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The accumulation of an aryloxylkylamidine (501C) and 5-hydroxytryptamine in human polymorphonuclear leucocytes: a quantitative electron microscopy study

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The uptake of anilino-*N*-2-m-chlorophenoxypropylacetamidine (501C) and 5-hydroxytryptamine (5-HT), in human polymorphonuclear leucocytes (PMN) was investigated by quantitative ultrastructural autoradiography. The major concentration of both drugs was in the cytoplasmic granules, 71·5 ± 6·8% for [^{3}H] 501C, and 68·75 ± 6·1% for [^{3}H]5-HT. Lesser quantities of both drugs were associated with the nucleus; 18·1 ± 4·8% for [^{3}H] 501C and 23·0 ± 4·2% for [^{3}H] 5-HT. Only small amounts of activity were recorded at other sites. The data suggests that there may be common binding sites for 501C and 5-HT in PMN. Furthermore the concentration of 501C in PMN granules may also account for the damaging effect of high concentrations of the drug on PMN.

Polymorphonuclear leucocytes (PMN) have been shown to concentrate a large number of basic drugs. These include amphetamine, codeine, desipramine, morphine (Marks & Medzihradsky 1974) and 5-hydroxytryptamine (5-HT) (Eliseeva & Stefanovitch 1982). Whereas 5-HT appears to have no significant effect on leucocyte function (for example see Trist & Weatherall 1982), the 5-HT antagonist 501C (anilino-*N*-2-m-chlorophenoxypropylacetamidine) inhibits mobility, phagocytosis and other activities of PMN when present in sufficient concentrations (unpublished data). We have found that 501C like other bases is also concentrated avidly by leucocytes.

A quantitative ultrastructural autoradiographic investigation has been carried out with PMN incubated with [^{3}H]501C and [^{3}H] 5-HT to ascertain where these drugs are accumulated within the cell. The experiments were directed to establish whether 501C and 5-HT have common binding sites within the cell and to provide further information on their mode of action on leucocyte function.

MATERIALS AND METHODS

Collection and separation of leucocytes

Human peripheral blood was obtained by venepuncture using heparin, 20U ml^{-1} (Pularin, Evans Medical Supplies). The red cells were sedimented by adding 2% methyl cellulose in phosphate buffered saline (7·5 ml/100 ml blood) and the leucocyte rich plasma removed. The leucocytes were then separated from the plasma by centrifugation, washed once with a saline medium (Na^+, 133; K^+, 5·4; Ca^{2+}, 1·5; Mg^{2+}, 0·8; Cl^{-1}, 131; HCO_3, 8·9; H_2PO_4, 1·0; $SO_4{}^{2-}$, 0·8; glucose, 5·5 mM, pH 7·4) and resuspended in saline medium at 6·2 × 10^6 cells ml^{-1}. Cell counts were estimated with a Coulter particle counter (Model FN).

Estimation of drug uptake

Initially experiments were carried out with [^{14}C]501C to follow the uptake of the drug in the same conditions employed for the electron microscopy study. Aliquots (1·0 ml) of human leucocytes at 4·1 × 10^6 ml^{-1} were incubated with [^{14}C]501C (20 μM, spec. act. 17 mCi mmol^{-1}) for 60 min at 36 °C. The cells were spun in a microcentrifuge (Quickfit) at 12 600*g* for 20 s, washed with 1 ml of ice-cold inactive medium containing 501C at 20 μM and resuspended in fixative (see below). After 30 min the leucocytes were centrifuged and resuspended in 0·1 ml of concentrated nitric acid for 1 or 2 h at room temperature (20 °C). The lysate produced was diluted to 1 ml with distilled water and 0·1 ml aliquots counted by liquid scintillation spectrometry.

Incubation of leucocytes with [^{3}H]501C and [^{3}H]5-HT

Aliquots of leucocyte suspensions (0·9 ml) were dispensed into polythene micro-centrifuge tubes and left to recover for 1 h at 36 °C. 0·1 ml aliquots of

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either [^{3}H]501C (200 μM, spec. act. 1·6 Ci mmol^{-1}) or [^{3}H]5HT (200 μM, spec. act. 18·2 Ci mmol^{-1}; Radiochemical Centre, Amersham) were added to the appropriate tubes and they were left for 1 h at 36 °C. The tubes were then spun in the microcentrifuge at 12 600*g* for 20 s. The supernatant was removed and the cells washed twice with ice-cold 'inactive' 5-HT or 501C at 20 μM. Finally the cells were resuspended in a fixative solution and processed for electron microscopy.

Preparation of leucocytes for electron microscopy
The leucocytes were fixed for 30 min in a freshly prepared mixture consisting of 0·8% glutaraldehyde and 0·6% osmium tetroxide in 0·1M cacodylate buffer pH 7·4 at 4 °C (Hirsch & Fedorko 1968). They were dehydrated in dimethoxypropane and embedded in Araldite (EM Scope Laboratories Ltd., Ashford, Kent).

