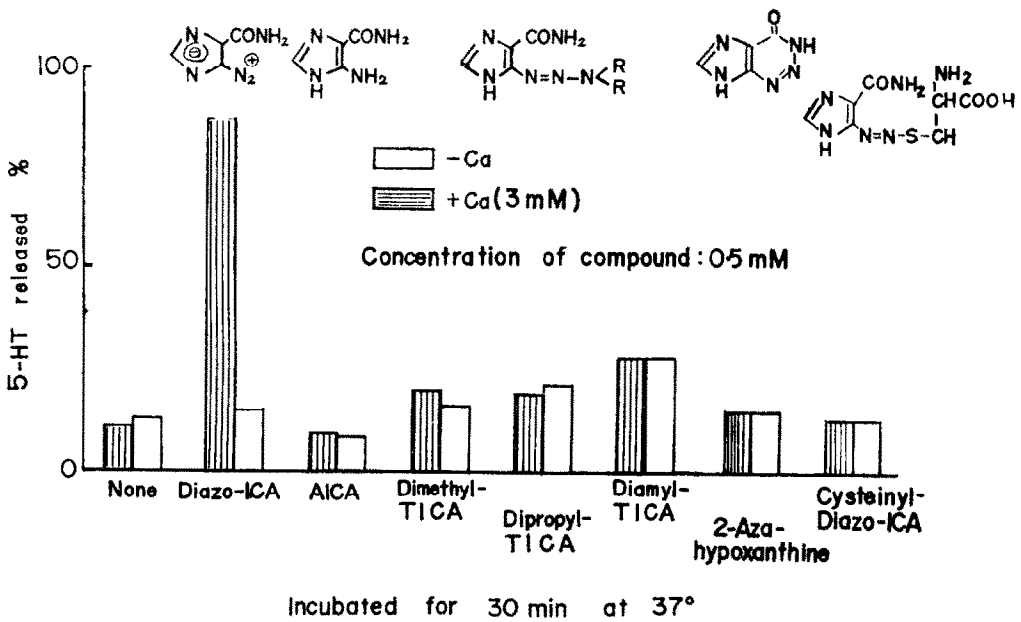

FIG. 5. Effect of Diazo-ICA and related compounds on 5-HT release from isolated platelets in presence and absence of calcium ion.
Diazo-ICA: 4(or 5)-diazoimidazole-5(or 4)-carboxamide
AICA: 4(or 5)-aminoimidazole-5(or 4)-carboxamide hydrochloride
Dimethyl-TICA: 4(or 5)-(dimethyltriazeno)imidazole-5(or 4)-carboxamide hydrochloride
Dipropyl-TICA: 4(or 5)-(dipropyltriazeno)imidazole-5(or 4)-carboxamide hydrochloride
Diamyl-TICA: 4(or 5)-(diamyltriazeno)imidazole-5(or 4)-carboxamide hydrochloride
Cysteinyl-Diazo-ICA: S-(5(or 4)-carbamoyl-4(or 5)-imidazolyl azo)cysteine.

Diazo-ICA caused release of 5-HT from the platelets in the presence, but not in the absence of calcium ion. However, the parent compound of Diazo-ICA, 4(or 5)-aminoimidazole-5(or 4)-carboxamide (AICA), 2-azahypoxanthine (a cyclized product of Diazo-ICA), S-[5(or 4)-carbamoyl-4(or 5)-imidazolyl azo]cysteine, prepared by coupling Diazo-ICA with cysteine caused no release in the presence or absence of

calcium. 4(or 5)-Dimethyltriazenoimidazole-5(or 4)-carboxamide (Dimethyl-TICA), 4(or 5)-dipropyltriazenoimidazole-5(or 4)-carboxamide (Dipropyl-TICA) and 4(or 5)-diamyltriazenoimidazole-5(or 4)-carboxamide (Diamyl-TICA), prepared by coupling Diazo-ICA with the corresponding dialkylamine, at a concentration of 0·5 mM caused slight, calcium independent release. Thus the diazonium group on the imidazole ring plays an important role in calcium dependent 5-HT release.

Rate of 5-HT release and effect of lowered temperature on release by Diazo-ICA

Liberation of 5-HT from platelets was determined at intervals during incubation of 0·36 mM of Diazo-ICA with platelets suspensions at 37°, room temperature (20°) and 4°. Figure 6 shows typical results of a number of experiments. Release of 5-HT from platelets by Diazo-ICA in the presence of calcium (3·0 mM) was very rapidly at 37°, being almost complete in 10 min. At the concentration used, Diazo-ICA released about 60 per cent of the 5-HT from platelets.

