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# CYTOTOXIC EFFECTS OF IMIPRAMINE ON PLATELETS*

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**Abstract**—Incubation for 30 min at 30° with 1 mM imipramine, chlorpromazine or phencyclidine produced increases in creatine phosphokinase (CPK) activity of rat platelet-rich plasma (PRP). Increases in lactic dehydrogenase (LDH) activity of human PRP were produced by imipramine at 0·5 mM. Ouabain, diphenylhydantoin, calcium chloride, EDTA and thrombin did not affect CPK activity of rat PRP. None of the dugs tested except 10 mM diphenylhydantoin affected CPK or LDH activity in platelet-poor plasma. Increases in enzyme activity of PRP produced by 1 mM imipramine were associated with a decrease in platelet count. The increases were independent of temperature of incubation from 0° to 30° and were maximal after a 5-min incubation. The release of LDH and CPK caused by the above drugs is not related to platelet aggregation or the platelet release reaction. The increases in LDH and CPK activity in PRP appear to be the result of platelet destruction or damage to the platelet plasma membrane with release of these enzymes.

HIGH CONCENTRATIONS of phenothiazines and imipramine *in vitro* and *in vivo* can cause, the efflux of a variety of intracellular substances. Dujovne *et al.*[1,2] have reported leakage of enzymes from liver cells grown in tissue culture after exposure to phenothiazines at concentrations of 1 mM. Meltzer[3] has reported that chlorpromazine (CPZ) can produce leakage of creatine phosphokinase (CPK) from rat skeletal muscle *in vitro*. CPZ releases epinephrine from the adrenal medulla under a variety of conditions.[4–7] Release of 5-hydroxytryptamine, histamine, potassium and nitrogen from platelets incubated with phenothiazines or imipramine has been observed.[8–12] The release of intracellular substances produced by these drugs has been interpreted by some to be the effects of a selective or general increase in membrane permeability [3,8,12] or an expression of cytotoxicity.[1,2,9,11,13] In support of the latter hypothesis, Solatunturi[13] observed damage to the platelet plasma membrane by electron microscopy after exposure to CPZ at concentrations of 0·5 to 1 mM.

The present study was designed to investigate the effects of CPZ and imipramine on the release of CPK from rat platelets and lactic dehydrogenase (LDH) from human platelets *in vitro*. Both enzymes are mainly soluble in the cytoplasm rather than present in organelles. After determining that both drugs could promote release of these enzymes from platelets, the mechanism of the imipramine-induced release of CPK and LDH from platelets was further studied by comparison of its action with agents which promote platelet aggregation and the platelet release reaction, as well

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as with other membrane-active drugs. The destructive effects of imipramine on platelets were considered to account only partially for its enzyme-releasing properties.

## MATERIALS AND METHODS

Male Sprague–Dawley rats, purchased from Sprague–Dawley, Inc., Madison, Wis., were used for these studies. Rats were housed in groups of six in a temperature-controlled room (22 ± 1°) with 12 hr light–dark cycle. Rat blood was obtained from the inferior vena cava immediately after anesthesia with thiopental, 100 mg/kg, i.p. Five ml of blood was withdrawn through No. 21 needles into plastic syringes, and, after removal of the needle, was immediately added to glass test tubes containing 7 mg EDTA. In some studies, the anticoagulant was 20 units sodium heparin/ml of blood. In human subjects, blood was drawn directly into EDTA Vacutainer tubes.

Platelet-rich plasma (PRP) was prepared by centrifugation of anti-coagulated whole blood at 300 *g* for 5 min at 20°. Platelet-poor plasma (PPP) was obtained from whole blood or PRP by centrifugation at 750 *g* for 15 min. Platelet counts were carried out by light microscopy in a standard counting chamber using a Unopette-diluting apparatus. Each PRP sample was counted to insure a minimal platelet count of 100,000 platelets/mm$^3$; any samples with platelet counts less than this were discarded. PRP platelet counts were generally 300,000 platelets/mm$^3$ or greater. Plasma was stored in an ice bath prior to experimentation, which was carried out within 4 hr of obtaining the blood sample. Platelet counts were also performed in the same manner after some of the incubations of PRP to be described subsequently.

To study the ability of drugs and chemicals to release CPK or LDH from platelets, 0·2 ml PRP or PPP from the same blood specimen was mixed with 0·1 ml normal saline containing varying concentrations of the agent, followed by incubation at 30° for 0·5 hr. In separate experiments, incubation time and temperature parameters were varied from 5 min to 4 hr and from 0° to 30° to investigate the effect of these variables on enzyme release. Immediately after incubation, PRP was centrifuged at 750 *g* for 15 min at 0° to remove all intact platelets. Enzyme activities of the resulting plasma and the original PPP sample were determined within 2 hr. The difference in enzyme activity between the supernatant PRP of the treated sample and that of the untreated samples was assumed to represent release of enzyme from the platelets. Measurement of enzyme activity of identically treated samples of PPP also insured that any direct effect of the drug or chemical on enzyme activity or assay procedure would be detected.

