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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—II

COMPARATIVE STUDIES WITH *N*-ETHYLMALEIMIDE *IN VITRO*

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Abstract—Studies were made on the association of the SH-blocking effect of Diazo-ICA with its activity in releasing 5-HT from isolated rabbit platelets, and the releasing activity was compared with that of NEM. High concentration of NEM, like Diazo-ICA, caused release of 5-HT from platelets, but, unlike Diazo-ICA, its action was independent of the calcium ion concentration in the incubation medium. The calcium-dependent release of 5-HT by Diazo-ICA was completely blocked by a small amount of NEM. The sulfhydryl compounds, cysteine, glutathione and BAL also blocked release of 5-HT by Diazo-ICA. ATP, ADP and inorganic pyrophosphate inhibited release of 5-HT by Diazo-ICA, but AMP and creatine phosphate did not.

These findings suggest that the sulfhydryl group and pyrophosphate structures are involved in the mechanism of release of 5-HT from rabbit platelets by Diazo-ICA.

4(OR 5)-DIAZOIMIDAZOLE-5 (OR 4)-CARBOXAMIDE (Diazo-ICA) is an analog of 4(or 5)-aminoimidazole-5(or 4)-carboxamide (AICA), the ribonucleotide of which is a precursor of nucleic acid.[1] This diazonium salt and related triazenoimidazoles were synthesized[2–6] and shown to be potent antitumor,[2–5] and antibacterial agents.[6] Some of them are now being tested clinically at several research centers and recently, Skibba *et al.* reported[7] the therapeutic effect on human melanoma of 4(or 5)-(dimethyltriazeno)imidiazole-5(or 4)-carboxamide, a biological masked compound of Diazo-ICA.[3,4]

Recently, we found that Diazo-ICA has potential pharmacological actions, such as in causing 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.[8] A hypothermic effect,[9] prolongation of hexobarbital induced hypnosis and antidiuretic activity were also observed after intraperitoneal administration of Diazo-ICA.[10]

These multiple pharmacological effects suggested that Diazo-ICA may have not only a direct effect, but also an indirect effect through release of biogenic amines, especially 5-hydroxytryptamine (5-HT) from storage sites. Accordingly, the effects of Diazo-ICA in release of 5-HT from rabbit platelets, urinary excretion of 5-hydroxyndole acetic acid (5-HIAA), a urinary end-product of 5-HT,[10] and monoamine

oxidase[11] were studied *in vivo* and *in vitro*. In addition, Iwata *et al.*[12] showed that Diazo-ICA has positive ino- and chronotropic actions on isolated guinea-pig atria and found that these actions of the diazonium salt were due to liberation of catecholamines from their cardiac depots.

Previously we found that Diazo-ICA caused release of 5-HT from isolated rabbit platelets *in vitro* and that this release was dependent on calcium.[13]

On the other hand, Iwata *et al.*[12] in studies on the biochemical reactivity of Diazo-ICA, using an amperometric method found that this compound, like *N*-ethylmaleimide (NEM), couples with sulfhydryl compounds such as cysteine, glutathione and cysteamine and sulfhydryl groups in biological preparations.

Therefore, the possible association between the SH-blocking action of Diazo-ICA and its activity in releasing 5-HT from platelets was investigated and the effects of Diazo-ICA were compared with those of NEM.

MATERIALS AND METHODS

Isolation of platelets

Platelets were isolated from rabbits of either sex as described in our previous paper.[13]

Incubation mixture and determination of 5-HT

Samples (2 ml) of platelet suspension in modified Tyrode's buffer with or without 3 mM Ca ($2{\cdot}6 \times 10^8$ platelets/ml of suspension) were placed in 15 ml polyethylene tubes, mixed with 0·5 ml of solution of the test compound in buffer and 0·5 ml of buffer and incubated for 10–30 min, at 37°. Then the platelets were separated from the incubation medium by centrifugation at 1000 *g* for 10 min at 4°.

The 5-HT in samples of platelets was determined spectrophotofluorometrically by the method of Weissbach *et al.* In this determination the activation and fluorescence wavelengths were 290 mμ and 550 mμ, respectively.

Materials

Diazo-ICA was prepared according to the method of Shealy *et al.*[4] in our laboratory. Sulfhydryl compounds and nucleotides were obtained from Nakarai Chemical Co., Ltd.