Microscopic examination of platelets after treatment with Diazo-ICA in the presence of calcium showed that they were morphologically essentially the same as in the control sample so that 5-HT release was not due to their disruption.

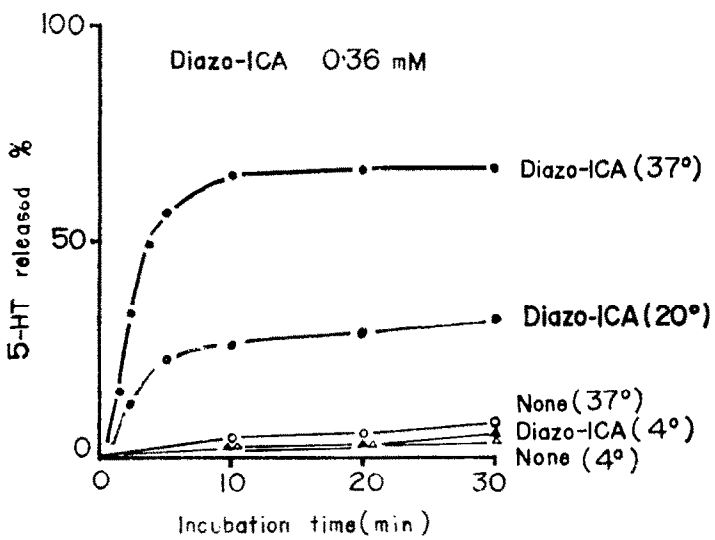

FIG. 6. Rate of 5-HT release and effect of lowered temperature on release by Diazo-ICA.

Liberation of 5-HT from the platelet suspension at 37°, without added Diazo-ICA, was usually negligible, but occasionally as much as 10 per cent of the 5-HT appeared in the medium, after more than 1 hr's incubation, presumably due to disruption of the platelets.

In the presence of calcium, on incubation for 30 min at room temperature platelets released only about 35 per cent of their 5-HT, while in an ice-bath (4°) there was negligible release.

This marked temperature-dependence suggests that a chemical rather than a physical mechanism is involved in 5-HT release.

Comparative effects of diazonium salts on 5-HT release from isolated platelets

Addition of Diazo-ICA to suspension of isolated platelets resulted in the appearance of considerable quantities of 5-HT in the medium containing calcium ions. Thus, it was examined whether the other diazonium salts also have the same effect on isolated platelets or not. Diazobenzene-sulfonamide benzene sulfonate and diazobenzene-sulfonate were prepared and evaluated for the release activity. Within 10 min incubation at 37°, both diazobenzene-sulfonamide and diazobenzene-sulfonate at relatively high doses (5×10^{-4} M) caused no or little liberation of 5-HT from isolated platelets suspended in modified Tyrode's solution with or without calcium ions. An experiment illustrating this is given in Fig. 7.

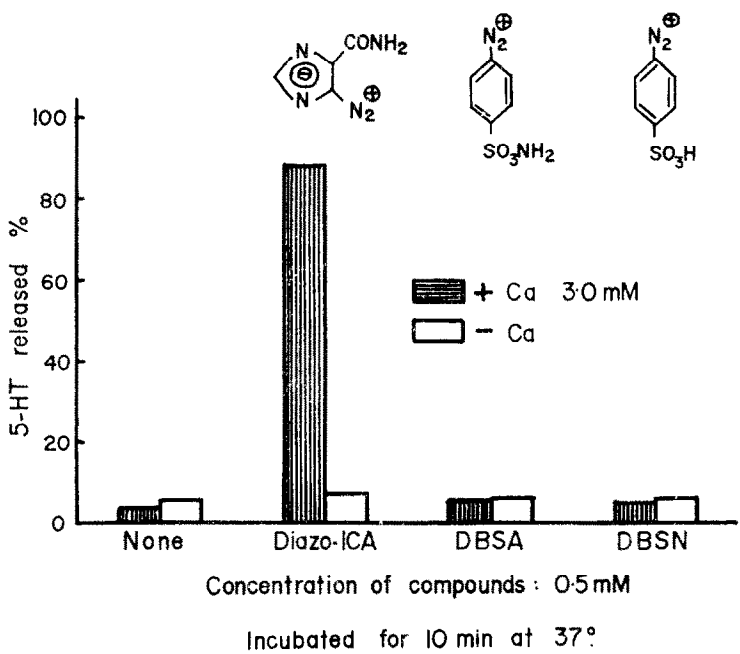

FIG. 7. Comparative effects of diazonium salts on 5-HT release from isolated platelets.
Diazo-ICA: 4(or 5)-diazoimidazole-5(or 4)-carboxamide
DBSA: diazobenzene sulfonamide benzenesulfonate
DBSN: diazobenzene sulfonate.

