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The uptake of mepacrine by horse polymorphonuclear leucocytes in vitro

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The uptake of mepacrine by isolated horse polymorphonuclear leucocytes (PMN) was measured using spectrophotofluorimetry. Two phases of uptake were observed, the first, rapid fraction, essentially complete by 10 min, and a second, slow fraction, which was still proceeding after 60 min. The appearance of mepacrine within the PMN was also visualized by fluorescence microscopy. Discrete yellow points of fluorescence were observed in the cytoplasm of PMN within 30 s. These discrete points corresponded both in size and number to the PMN granules. After 5 min, the nuclei showed faint fluorescence which increased in intensity with time. It was concluded that the accumulation of mepacrine by horse PMN consists of at least two phases, an initial rapid component which represents uptake of the drug through the plasma membrane into the cytoplasmic granules, and a subsequent slower component, possibly representing nuclear binding.

Mepacrine, an antimalarial acridine, has been shown to be highly concentrated by peripheral blood leucocytes in vivo (Shannon et al 1944), and in vitro the drug inhibits the ability of these cells to phagocytose (Baker et al 1976), transport potassium (Trist 1979) and randomly migrate (Palmer & Weatherall 1977). Mepacrine appears to act rapidly inhibiting potassium uptake within 2 min and phagocytosis within 5 min. Inhibition of potassium uptake suggests an action on the cell membrane. However, mepacrine has also been shown to be accumulated in lysosomes in a variety of mammalian cells (Allison & Young 1964) and to form a molecular complex with DNA (Kurnick & Radcliffe 1962). The purpose of the present study was to ascertain the rapidity of mepacrine uptake by PMN and determine the cellular distribution of the drug in order to understand the mechanism of its action.

MATERIALS AND METHODS

PMN isolation from horse peripheral blood

Peripheral blood was obtained by venepuncture from either geldings or mares. Clotting was prevented by the addition of preservative-free heparin, 30 U ml^{-1} (Pularin, Evans Medical Supplies), and leucocyte-rich plasma was obtained by gravity sedimentation of erythrocytes at room temperature (20°–25 °C) for 20 min. The settled plasma was passed through a water-jacketed glass-wool column (lightly packed quartz wool 2 to 40 μm diameter, Jencons Ltd) maintained at 28 °C containing 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). PMN were adsorbed by the glass-wool and were subsequently eluted with ice-cold sodium citrate buffer (tri-sodium citrate, 71·4; citric acid, 0·21; glucose, 81·7; KCl, 5·4mM; pH 7·4), at a column temperature of 4 °C. The PMN were resuspended in the saline medium at either 5×10^6 ml^{-1} or 5×10^7 ml^{-1}. Cell counts were estimated with a Coulter model FN particle counter.

Mepacrine uptake measured by spectrophotofluorimetry

PMN recovered from the glass-wool columns were dispensed into polythene microcentrifuge tubes (Precision Machining Engineers Ltd) and were kept at 36 °C for at least 1½ h before any experiments were performed. The cells were incubated with mepacrine in saline, 1 ml, and at set intervals the microcentrifuge tubes were spun in a microcentrifuge (Quickfit) at 12 600 g for 30 s. The supernatant was removed and the mepacrine concentration was measured in a Perkin Elmer/Hitachi spectrophotofluorimeter, model MPF 44A (excitation 360 nm; emission 502 nm), which had been calibrated with known concentrations of mepacrine base.

The cellular uptake of mepacrine was calculated from the disappearance of the drug from the supernatant, using the original cell count measured before the incubation. No correction was applied for any cell loss during the experiment.

Estimation of cell water

The PMN cell water was determined from the wet and dry weights of the purified PMN using [^{14}C] sorbitol (Radiochemical Centre, Amersham) as an extra-cellular marker.

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Fluorescence microscopy

The appearance of mepacrine within the PMN was observed by fluorescence microscopy. Coverslips coated with polyvinyl formal (Formvar 15/95E, BDH) were placed horizontally in Petri dishes containing the horse granulocytes prepared and suspended in the saline medium as described above. The Petri dishes were placed in a CO_2 incubator at 37 °C for 1 h after which the coverslips were removed and washed with saline medium to remove any non-adherent cells. The coverslips were then incubated for set intervals in saline medium containing mepacrine (10 μM) at 37 °C, washed and inverted on a drop of drug-free salt medium on a microscope slide and immediately examined with the fluorescence microscope.

In other experiments coverslips with adherent cells were inverted on a drop of saline medium, and whilst being examined with the fluorescence microscope, a drop of saline medium containing mepacrine (10 μM) placed at the edge of the coverslip was drawn under the coverslip by capillarity by applying a piece of filter paper to the opposite edge of the coverslip.

A Vickers M41 Photoplan fluorescence microscope was used, fitted with a HBO 200 high pressure mercury vapour lamp and a dichroic reflector. Blue excitation light was selected with a 3 mm B.G.12 exciter filter (transmission 330–480 nm, max. 400 nm). A barrier filter O.G.1 1·5 mm + G.G.9 1·5 mm (transmission starts at approximately 515 nm) was used to block stray blue light after excitation. In order to verify the position of the points of fluorescence within the cell, a white light phase image was superimposed on the fluorescent image. Observations were carried out using a ×40 objective and a ×100 oil immersion objective. Photographs were taken using Ilford black and white panchromatic film FP4-135 ASA-125.