RESULTS

Comparison of the calcium requirements for release of 5-HT from isolated rabbit platelets by Diazo-ICA and NEM

Yamamoto and Iwata showed that calcium was required for release of 5-HT from rabbit platelets by Diazo-ICA,[13] and further demonstrated that Diazo-ICA, like NEM, readily combined with the sulfhydryl residues of mercapto compounds and with those of biological preparation.[12] Thus the possibility that the SH-blocking property of Diazo-ICA was associated with its calcium-dependent activity in releasing 5-HT from platelets was investigated and results were compared with those on NEM.

The release of 5-HT from isolated rabbit platelets was stimulated on addition of Diazo-ICA (0·1–0·5 mM) in the presence of calcium (3 mM), while calcium itself did not cause 5-HT release. In medium without calcium Diazo-ICA caused no appreciable release of 5-HT.

NEM was also shown to stimulate 5-HT release from platelets, but its activity was unaffected by the presence of calcium in the incubation medium.

Addition of NEM and Diazo-ICA (0·4 mM) to incubation medium containing 3 mM calcium resulted in 20 and 70 per cent release of 5-HT, respectively, on incubation at 37° for 10 min.

5-HT release by Diazo-ICA was very rapid and on incubation release was complete within 10 min. On the contrary, the release induced by NEM increased with the incubation time and approximately 60 per cent of the 5-HT was liberated from platelets after 30 min incubation in medium containing calcium.

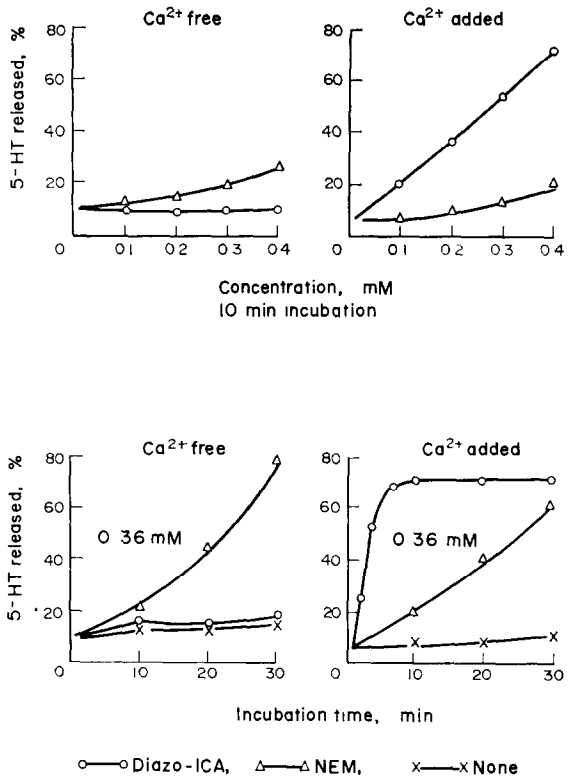

FIG. 1. Comparison of the calcium requirements for release of 5-HT from isolated rabbit platelets by Diazo-ICA and NEM.

Effect of sulfhydryl compounds on release of 5-HT from isolated rabbit platelets by Diazo-ICA in the presence of calcium

The mercapto compounds, cysteine, glutathione and BAL at the concentrations tested caused complete inhibition of release of 5-HT by Diazo-ICA from isolated platelets incubated in modified Tyrode solution with calcium ions for 30 min at 37° (Fig. 2).

No difference was found in the extent of inhibition of these mercapto compounds, under the present conditions. On the contrary, under similar conditions cystine

(1·5 mM) caused practically no inhibition of the release of 5-HT by Diazo-ICA. There was no release in the presence of mercapto compounds alone.

Effect of NEM and AMP on the release of 5-HT from isolated platelets by Diazo-ICA in the presence of calcium

NEM at concentrations above 0·5 mM caused release of 5-HT from platelets by some unknown mechanism, not requiring calcium. However, release of 5-HT by Diazo-ICA was found to be completely inhibited by simultaneous addition of 0·22 mM NEM, which alone had no releasing activity. These results are shown in Fig. 3. ATP has been shown to release amines from adrenal granules and AMP, like NEM, blocks this release. Therefore, the effect of AMP on the release of 5-HT by Diazo-ICA was examined. It was found that even a high concentration (3 mM) of AMP did not affect the release of 5-HT by Diazo-ICA, as shown in Fig. 3.