DISCUSSION

Previous work has shown that Diazo-ICA impairs the capacity of platelets to store 5-HT *in vivo*.[2] The present studies on the release of 5-HT show that Diazo-ICA induces release of 5-HT from platelets in whole blood *in vitro* or from isolated platelets in the absence of plasma by a mechanism that is absolutely dependent upon calcium ion in the medium.

Takagi *et al.* have reported recently that release of 5-HT from isolated rabbit platelets by lipopolysaccharide is dependent upon the presence of both calcium ion and plasma.[6] On the other hand, it is known that the release by reserpine is

independent of calcium ion, but requires a component of plasma.[4, 6] The authors suggested that the site of action of lipopolysaccharide might be different from that of reserpine, because of the synergistic effects of lipopolysaccharide and reserpine.

The present experiments showed that 5-HT release by the diazonium salt was absolutely dependent upon calcium ion, but required no plasma component. Consequently, the mechanism of action of Diazo-ICA seems to differ from those of reserpine and lipopolysaccharide, which both required a plasma component. Thus Diazo-ICA may be useful in *in vitro* studies on 5-HT storage and release.

The rapid liberation of 5-HT *in vivo* and *in vitro* and the fact that 2-azahypoxanthine, a cyclized product of Diazo-ICA, does not cause release of 5-HT, suggest that Diazo-ICA itself, rather than a metabolic or modified product of it, causes release of 5-HT from platelets.

The ability of Diazo-ICA to release 5-HT *in vitro* depends greatly upon temperature. At 37° almost all the 5-HT was liberated from platelets, while negligible amounts were released at 4°. This marked temperature-dependence suggests that a biochemical, rather than a physical mechanism is involved in release. Carlson *et al.* also showed that release of 5-HT from platelets by reserpine was negligible at 4°.[4]

In the presence and absence of calcium ions, we tested the effects of several triazenoimidazoles and S-[5(or 4)-carbamoyl-4(or 5)-imidazolyl azo]cysteine, which prepared by coupling with Diazo-ICA and the corresponding dialkylamine and cysteine, respectively. These triazene derivatives had been found to have antitumor and antibacterial activities,[7–9] however, caused no or little release of 5-HT. S-[5(or 4)-carbamoyl-4(or 5)-imidazolyl azo]cysteine which had been demonstrated to inhibit xanthine oxidase,[10] also caused no release of 5-HT from isolated platelets. Thus, it was confirmed that only Diazo-ICA which had been shown to release catecholamine from guinea-pig atrium[3] caused potent calcium dependent release of 5-HT. Therefore, these results suggest that the diazonium group of the imidazole is directly involved in the mechanism of release of 5-HT together with calcium ion.

Two other diazonium compounds tested, diazobenzene sulfonate and diazobenzeneamide, caused no release. From this result it is suggested that diazonium group on imidazole ring but not on benzene ring can contribute to the release mechanism of 5-HT from platelets.

Several authors have reported that platelet aggregation in clotting causes 5-HT release,[11, 12] but we observed no aggregation of platelets by Diazo-ICA in the present conditions.

Diazonium salts usually cause chemical modification of enzymes and proteins by reaction with tyrosyl, histidyl, or leucyl residues.[13–16] However, using an amperometric method, Iwata *et al.* showed that Diazo-ICA couples with SH-compounds, such as cysteine, cysteamine, glutathione and BAL and sulfhydryl groups in biological preparations.[3] Recently, Yamashita *et al.* reported from experiments on myosin A adenosine triphosphatase coupled with diazobenzene-*p*-sulfonic acid, that the sulfhydryl residues in the active site of myosin reacted with the diazonium compound, resulting in inhibition of EDTA-ATPase activity without any essential change in activity or catalytic properties of Ca-ATPase.[17]

Some sulfhydryl group of 5-HT storage granules in platelets or their membranes, involved in 5-HT release seems to be very reactive with Diazo-ICA. This group can be blocked by the sulfhydryl reagent, *N*-ethylmaleimide (NEM) which at high, but not

low, concentration also releases 5-HT by destruction of platelets.[18] Blocking by NEM resulted in inhibition of 5-HT release by Diazo-ICA in the presence of calcium ion. This suggests that the site of action of Diazo-ICA for 5-HT release from the platelets was modified by addition of the sulfhydryl reagent, NEM. Therefore, the present results indicate that the mechanism of release involves coupling of Diazo-ICA with a reactive sulfhydryl group and that the mechanism of release is activated in some unknown biochemical way by calcium ion.

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