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SHORT COMMUNICATIONS

Chlorpromazine makes the platelet plasma membrane permeable for low-molecular weight substances and reduces ATP production

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At concentrations of 50 μM and higher, trifluoperazine (TFP) [1] and chlorpromazine (CPZ) [2] cause loss of radioactive ATP and ADP from platelets prelabelled with [^{14}C]adenine. A number of studies on the inhibitory action of these phenothiazines on agonist-induced responses in intact platelets have been done with these concentrations [3–9]. Therefore, the inhibitions studied may have been due to energy depletion.

The mechanisms underlying the loss of adenine nucleotides from platelets by CPZ are not understood. Since these nucleotides released from ^{14}C-labelled platelets were radioactive, they clearly originated from the cytoplasm [10]. The present study was aimed to see whether the adenine nucleotide released from platelets by CPZ is selective or whether other low-molecular and high-molecular substances are also released. We also wanted to establish whether the CPZ-induced loss of metabolic adenine nucleotides affected energy production in the cells.

Materials and Methods

CPZ, reduced nicotinamide adenine dinucleotide, antimycin A and 2-deoxy-D-glucose were from the Sigma Chemical Co. (St Louis, MO). Other chemicals were reagent grade. Thrombin (bovine, "Topical", La Roche) was stored in a small aliquots at 100 units/mL in 0.15 M NaCl. Lactate dehydrogenase (Type I from rabbit muscle) was from Sigma. [^{32}P]Orthophosphate (carrier-free, code PBS) [11] and [^{14}C]adenine (300 mCi/mmol, code CFA 436) were from Amersham (Amersham, U.K.).

Platelet-rich plasma (PRP) was prepared from human blood [11]. The PRP was subsequently incubated at 37° with either [^{32}P]orthophosphate (0.1 mCi/mL) or [^{14}C]adenine (0.8 μM) and then gel-filtered as described previously [12]. ^{32}P- or ^{14}C-labelled GFP was equilibrated at 37° and CPZ in various concentrations was added. After 60 sec, 0.2 units/mL thrombin or a corresponding volume of 0.15 M NaCl was added and the mixtures were incubated further. Ninety seconds after addition of thrombin or 0.15 M NaCl, 200-μL aliquots of the incubation mixture were transferred to 30 μL of 1 M formaldehyde in 50 mM Na-EDTA (pH 7.4) on ice. These samples were then centrifuged for 2 min at 12,000 *g* (ambient temperature). One hundred microliters of the supernatants were mixed with 100 μL of 10 mM EDTA in 86.4% ethanol for analysis of [^{14}C]nucleotides by high voltage paper electrophoresis [13] and ethanol-soluble [^{32}P]metabolites by two-dimensional paper chromatography [14]. Lactate dehydrogenase was determined [15] in these supernatants or in unfixed supernatants obtained by centrifugation through an oil layer [16]. β-Hexosaminidase was determined with a 4-methyl-umbelliferyl *N*-acetyl-β-D-glucosaminide as substrate [17].

Non-centrifuged aliquots of the formaldehyde-fixed incubation mixtures were also prepared for determination of the total amount of substances or activity of enzymes in the platelets. These were extracted with ethanol-EDTA as above for adenine nucleotides and ^{32}P-labelled substances, and lysed with 0.1% Triton X-100 for the enzymes before further analysis.

ATP production was determined as the rate of lactate production in the platelets when mitochondrial respiration was abolished. Aliquots (1.0 mL) from the incubation mixtures (above) were mixed with 10 μg/mL of antimycin A and 200-μL samples were mixed with EDTA-ethanol (above) before and after incubation for 60 min at 37°. Lactate were determined in the ethanol extracts [18].

The term "release" defines transfer of a substance from the cell to the medium by any mechanism but exocytosis, which is termed "secretion". This distinction is particularly important for the adenine nucleotides in platelets. Two-thirds of these are sequestered in the dense granules as a non-metabolic pool which is *not* labelled by incubation of the cells with [^{32}P]P_i or [^{14}C]adenine; this nucleotide pool is secreted when platelets are treated with thrombin. The remaining one third is present in the cytoplasm, participates in metabolism and is labellled by the radioisotopes; these are *not* secreted by thrombin [10]. Any transfer to the medium of the labelled nucleotides (or glycolytic intermediates) is referred to as "release".

Each figure shown is representative of three to five experiments.