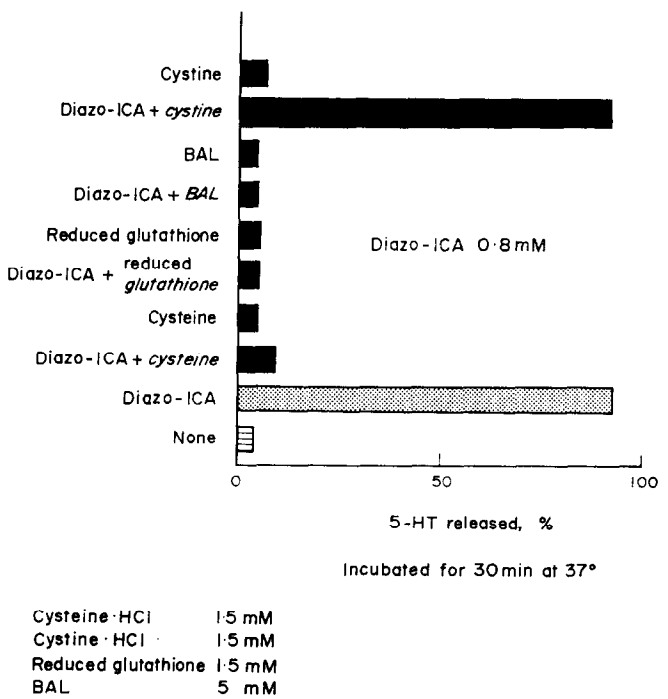

FIG. 2. Effect of sulfhydryl compounds on release of 5-HT from isolated rabbit platelets by Diazo-ICA in the presence of calcium.

Effect of ATP and other nucleotides on release of 5-HT from rabbit platelets by Diazo-ICA in the presence of calcium

Figure 4 shows that the release of 5-HT from isolated platelets by Diazo-ICA (0·36 mM) was completely blocked by ATP at a concentration of 3 mM. ADP had a similar but weaker effect while AMP had no inhibitory affect. At the concentrations tested none of these nucleotides themselves caused 5-HT release.

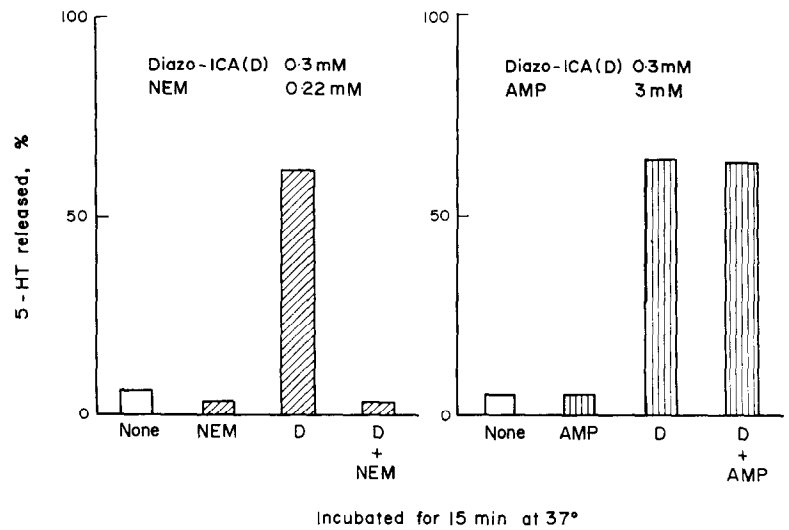

FIG. 3. Effect of NEM and AMP on the release of 5-HT from isolated platelets by Diazo-ICA in the presence of calcium.