Preparation of electron microscope autoradiographs
Ultrathin sections (approx. 80 nm) were cut on an LKB ultratome III (LKB Bromma, Sweden) and collected on formvar/carbon coated 200 mesh copper grids. The grids were coated with a monolayer of Ilford L4 nuclear track emulsion applied by the loop method (Williams 1977). After nine months at 4 °C the preparations were developed in Microdol-X (Kodak) for 3 min, washed and fixed for 8 min in Kodafix (Kodak). After a further wash the sections were stained with saturated alcoholic uranyl acetate and lead citrate and examined in a Phillips 300 electron microscope at an accelerating voltage of 80 KV.

Analysis of the electron microscope autoradiographs
Visual examination of the sections in the microscope provided very little information on the site and amount of drug accumulation for either 5-HT or 501C. All that it showed was a scatter of silver traces across the cells with no apparent concentrations in any one area or organelle. The greatest difficulty in interpreting results such as these is locating exactly the source of disintegration. At the magnification used in electron microscope autoradiography, the source of the radionucleotide in the section may be far removed from the actual silver grain which may of course be situated over another organelle. This effect is termed the cross scatter. The method we used for the analysis of the autoradiographs was the simplified method of "hypothetical grain" analysis developed by Blackett & Parry (1977). This technique enables the radioactivity in different structures to be estimated. It takes into account the cross scatter that would be expected between neighbouring structures which may be labelled to different extents within a section. Also with this technique there is no need to make any assumptions about the size, shape and arrangement of the structures within the autoradiographs being analysed.

There are three steps in the analysis. The first step involves the generation of a hypothetical distribution of grains for determining the amount of cross scatter expected in the sample in which the radioactivity is theoretically distributed evenly throughout. The second step is the estimation of the location of the real silver grains on the section. In the third and final step, the two sets of values produced in steps one and two are compared to obtain estimates of the activity in different organelles.

Approximately two hundred PMN from each drug treatment were photographed and prints made at the appropriate magnification determined by the experimental procedures in this case × 23 400. The source and site of hypothetical grains and real grains were determined by use of a transparent overlay screen designed specifically for use with autoradiographs produced with tritiated material. This overlay was used to determine the position of approximately 800 hypothetical grains and 300 real grains each for 501C and 5HT treated cells.

The real grain analysis was compared to the hypothetical grain analysis using a minimizing subroutine. This gave values for the relative activity of labelled drug at various sites within the cells expressed as a percentage (mean ± s.e.) of the total activity in the section. The use of the transparent overlay screen and the computer program used to analyse the data have been described in detail by Blackett & Parry (1977).

RESULTS

After 60 min incubation in 20 μM 501C at 36 °C, 10^6 cells accumulated 402·9 ± 38·7 pmol (m ± s.e., n = 3): in a second experiment 10^6 cells accumulated 510·0 ± 56·0 pmol (m ± s.e., n = 3). Assuming a cell volume of 360 fl (Trist 1979) then the concentration of 501C in total cell water was 1·12 mM and 1·417 mM or 56 times and 71 times that in the medium respectively. Examination of the electron micrographs confirmed that with both drugs radioactivity occurs inside the PMN (Fig. 1A, B). However, it was not possible by purely visual inspection to interpret exactly the source of disintegration, i.e. the site of drug concentration. This was only made possible by using "hypothetical grain analysis".

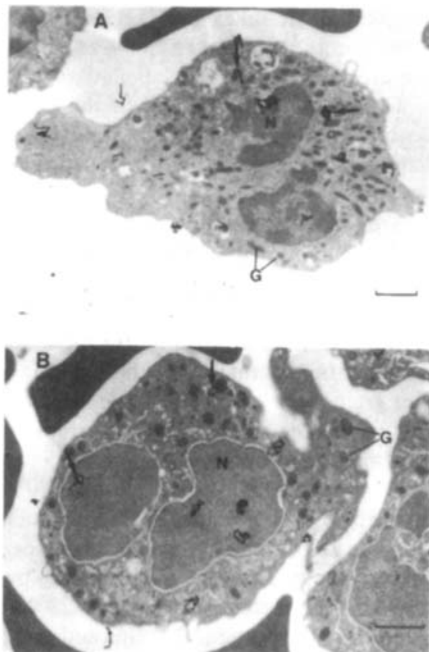

FIG. 1A, B. Autoradiographs of PMNs incubated in-vitro for 60 min with 20 μM [^{3}H]501C (A) and 20 μM [^{3}H]5-HT (B). Silver grains (arrows) are seen over the nuclei (N), granules (G) and cytoplasm of the PMN. A few grains are associated with erythrocytes (Bar = 1 μm).

The source and site of the hypothetical grains are shown in Table 1. Each row represents the position of the source of the hypothetical radioactive disintegration and each column represents the site of the hypothetical grains. The cross scatter of grains that would be expected between different regions of the PMN if the radioactivity were evenly distributed was estimated from these data. This is necessary as the site of the real grains may not be exactly over the site of the disintegration. Adding up the grains in each column gives the relative frequency of grains at various sites if the labelled drugs had been distributed uniformly throughout the cells. The same hypothetical grain data was used for comparison with the real grain data for both 501C and 5-HT drug treatments (Tables 1, 2).