The following drugs or chemicals were tested: CPZ, imipramine, phencyclidine, ouabain, diphenylhydantoin, calcium chloride, EDTA and thrombin. All drugs were tested in the following concentrations: 10 mM, 1 mM, 0·01 mM and 0·001 mM. Thrombin was tested in concentrations of 0·0001 to 100 units/ml. Imipramine was tested with a more extensive range of concentrations: 10 mM, 7·5 mM, 5 mM, 2·5 mM, 1 mM, 0·75 mM, 0·5 mM, 0·25 mM, 0·01 mM and 0·001 mM.

CPK activity in rat platelet studies and LDH activity in human platelet studies were determined spectrophotometrically by the methods of Rosalki[14] and Wacker *et al.*[15] respectively. Reagents were obtained in kit form from Calbiochem.

CPZ was a gift from Smith, Klein & French Laboratories, Philadelphia, Pa. Imipramine was a gift from Ciba–Geigy Corp., Basel, Switzerland. Phenycyclidine and diphenylhydantoin were gifts from Parke, Davis & Co., Detroit, Mich. Ouabain and

thrombin (bovine, grade III) were obtained from Sigma Chemical Co., St. Louis, Mo. Calcium chloride and EDTA were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J.

TABLE 1. PER CENT CHANGE IN CPK ACTIVITY OF RAT PRP PRODUCED BY CHEMICAL AGENTS COMPARED TO UNTREATED PRP

| Drug tested | Concn of drug tested (mM) 10 | 1 | 0·1 |
|---|---|---|---|
| Imipramine | +85* | +163 | 0 |
| Chlorpromazine | † | +110 | +4 |
| Phencyclidine | +99 | +33 | −6 |
| Ouabain | −4 | +9 | −3 |
| Diphenylhydantoin | † | −7 | −5 |
| $CaCl_2$ | −20 | −8 | −11 |
| $MgCl_2$ | −4 | −2 | +3 |

* Per cent increase in CPK activity in rat PRP treated by the indicated drug concentration. Percentage is based on CPK activity of PRP not treated by drug but handled in an identical manner. Values are means of two or more experimental determinations.

† Chlorpromazine and diphenylhydantoin at 10 mM concentration interfered with the assay of CPK in both PRP and PPP.

## RESULTS

Imipramine, CPZ and phencyclidine in concentrations of 1 mM or higher produced increases in CPK activity of rat PRP (Table 1). Imipramine produced quantitatively the greater increase of these three agents, phencyclidine the least. None of the other drugs tested produced an increase in CPK activity of rat PRP even at the highest concentration tested. This included thrombin in concentrations ranging from 0·0001 to 100 units/ml. Diphenylhydantoin, 10 mM, decreased CPK activity in PRP samples, but a similar effect was observed in PPP, indicating that it interferes with the assay procedure at this concentration. None of the other drugs tested had any effect on the CPK assay.

The effects of a wider range of concentration of imipramine on LDH activity of human PRP are summarized in Table 2. The increase in LDH activity produced by

TABLE 2. EFFECT OF IMIPRAMINE ON LDH ACTIVITITY OF HUMAN PRP

| Concn of imipramine (mM) | No. of samples | Per cent change in CPK activity compared to untreated PRP (mean ± S. E. M.) |
|---|---|---|
| 10 | 6 | +162 ± 32 |
| 7·5 | 6 | +267 ± 43 |
| 5·0 | 8 | +384 ± 43 |
| 2·5 | 7 | +359 ± 74 |
| 1·0 | 11 | +95 ± 24 |
| 0·75 | 8 | +89 ± 15 |
| 0·50 | 11 | +46 ± 16 |
| 0·25 | 12 | +17 ± 13 |
| 0·10 | 7 | −10 ± 8 |
| 0·05 | 8 | +9 ± 11 |
| 0·01 | 6 | 0 ± 3 |

imipramine was maximal at 5 mM concentration, but a detectable increase was produced by concentrations as low as 0·5 mM. This concentration produced a mean increase in LDH activity of PRP of about 40 per cent. The mean per cent increase in LDH activity of human PRP treated by 1 mM imipramine was 90 per cent. The same concentration of imipramine resulted in a 160 per cent increase in CPK activity of rat PRP compared to untreated PRP.

Platelet counts and CPK activity of rat PRP after incubation for 30 min at 30° with 1 mM imipramine are given in Table 3. A decrease in the platelet count was accompanied by a substantial increase in CPK activity. In a previous study of rats of the same strain and weight, the CPK activity of platelets after lysis by repeated freezing and thawing was 610 mU/$10^9$ platelets.[16] The calculated increase of CPK in the PRP based on platelet loss only is 58 per cent of the observed increases in CPK activity of imipramine-treated PRP (Table 3).

The increases in CPK activity of rat PRP produced by 1 mM imipramine at 0, 15, 25 and 30° were not significantly different (data not presented). The increase in CPK of PRP was maximal after a 5-min incubation at 30° (data not presented). The CPK increases caused by 1 mM imipramine were not significantly different in PRP anticoagulated with either heparin or EDTA (data not presented).