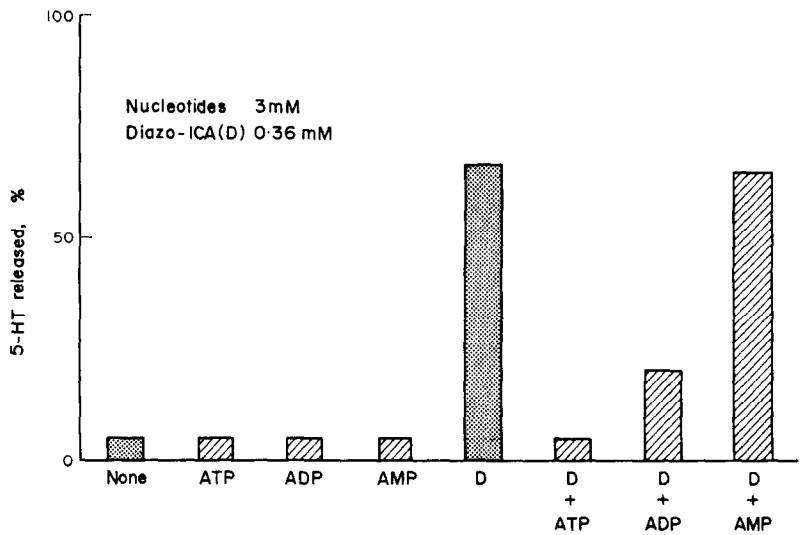

FIG. 4. Effect of ATP and other nucleotides on release of 5-HT from rabbit platelets by Diazo-ICA in the presence of calcium.

Effect of phosphate, inorganic pyrophosphate, creatine phosphate and ATP on release of 5-HT from rabbit platelets by Diazo-ICA in the presence of calcium

ATP completely blocked the release of 5-HT by Diazo-ICA. Therefore, the relation of high energy compounds with the release of 5-HT by Diazo-ICA was examined.

When platelets were suspended in modified Tyrode solution containing calcium and incubated for 10 min at 37°, negligible 5-HT was released from them. However, when they were incubated with 0·3 mM Diazo-ICA in the same system, 61 per cent of their 5-HT was released. This liberation of 5-HT by Diazo-ICA was completely inhibited

TABLE 1. EFFECT OF PHOSPHATE, INORGANIC PYROPHOSPHATE, CREATINE PHOSPHATE, AND ATP ON RELEASE OF 5-HT FROM RABBIT PLATELETS BY DIAZO-ICA IN THE PRESENCE OF CALCIUM

0·3 mM	5-HT Released (%)	
3 mM	None	Diazo-ICA
Phosphate	4	59
Inorganic pyrophosphate	3	4
Creatine phosphate	4	58
ATP	4	3
None	3	61

Incubated for 10 min at 37°.

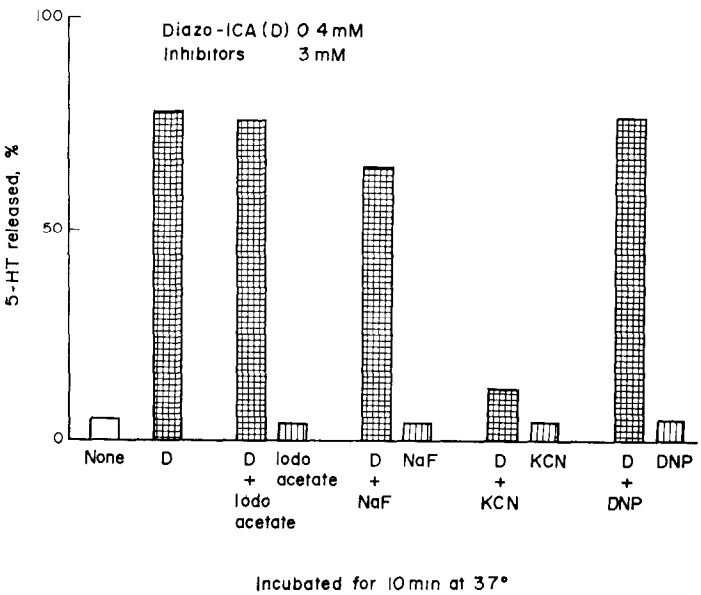

FIG. 5. Effect of metabolic inhibitors on 5-HT release from rabbit platelets by Diazo-ICA in the presence of calcium.

by the presence of inorganic pyrophosphate or ATP at a concentration of 3 mM. However, creatine phosphate and inorganic phosphate were ineffective. These results are shown in Table 1.

Effect of metabolic inhibitors on 5-HT release from rabbit platelets by Diazo-ICA in the presence of calcium

To elucidate the energy producing system involved in the mechanism of 5-HT release from isolated rabbit platelets by Diazo-ICA, the effects of the metabolic inhibitors, monoiodoacetate, NaF, KCN and DNP were investigated. As shown in Fig. 5, only KCN inhibited the release, and at a concentration of 3 mM, it caused almost complete inhibition. None of the metabolic inhibitors tested themselves caused release of 5-HT.