The sites of the real autoradiographic grains for each drug treatment are presented in Table 2. Comparison of the sets of data for real and hypothetical grains (Table 3) showed that the major activity of both drugs was in the PMN cytoplasmic granules; 71·5 ± 6·8% (m ± s.e.) for 501C and 68·7 ± 6·1% (m ± s.e.) for 5-HT. Smaller amounts were found associated with the nucleus, 18·1 ± 4·8% (m ± s.e.) for 501C; and 23·0 ± 4·2% (m ± s.e.) for 5-HT. Only small amounts of activity were found at the other sites (Tables 3, 4).

Table 1. Source and site of the hypothetical grains determined by the analysis of the autoradiographs using the transparent overlay screen. Each row represents the position of the source of the hypothetical radioactive disintegration and each column represents the site of the hypothetical grains.

	C	N	G	C N	C G	C G N	C E
Cytoplasm (C)	43	18	1	28	73	34	78
Nucleus (N)	3	62	0	42	7	34	5
Granules (G)	2	9	0	3	96	20	12
Exterior (E)	18	1	0	3	48	4	56

Table 2. The sites of the real autoradiographic grains for each drug treatment BW501C and 5-HT. C (cytoplasm), N (nucleus), G (granules) and E (exterior).

	C	N	G	C N	C G	C G N	C E
501C	9	34	0	36	199	42	37
5-HT	0	53	0	20	175	64	44

Therefore the granules contained approximately 75% of the total activity of [^{3}H]501C and [^{3}H]5-HT detected in the cells. The relative area of each source was calculated using the hypothetical grain data and showed that granules take up approximately 20% of the total area of the sectioned cells (Table 4).

DISCUSSION

The results and literature show that PMN concentrate 501C and 5-HT to a high concentration compared with that of the suspending medium

Table 3. Results obtained from the comparison of the hypothetical and real grain distribution for each drug treatment 501C and 5-HT.

	Percentage (m ± s.e.) of total activity	
	501C	5-HT
Cytoplasm	5·5 ± 6·4	0·0 ± 3·9
Nucleus	18·1 ± 4·8	23·0 ± 4·2
Granules	71·5 ± 6·8	68·7 ± 6·1
Exterior	4·9 ± 6·3	8·3 ± 6·1

Table 4. The relative area of each source calculated using the hypothetical grain data.

	Percentage (m ± s.e.) of total area
Cytoplasm	39·3 ± 1·9
Nucleus	21·9 ± 1·5
Granules	20·3 ± 1·5
Exterior	18·6 ± 1·4

in-vitro. Autoradiography has also shown that the major accumulation of the drugs is in the cytoplasmic granules and to a lesser extent in the nuclei.

There are two main types of PMN cytoplasmic granule, azurophilic and specific, which differ in morphology and contents (Bainton et al 1971). The azurophilic granules contain peroxidase and various lysosomal enzymes and thus qualify as lysosomes. The contents of the specific granules remains largely undetermined; they contain alkaline phosphatase and basic proteins and they lack lysosomal enzymes. At present we have no evidence as to whether these drugs are concentrated in one particular type of granule. Mammalian cells in culture, including PMN, have been shown to take up basic dyes and drugs such as chloroquine and mepacrine to many times the concentration of the surrounding medium in a matter of minutes, and there is both morphological and biochemical evidence that they are taken selectively into lysosomal structures (Allison & Young 1964; Fedorko et al 1968a, b; Read & Trist 1978, 1982).

It has been suggested that this lysosomotropic property of the drugs bears an intimate relationship to their pharmacological, therapeutic and pathogenic effects (DeDuve et al 1974). In addition to their selective concentration in existing lysosomes, they appear to stimulate the formation of additional lysosomal structures (Fedorko et al 1968a, b). It is evident that very high concentrations of 501C and 5-HT will be attained within the PMN granules. For example with a 20 μM concentration in the medium, the estimated final concentration in the granules will be in the range 1–2 mM. The accumulation of high concentrations of 501C within the granules could account for some of the changes seen in PMN ultrastructure incubated in-vitro. The granules of PMN incubated for 20 min with 20 μM 501C show to a varying degree a loss of their homogeneous matrix. The membranes of these granules appear irregular, attenuated and in places broken. In addition, increased numbers of cytolysosomes are noted, which also show changes to their membranes. Therefore, the concentration of 501C within the granules may have a damaging effect on the granule membranes, resulting in release of the granule content. Intracellular focal injury of this kind stimulates the formation of cytolysosomes.

This investigation illustrates the value of electron microscope autoradiography for identifying the sites of accumulation of drugs in cells and so provide further information on their possible mode of action. The hypothetical grain analysis enabled the accurate localization and quantification of activity which would have been impossible by visual inspection of the micrographs.

The application of these techniques has shown that 5-HT and 501C both accumulate in PMNs at the same sites, the major concentration of both drugs being found in the cytoplasmic granules and lesser quantities associated in the nucleus. Therefore, there may be common binding sites for the two drugs. As 5-HT and 501C have differing effects on leucocyte function it would appear from these results that granular and nuclear binding are not pre-requisites for inhibiting activity.

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