may thus increase the effects of various drugs by enhancing their absorption or passage through the body membranes (Wisniewski & Malyszko 1966; Leblanc 1960). It is possible that in alloxan-induced diabetes, where there is a deficiency of insulin in the body, the permeability of various biological membranes to paraoxon might be impaired. This would reduce access of paraoxon to cerebral cholinesterase and hence may reduce the degree of inhibition. Support for this suggestion is gained from the finding that the i.c.v. administration of paraoxon resulted in an almost equal degree of cholinesterase inhibition in diabetic and normal animals. Additional support is also obtained from our results that the administration of insulin along with paraoxon given intraperitoneally, enhanced its cholinesterase inhibiting activity to a level close to that seen in normal animals (Table 1). Since the inhibition of cholinesterase is directly related to the toxicity of organophosphorous compounds (Holmstedt et al 1967; Holmstedt 1959), it is possible that the diabetic state may in some way influence or modify the toxicity of organophosphorous compounds.

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Histamine receptor agonists and antagonists on granulocyte adherence

BRYAN V. ROBINSON, *Department of Pharmacology, Guy's Hospital Medical School, London SE1 9RT*

Over recent years it has become apparent that histamine, apart from having a role as a mediator of the inflammatory changes in blood vessels, may have an equally important role as a regulator of leucocyte function. For example it has been shown to inhibit antigen-induced release of histamine from basophils (Lichtenstein & Gillespie 1973) and to inhibit zymosan-induced lysosomal enzyme release from cytochalasin B pretreated leucocytes (Busse & Sosman 1976). Others have demonstrated that directional movement towards endotoxin-activated serum, either or neutrophils or of purified eosinophils, can be inhibited by histamine (Melmon et al 1972; Wadee et al 1980; Radermecker & Maldague 1981). The evidence suggests that inhibition of secretion and inhibition of chemotaxis by histamine are both H_2-receptor mediated phenomena (Lichtenstein & Gillespie 1973; Radermecker & Maldague 1981), and are accompanied by a rise in intracellular cyclic 3′, 5′adenosine monophosphate (cAMP) levels (Lichstenstein & Gillespie 1973; Wadee et al 1980). The present study has looked at the effect of histamine on another cell function, namely in vitro granulocyte adherence, and investigates the type of receptor involved. The effect of clonidine which has been shown to stimulate H_2-receptors in guinea-pig atria (McCulloch et al 1980) and in the central nervous system (Karppanen et al 1976) was also studied.

Methods

Measurement of granulocyte adherence. Adherence was determined by a modification of the method of Kvarstein (1969) using freshly donated human blood. 20 ml of blood were taken by venepuncture from a vein in the antecubital fossa and placed in a plastic universal tube containing 1 ml of preservative-free heparin (1000 u ml^{-1}). Following incubation with drugs at 37 °C, 0·5 ml aliquots of blood were pipetted into pre-warmed columns of glass beads. The blood was blown into the beads so that the top of the blood was level with the top of the beads, and the column was placed vertically in a rack in an incubator. The top of the column was then connected to a constant infusion pump (Palmer) via a length of polythene tubing. The pump was set up with ten 1 ml syringes in parallel so that ten samples could be perfused at the same time. The speed was adjusted to move the plungers at a rate of 1 inch min^{-1} (equivalent to 0·45 ml min^{-1}). Further columns were set up at 1 min intervals as the drug incubation period ended. Blood samples were collected from the bottom of each column into plastic blood tubes. These were stored at 5 °C until the haematology was performed.

Preparation of columns. Short (150 mm) glass Pasteur pipettes (Bilbate) and glass ballotini (Grade No. 8) with an approximate size range of 440—530 μm (Jencons) were

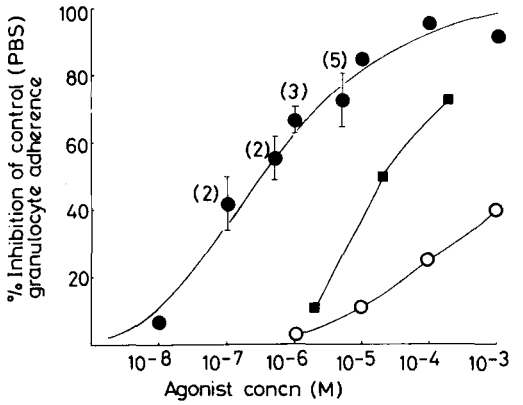

FIG. 1. The effect of increasing molar concentrations of histamine and selective H_2-receptor agonists on granulocyte adherence to glass beads in vitro. Adherence is expressed as the percentage inhibition of the adherence achieved in control (PBS) groups. The histamine curve (●) obtained from nine separate experiments using blood from a single volunteer. The figures in parentheses show the number of experiments in each case. The ametazole (○) and clonidine (■) results were each obtained from single separate experiments again using blood from the same volunteer. The bars indicate means ± s.e.