DISCUSSION

There is evidence that an energy-requiring process is involved in release of 5-HT from platelets by drugs,[15] but the mechanism of release is unknown. We found that calcium was required for 5-HT liberation from platelets by Diazo-ICA.[13] Iwata *et al.*[12] showed that Diazo-ICA readily combines with sulfhydryl compounds, such as cysteine and glutathione, so the actions of NEM and Diazo-ICA in liberation of 5-HT from platelets were compared. High, but not low concentrations of NEM caused 5-HT release even in the absence of calcium in the incubation medium. Moreover, 5-HT release by both NEM and Diazo-ICA was prevented by sulfhydryl compounds such as cysteine, gluthione or BAL. Release of 5-HT by NEM has been suggested to be due to destruction of platelets.[5]

Diazo-ICA does not cause cell destruction.[13] Therefore, the calcium requirement for 5-HT release from platelets by Diazo-ICA may be associated with the absence of cell destruction. Unexpectedly we found that 5-HT liberation by Diazo-ICA was blocked by a low concentration of NEM, which itself caused no release of 5-HT from the platelets. This suggests that the site of action of Diazo-ICA for release of 5-HT from the platelets is modified by addition of the sulfhydryl reagent, NEM.

Trifaró *et al.*[16] reported that SH groups may be associated with catecholamine release from adrenal granules, but the nature of the association is unknown.

The ability of Diazo-ICA to release 5-HT *in vitro* depends greatly upon the temperature.[13] Thus during incubation at 37° almost all the 5-HT was liberated, while negligible amounts were released at 4°. This marked temperature-dependence suggests that some enzyme may participate in the release. Many workers have suggested that ATPase may be important in the release of biological amines. To elucidate the role of ATPase in the release of 5-HT by Diazo-ICA, the influences of the ATPase inhibitors, AMP and NEM were examined. Incubation of isolated platelets with NEM resulted in complete inhibition of the release by Diazo-ICA, while AMP did not, although the release of catecholamines by ATP is blocked by both AMP and NEM.

On the other hand, ATP and ADP blocked the release of 5-HT from platelets by Diazo-ICA. This suggests that the inhibition of ATPase by Diazo-ICA was prevented by ATP or ADP in medium containing calcium. However, it is also possible that ATP or ADP, but not AMP, chelates with calcium resulting in a decrease in the amount of free calcium in the medium and consequent reduction in the release of 5-HT by Diazo-ICA.

REFERENCES

1. H. E. Skipper and L. L. Bennett, Jr., *Annual Review of Biochemistry*, Annual Reviews, Inc., Palo Alto, Calif., 1958 Vol. 27. pp. 137.
2. K. Hano, A. Akashi, I. Yamamoto, S. Narumi, Z. Horii and I. Ninomiya, *Gann* **56**, 417 (1965).
3. K. Hano, A. Akashi, I. Yamamoto, S. Narumi and H. Iwata, *Gann* **59**, 207 (1968).
4. Y. F. Shealy, R. F. Struck, L. B. Holum and J. A. Montgomery, *J. org. Chem.* **26**, 2396 (1961).
5. Y. F. Shealy, C. A. Krauth and F. A. Montgomery, *J. org. Chem.* **27**, 2150 (1962).
6. I. Yamamoto, *Biochem. Pharmac.* **18**, 1463 (1969).
7. J. L. Skibba, G. Ramires, D. D. Beal and G. T. Bryan, *Cancer Res.* **29**, 1944 (1969).
8. K. Hano, A. Akashi, Y. Suzuki, I. Yamamoto, S. Narumi and H. Iwata, *Jap. J. Pharmac.* **17**, 668 (1967).
9. H. Iwata, T. Dohi and I. Yamamoto, *Jap. J. Pharmac.* in press.
10. H. Iwata and I. Yamamoto, in preparation.
11. I. Yamamoto, M. Oka and H. Iwata, *Biochem. Pharmac.* **19**, 1831 (1970).

12. H. IWATA, I. YAMAMOTO and M. OKA, *Jap. J. Pharmac.* **18,** 471 (1968).
13. I. YAMAMOTO and H. IWATA, *Biochem. Pharmac.* **19,**1541 (1970).
14. H. WEISSBUCH, T. P. WAALKES and S. UDENFRIEND, *J. biol. Chem.* **230,** 865 (1958).
15. A. PLETSCHER, G. BARTHOLINI and M. DA PRADA, *Mechanism of Release of Biogenic Amines*, p. 165, Pergamon Press, Oxford (1965).
16. J. M. TRIFARÓ and A. M. POISNER, *Molec. Pharmac.* **3,** 572 (1967).