## DISCUSSION

Incubation of rat PRP with CPZ, imipramine and phencyclidine at 1 mM concentration increased the activity of CPK in the plasma which remained after removal of platelets by centrifugation. Imipramine produced a similar effect on LDH activity in human PRP. There are a number of possible causes of release of enzymes by platelets which are worthy of consideration.

We first considered that CPK or LDH release from platelets might occur during the platelet release reaction or platelet aggregation, in which release of some enzymes has been previously reported.[17] However, platelet aggregation is inhibited rather than facilitated by both CPZ and imipramine at the millimolar to micromolar concetration range.[18,19] Moreover, aggregation is characteristically produced by thrombin,[17] which we found to have no effect on enzyme release. Therefore, it seems unlikely that LDH or CPK release is related to platelet aggregation. The platelet release reaction differs from platelet aggregation in that during the platelet release reaction, adenine nucleotides (especially adenosine diphosphate), serotonin, amino acids and proteins including some enzymes are released from the platelet.[17,20] Release of CPK from rat platelets during the release reaction has not been studied; however, LDH is not released from human platelets during the release reaction.[21] Imipramine and amitryptiline inhibit the release reaction in concentrations of 0·01 to 0·04 mM.[22,23] The effect of phenothiazines on the platelet release reaction is also inhibitory.[18,22] Thus, the platelet release reaction appears to be a different phenomenon from that reported here.

The decrease in platelet count and increase in CPK activity of rat PRP after incubation with 1 mM imipramine suggests that imipramine at this concentration causes platelet destruction. Our calculations indicate that the total amount of CPK activity present in the platelets which disintegrate during imipramine treatment is 58 per cent

TABLE 3. EFFECT OF IMIPRAMINE (1 mM) ON PLATELET COUNT AND CPK ACTIVITY OF RAT PRP

| Sample No. | (A) Saline-treated plasma fraction platelet count/mm$^3$ | (A) Saline-treated plasma fraction CPK activity (mU/ml) | (B) Imipramine-treated plasma fraction platelet count/mm$^3$ | (B) Imipramine-treated plasma fraction CPK activity (mU/ml) | Loss in platelet count (A–B) /mm$^3$ | Predicted increase in CPK activity from lost platelets* | Observed increase in CPK activity (B–A) |
|---|---|---|---|---|---|---|---|
| 1 | 546,000 | 43 | 347,000 | 270 | 199,000 | 121 | 227 |
| 2 | 650,000 | 85 | 368,000 | 434 | 282,000 | 171 | 349 |
| 3 | 585,000 | 60 | 282,000 | 297 | 297,000 | 180 | 237 |

* Activity calculated on loss of platelets, assuming 610 mU CPK activity/$10^9$ platelets.[16]

of the observed increases in CPK activity of treated PRP. Thus, destruction of platelets, with loss of enzyme contents, cannot account for the entire increase in CPK activity in the plasma fraction. This indicates that additional efflux of CPK from the surviving platelets, perhaps through increased cell membrane permeability or exocytosis, must also occur.

The drug concentrations at which enzyme release was observed in this study are similar to those reported to cause cytotoxicity or increased permeability by Solatunturi,[13] Langslet *et al.*,[24] Dujovne *et al.*[1,2] and Meltzer.[3] Other effects produced by similar concentrations of CPZ or imipramine are decreased oxygen consumption and release of 5-hydroxytryptamine from platelets (CPZ and imipramine, 0·1–1·0 mM),[9] release of epinephrine from isolated perfused bovine adrenal glands,[4] adrenal medullary slices[5,6] and adrenomedullary granules[7] (CPZ and imipramine, 0·5–5 mM),[25] inhibition of mitochondrial enzyme activity,[26] and release of calcium and epinephrine from the perfused bovine adrenal medulla (CPZ, 1 mM).[27] It seems likely that cytotoxic effects of the drugs play at least a partial role in the above observations.

Boullin and O'Brien[28] have reported that imipramine, 0·001 mM, is concentrated at the outer membrane of platelets in suspension. The rapid onset of the cytotoxic effects of imipramine and lack of dependence on incubation temperature observed in this study suggest that these results may be due to a direct action of the drug on the platelet membrane. Although low concentrations of CPZ and imipramine (0·001 to 0·01 mM) have a membrane-stabilizing effect, this stabilizing effect is reversed at higher doses for both drugs.[25,29,30] Relatively high concentrations of the drug which would be present at the platelet surface in this study could have damaged the plasma membrane.

Since CPZ and imipramine produced cytotoxic effects at concentrations from 0·1–1·0 mM which are higher than those which occur *in vivo*, it does not seem likely that this effect has any significance physiologically. No increases in plasma LDH activity or decreases in platelet count have been observed in psychiatric patients treated with therapeutic doses of CPZ or imipramine for 2–4 months.*

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