siliconized with Repelcote (Hopkins & Williams), dried, washed twice with distilled water to remove traces of solvent and dried again. Small cores of polyester foam sponge, obtained using a 6 mm cork borer, were placed in the neck of each pipette and 3 g of siliconized beads placed on top. Between 20–30 columns were needed for each experiment which were placed in a rack in an incubator overnight to warm to 37 °C.

Drug incubation procedure. 0·6 ml aliquots of fresh heparinized blood were pipetted into 4 ml flat-bottomed plastic blood tubes and incubated in a water bath at 37 °C. Using a random sequence of addition 20 µl amounts of agonist drugs or phosphate buffered saline (PBS) were added in turn at 1 min intervals to each of the tubes, which were then gently mixed and returned to the water bath. At least duplicate, but more commonly triplicate, tubes were set up for each drug level or drug combination. A minimum of four PBS controls were also prepared. 10 min after the drug addition each tube was mixed again and 0·5 ml of blood was pipetted onto a pre-warmed column of glass beads to assess the adhesiveness of the granulocytes. Where antagonist drugs were also used the blood samples were pre-incubated for 10 min in the presence of 10 µl of antagonist or PBS before addition of the agonist.

Haematology. Blood smears were prepared for all blood samples and stained using May-Grunwald Giemsa stain by the Haematology Department, Guy's Hospital. Differential cell counts were performed by counting 100 cells on each slide. Total white blood cell counts were made in duplicate on a ZF Coulter counter by diluting 40 µl of blood in 20 ml of Isoton (Coulter) and adding 6 drops of Zaponin (Coulter) to haemolyse the red blood cells.

Calculation of granulocyte adherence. The percentage granulocyte adherence was calculated from the following formula:

$$\frac{\left(\begin{array}{c}\text{Total WBC}\\ \text{count in}\\ \text{whole}\\ \text{blood}\end{array} \times \begin{array}{c}\%\ \text{number}\\ \text{of}\\ \text{granulo-}\\ \text{cytes}\end{array}\right) - \left(\begin{array}{c}\text{WBC}\\ \text{count in}\\ \text{effluent}\\ \text{blood}\\ \text{from}\\ \text{column}\end{array} \times \begin{array}{c}\%\ \text{number}\\ \text{of}\\ \text{granulo-}\\ \text{cytes}\end{array}\right)}{\left(\begin{array}{c}\text{Total WBC}\\ \text{count in}\\ \text{whole}\\ \text{blood}\end{array} \times \begin{array}{c}\%\ \text{number}\\ \text{of}\\ \text{granulo-}\\ \text{cytes}\end{array}\right)}$$

The effects of the various drugs were assessed either by comparing the number of granulocytes recovered in each treatment group with the number in whole blood; or by comparing the adherence in the treatment group with that in the control group to which a similar volume of PBS had been added.

Drugs. Preservative-free heparin (mucous) injection B.P. (Leo) containing 1000 u ml^{-1} sodium heparin was used as an anticoagulant in all experiments, and all drugs were prepared in phosphate buffered saline prepared from Dulbecco A buffer tablets (Oxoid). Histamine acid phosphate (BDH), clonidine hydrochloride (Boehringer-Ingelheim) and cimetidine (Smith, Kline and French Laboratories) were available as pure compounds and were dissolved in PBS to the appropriate molar concentration. Mepyramine maleate, 25 mg ml^{-1} (Anthisan—May & Baker) and ametazole (betazole) hydrochloride, 50 mg ml^{-1} (Histalog—Eli Lilly) were available as solutions for injection and were diluted as required in PBS.