Results

Both [^{14}C]adenine- and [^{32}P]orthophosphate-labelled ATP and ADP appeared extracellularly when GFP was incubated for 150 sec with CPZ in concentrations above 75 μM (Fig. 1). The fraction of these cytoplasmic, low-molecular substances that was released, increased as the concentration of CPZ was raised, reaching a maximal value of 80% at 500 μM. Presence of thrombin during the last 90 sec of incubation made no difference (Fig. 1A). ^{32}P-Labelled fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate were also released by CPZ, and at lower concentrations than the adenine nucleotides (Fig. 1B). The radioautograms showed the presence of all ethanol-soluble, intracellular, ^{32}P-labelled substances from platelets on the chromatograms (see Ref. 14) of the medium after incubation of GFP with CPZ. Prolonging the incubation of GFP with each concentration of CPZ from 150 sec to 15 min did not cause more release of these substances (results not shown).

CPZ caused progressive inhibition of thrombin-induced secretion of ATP + ADP when its concentration was increased above 15 μM and abolished secretion at 50 μM. Raising the concentration of CPZ above 50 μM caused the same increase in the mass of ATP + ADP extracellularly as found for the radioactive adenine nucleotides (data not shown). This bi-phasic effect of CPZ on ATP + ADP (mass) was identical to that found for TFP [1].

Lactate dehydrogenase was not released either in the absence or presence of thrombin by any concentrations of CPZ up to 2000 μM (Fig. 2). This was not due to inhibition of extracellular lactate dehydrogenase by the formaldehyde fixation, since supernatants made without formaldehyde (oil layer centrifugation) did not contain measurable lactate dehydrogenase as well. Control experiments showed that neither formaldehyde nor CPZ in the concentration used had any effect on the lactate dehydrogenase activity in extracts made by making GFP 0.1% with respect to Triton X-100.

β-Hexosaminidase, on the other hand, was released pro-

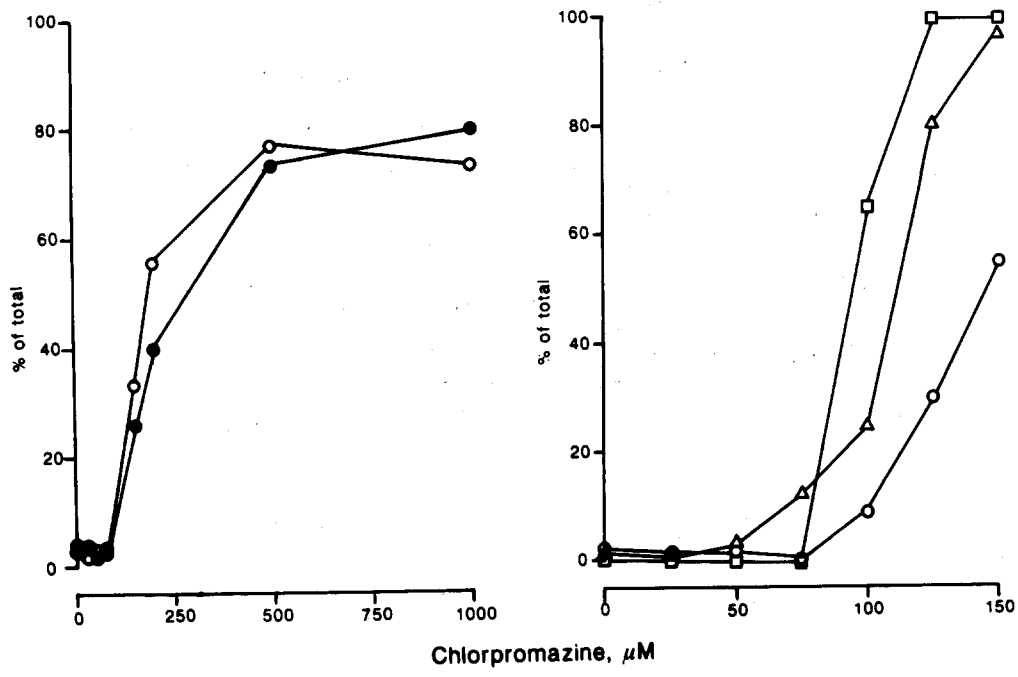

Fig. 1. Effect of CPZ on the extracellular appearance of labelled adenine nucleotides and glycolytic intermediates in GFP. Aliquots of GFP prepared from PRP labelled with [^{14}C]adenine (left panel) or [^{32}P]P_i (right panel) were incubated with CPZ in the concentrations given on the abscissas for 60 sec and then for a further 90 sec period with (closed symbols) or without (open symbols) 0.1 units/mL of thrombin. The platelets were then fixed and centrifuged, and the content of [^{14}C]ATP + ADP (○, ●), [^{32}P]ATP + ATP (○), [^{32}P]fructose 1,6-biphosphate (△) and [^{32}P]glyceraldehyde 3-phosphate (□) were determined in the supernatants and non-centrifuged samples.