DL-Mepacrine HCl was obtained from K & K Laboratories Inc., Plainview, New York, U.S.A., and was shown to be pure by thin layer chromatography, using silica gel G with ethanol (95%)/ NH_4OH (25%) 4:1 as the solvent.

RESULTS

Characterization of purified PMN

The PMN obtained from the column-separation were 95·0 ± 0·7% (mean ± s.e., n = 9) pure when compared with all other cell types; they represented 34·1 ± 9·1% (s.e., n = 9) of the PMN applied to the column. The cells at 37 °C were able to phagocytose opsonized yeast cells, migrate from capillary tubes, and to exclude the vital dyes Trypan blue and Eosin Y.

Uptake determined by spectrophotofluorimetry

The uptake of mepacrine (3·2–32 μM) at 36 °C increased with time, and two phases were observed (Fig. 1): the first being a rapid fraction, which was essentially complete by 10 min, and the second, a slower fraction, which was still proceeding after 60 min. Since the termination of uptake was by centrifuging the cells at 12 600 *g* for 10 s and removing the supernatant as quickly as possible, the initial uptake was too rapid to be estimated accurately by this technique.

The entry from 10 μM mepacrine in the medium reached 1·6 fmol cell^{-1} after 60 min, and as one granulocyte has a cell water content of 270·8 ± 18·4 fl (mean ± s.e., n = 8), derived from wet and dry weight determinations, the concentration of mepacrine in total cell water would be 5·9mM or 590 times that in the medium.

In some experiments after 60 min incubation mepacrine was extracted from the PMN with 1,2-dichloroethane. The extracted drug co-chromatographed with a standard mepacrine solution when run on a thin layer chromatogram (silica gel G into ethanol (95%)/NH_4OH (25%) 4:1 (v/v)).

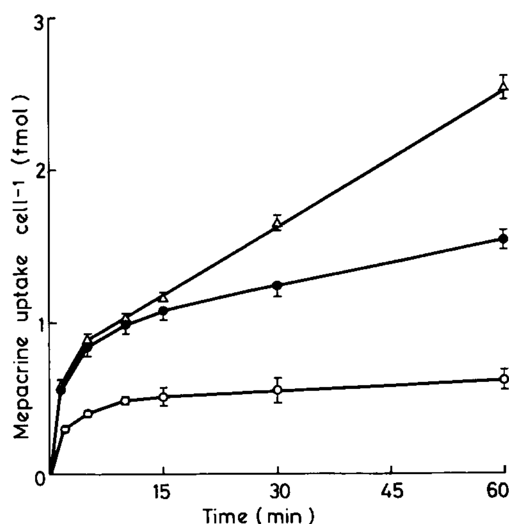

FIG. 1. Uptake of mepacrine by horse granulocytes (fmol/cell) versus time (min). Cells were suspended at 36 °C in media containing 32 μM mepacrine (△); 10 μM mepacrine (●) or 3·2 μM mepacrine (○). The drug uptake was calculated by measuring its disappearance from the medium. Each point represents the mean of two replicates and the vertical bars are the standard deviation.

Uptake observed by fluorescence microscopy

The uptake of mepacrine (10 μM) was studied by fluorescence microscopy in 10 experiments. In each experiment a minimum of 200 cells were observed and representative photographs were taken.

As rapidly as the coverslips could be examined under the microscope (approximately 30 s after the commencement of exposure to mepacrine) discrete yellow-green points of fluorescence were observed within the cytoplasm of the PMN (Fig. 2a). After 5 min, faint fluorescence of nuclei (identified by their multi-lobate appearance) was also observed in the cells. By 10 min marked nuclear fluorescence could clearly be seen, together with the original discrete points of fluorescence within the cytoplasm (Fig. 2b). As the nuclear fluorescence became stronger many cells lost the specific cytoplasmic fluorescence, so that after 1–2 h only the nucleus was evident (Fig. 2c). By this stage some of the PMN had also developed fluorescent vacuoles. The magnification of the microscope was not sufficient to determine whether the vacuoles had formed by coalescence of the original discrete points of fluorescence observed in the cytoplasm.

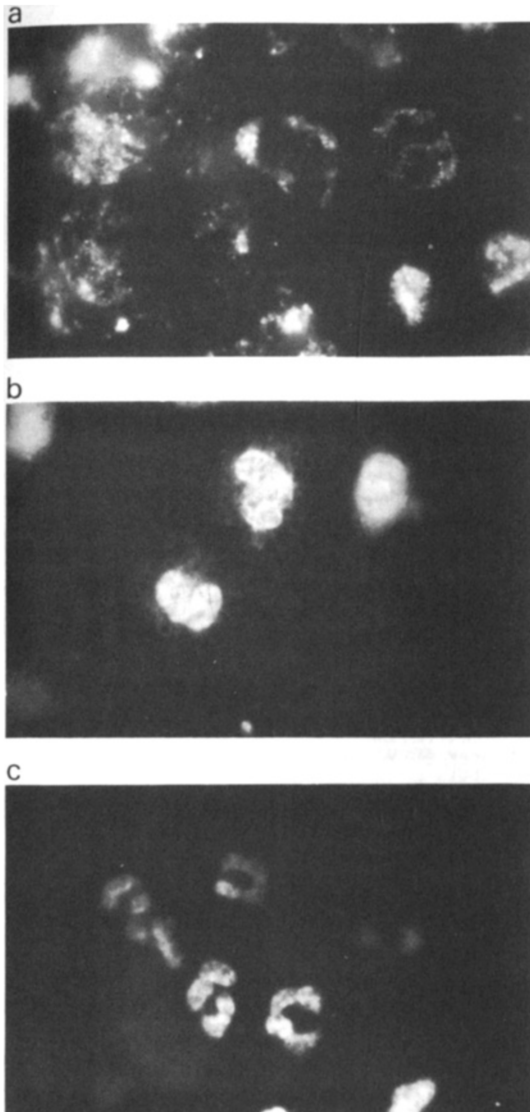

FIG. 2. Fluorescence photomicrographs of horse granulocytes attached to coverslips for varying intervals in saline medium containing mepacrine (10 μM) at 37 °C. (a) 2 min incubation, PMNs showing discrete points of fluorescence within the cytoplasm (×1200). (b) 10 min incubation, PMNs showing discrete points of fluorescence within their cytoplasm and also fluorescence of their nuclei (×1200). (c) 2 h incubation, PMNs showing only nuclear fluorescence (×900).

DISCUSSION

It is well known that basic dyes are taken up by almost any cell in a matter of minutes, and there is clear evidence, both morphological and biochemical, that these dyes are taken up selectively into lysosomal structures (De Duve et al 1974). Antimalarials, such as chloroquine (De Duve et al 1974) and mepacrine (Allison & Young 1964) are also concentrated in lysosomes in mammalian tissues. In horse PMN mepacrine appears to be accumulated initially into the cytoplasmic granules. The discrete points of fluorescence observed within the cytoplasm of the PMN correspond both in size and number to the granules of the PMN.

There are two main types of PMN cytoplasmic granule, azurophilic and specific, which differ in morphology and contents (Bainton et al 1971; Tschudi et al 1977). The azurophilic granules contain peroxidase and various lysosomal enzymes and thus qualify as lysosomes. Whilst, the contents of the specific granules remains largely undetermined; they lack lysosomal enzymes and contain alkaline phosphatase and basic proteins. At present we have no evidence as to whether the mepacrine is concentrated in one particular type of granule.

The rapidity of the appearance of fluorescence in the granules (within 30 s) suggests that the fast component of the mepacrine uptake determined by spectrophotofluorimetry represented selective accumulation of the drug by the granules from the cytoplasm after passage through the plasma membrane.

The slow component of the mepacrine uptake was associated with the appearance and gradual increase in nuclear fluorescence. Mepacrine forms a molec-

ular complex with DNA in vitro, in which it combines stoichiometrically with DNA in the proportion of approximately 1 dye molecule per four nucleotides (Kurnick & Radcliffe 1962). There are two other possible contributory factors to the slow component of mepacrine uptake. First, the formation of additional lysosomal bodies (secondary lysosomes) may provide new sites for drug accumulation. Second, as proposed for chloroquine by Homewood et al (1972) the acid condition of the lysosomal bodies (in this case azurophilic granules and secondary lysosomes) will trap the mepacrine by protonation (pKa_1 10, pKa_2 7·7; Irvin & Irvin 1950) and deplete the lysosomes of hydrogen ions. Uptake of further drug will cease unless more protons are made available. The presence of a proton pump in the lysosomal membrane (De Duve et al 1974) may provide hydrogen ions as a driving force for the continuing but slow uptake.

In some cells there was a gradual loss of granule fluorescence with time, which may indicate that the drug was being released from the granules and was therefore available for other binding sites. Alternatively, the loss of fluorescence could be explained by conversion of the drug in the granule to a non-fluorescent form, either by metabolism or by the action of prolonged u.v. irradiation. If this were the case it is in a form not sufficiently different from mepacrine to be seen in t.l.c.

Thus, the accumulation of mepacrine by horse polymorphonuclear leucocytes consists of at least two phases, an initial rapid and a subsequent slower component. The intracellular distribution of the drug suggests that the rapid component represents uptake of the drug through the plasma membrane into cytoplasmic granules. This corresponds to the onset of inhibition of PMN activity such as potassium uptake. It would seem unlikely that the concentration of the drug in the granules would affect plasma membrane function. It is more likely that the drug either acted directly on the plasma membrane itself or indirectly such as on enzymes involved in supplying energy. However, little fluorescence was seen in the plasma membrane or cytoplasm, suggesting that if mepacrine is acting at these sites the concentration is too low to be detected by fluorescence microscopy.

The slower component of mepacrine uptake possibly represents nuclear binding.

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