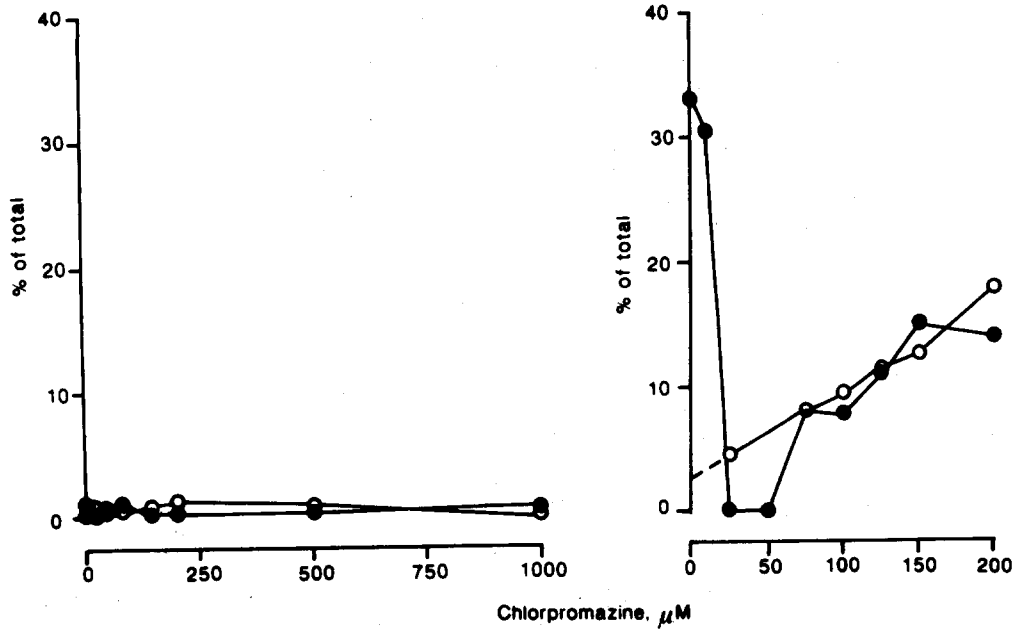

Fig. 2. Effect of CPZ on the extracellular appearance of lactate dehydrogenase (left) and β-hexosaminidase (right) in GFP. The experiment was performed and the open/closed symbols are defined as in Fig. 1, except that the enzymes were determined.

gressively as the CPZ concentration was increased above 50 μM in the absence of thrombin and to an extent of 18% at 200 μM (Fig. 2). Thrombin causes secretion of β-hexosaminidase from platelet lysosomes [19], which is seen in Fig. 2 in the absence of CPZ. When the concentration of CPZ was increased the thrombin-induced secretion was progressively inhibited and abolished at 25 μM. Increase in the CPZ concentration above 50 μM in the presence of thrombin caused appearance of the same amounts of extracellular β-hexosaminidase as in the absence of thrombin (Fig. 2). This inhibition of thrombin-induced β-hexosaminidase secretion at low and release of β-hexosaminidase at high concentrations of CPZ was similar to the effects of TPF [1].

CPZ in concentrations up to 75 μM had no effect on the lactate production in the presence of antimycin A (Fig. 3). However, as the CPZ concentration was increased from 75 to 200 μM, i.e., which causes progressive leakage of cytoplasmic nucleotides (see above), lactate production was gradually inhibited and reached 35% of the control value at 200 μM. Thrombin alone (Fig. 3, ordinate) increased lactate production by 100%; this elevated rate was not altered by CPZ in the 0 to 50 μM range, but lactate production was progressively inhibited as the drug concentration was increased, reaching 17% of initial value at 200 μM (Fig. 3).

Discussion

CPZ in concentrations above 50–70 μM causes rapid leakage of low-molecular weight substances from platelets. Since these are metabolic intermediates localized in the cytoplasm, CPZ must render the *plasma membrane* permeable for low-molecular ($M_r < 2000$) substances. The other cytoplasmic marker used, lactate dehydrogenase with $M_r = 170{,}000$ [20], was not released to the medium by CPZ below 2000 μM. This strongly suggests that CPZ perforates the platelet plasma membrane in such a way that it becomes permeable to low-molecular and impermeable to high-molecular weight substances. Such selective permeabilization of the plasma membrane has been shown with electric discharge [21] and for detergents such as saponin [22].

β-Hexosaminidase is mainly localized in the platelet lysosomes [23, 24] and only 60% of the enzyme is secreted with maximal stimulation [19]. Variable proportions of β-hexosaminidase are also associated with platelet microsomes after subcellular fractionation [25, 26]. Thus, the platelet plasma membrane may contain bound β-hexosaminidase which could be solubilized by CPZ and released to the medium. This contention is supported by our recent finding (P. Tharmapathy and H. Holmsen, unpublished) that the plasma membrane-located acid 4-methylumbelliferyl phosphatase [24–26], which is not secretable [19], is released to the medium by 50–200 μM chlorpromazine to a much greater extent than β-hexosaminidase.

CPZ in permeabilizing concentrations (50–500 μM) abolishes secretion of serotonin [3, 6] which is localized in the dense granules. This is in accordance with the complete retention of all organelles in platelets treated with 140 μM CPZ [9]. CPZ therefore seems to act specifically on the *plasma membrane* in which it forms small pores and releases membrane-bound enzymes.

CPZ in non-permeabilizing concentration (<50 μM) had no effect on ATP turnover. However, within this concentration range CPZ (Ref. 2, present work) and TFP [1] both inhibit platelet responses. Hence, this inhibition is not due to lowering of ATP availability. When the platelets were permeabilized by CPZ (>50 μM), the ATP turnover did decrease which could contribute to inhibition of platelet responses and accompanying signal transduction processes that utilize ATP directly such as protein phosphorylation [1, 5, 7, 9].

Ferrell *et al.* [9] showed that 140 μM CPZ causes despiculation and sphering of platelets and suggested that this is caused by intercalation of CPZ with the inner layer of the plasma membrane, thus altering the "bilayer balance". We have recently shown (H. Klausen and H. Holmsen, unpublished) that reduction of ATP availability by incubation of platelets with antimycin A and deoxyglucose also causes sphering and vacuolization of the platelets. It is therefore uncertain whether the sphering caused by permeabilizing doses of CPZ is a direct effect of the drug or of the accompanying ATP depletion resulting in decreased ATP availability.

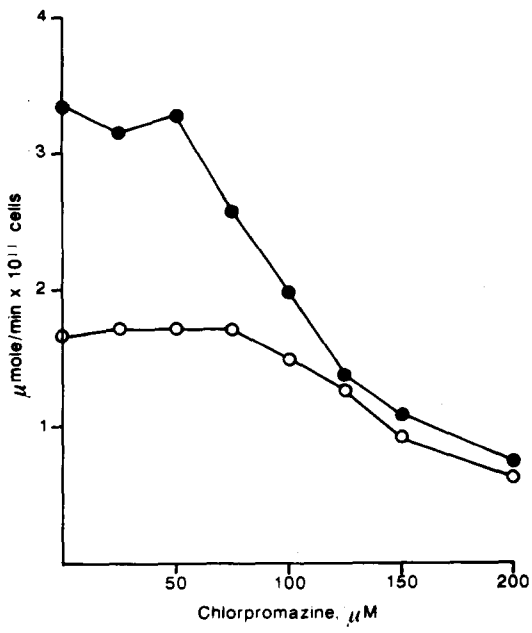

Fig. 3. Effect of CPZ on ATP production in GFP. The experiment was performed and the open/closed symbols are defined as in Fig. 1, except that after the 150 sec total incubation, antimycin A (5 μg/mL) was added to the samples which were further incubated for 60 min. Then EDTA-ethanol was added and lactate determined.

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Influence of volatile anesthetics on muscarinic regulation of adenylate cyclase activity

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General anesthetics have profound effects on several aspects of cardiovascular performance under autonomic control involving muscarinic cholinergic receptors [1], and it is reasonable to suspect that a disruption of cholinergic transmission in the heart contributes to the development of certain aspects of anesthetic action. We have demonstrated that volatile anesthetics alter the ability of guanine nucleotides to regulate muscarinic agonist binding in both rat cardiac and brainstem membranes, indicating that volatile anesthetics disrupt muscarinic receptor–G protein interactions [2–4]. Halothane was also found to decrease muscarinic control of adenylate cyclase activity [5]. The present studies were designed to determine if disruption of muscarinic modulation of adenylate cyclase activity is a common property of volatile anesthetics. Our results demonstrate that muscarinic control of adenylate cyclase is inhibited by numerous volatile anesthetics.

Materials and Methods

Adult male Wistar rats were killed by decapitation. The hearts were removed and cleaned of fat and blood vessels, and homogenized in TED buffer, pH 7.5 (10 mM Tris–