Results

Histamine produced a graded inhibition of polymorphonuclear leucocyte adherence to glass beads when added to heparinized blood at concentrations in the range 10^{-7}–10^{-3} M (Fig. 1). This was a reproducible finding and although the extent of the binding of granulocytes to the beads from a single individual varied over a period of time from 45% to 81% adherence (mean 69 s.d. 12); the extent of inhibition produced by histamine was relatively constant. Fig. 1 shows the concentration effect curve compiled from nine separate experiments. The ED50 of histamine was calculated from this as 3×10^{-7} M.

To investigate the type of receptor involved the blood was incubated in the presence of either the H_1-histamine antagonist, mepyramine maleate, or, the H_2-histamine antagonist, cimetidine hydrochloride for 10 min before addition of the histamine. Because only a limited number of samples could be dealt with at any time it was not practicable to carry out the conventional procedure in which the effect of increasing amounts of antagonist were observed on the complete dose-response curve. Instead a single concentration of histamine (5×10^{-7} M) was used which was selected as being in the middle of the range of efficacy, and the effect of various concentrations of antagonist on this histamine response assessed.

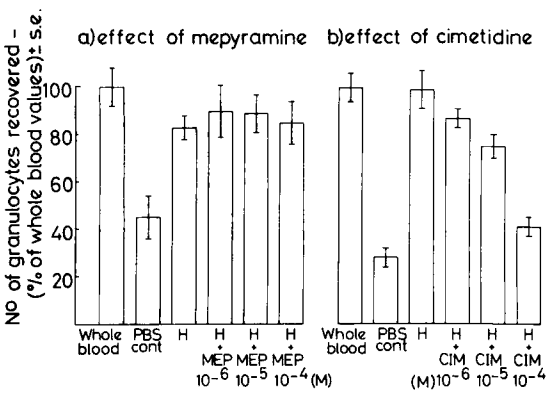

FIG. 2. The effect of mepyramine maleate (10^{-6}–10^{-4} M) and cimetidine hydrochloride (10^{-6}–10^{-4} M) on the inhibitory action of histamine (5×10^{-7} M) on granulocyte adherence. Results are the number of granulocytes in blood after passing through a column of glass beads, expressed as a percentage of the total number of granulocytes in whole blood. The bars indicate the mean values of three or four estimates ± s.e.

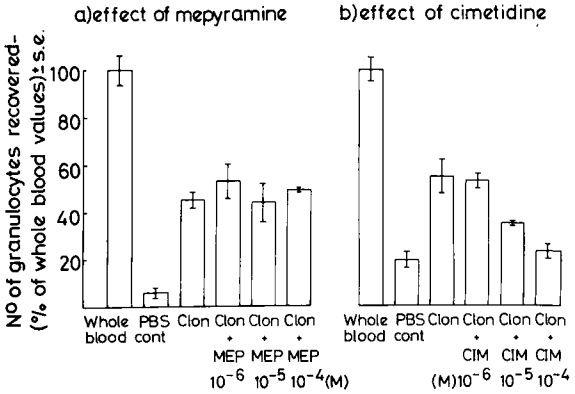

FIG. 3. The effect of mepyramine maleate (10^{-6}–10^{-4} M) and cimetidine hydrochloride (10^{-6}–10^{-4} M) on the inhibitory action of clonidine (2×10^{-4} M) on granulocyte adherence. Results are the number of granulocytes in blood after passing through a column of glass beads, expressed as a percentage of the total number of granulocytes in whole blood. The bars indicate the mean values of three or four estimates ± s.e.

Fig. 2(a) shows that mepyramine maleate over the concentration range 10^{-6}–10^{-4} M did not significantly reverse the inhibition of granulocyte adherence by histamine. On the other hand (Fig. 2(b)) cimetidine over a similar concentration range did reduce the inhibition of adherence caused by histamine. Cimetidine 10^{-6} M produced 17% reduction of the histamine response whilst 10^{-4} M cimetidine reduced it by 82%, in other words almost back to the level of adherence achieved in the PBS control group. Cimetidine was shown in a separate experiment not to modify significantly the level of granulocyte adherence when used alone. Compared with the PBS control group the percentage adherence was 103% at a concentration of 10^{-6} M, 96% at 10^{-5} M and 97% at 10^{-4} M. None of these values was statistically different from the control.

The presence of H_2-receptors on the granulocyte may also be inferred from the observation that the selective H_2-receptor stimulant, ametazole, inhibited adherence, albeit at much higher concentrations (Fig. 1). The dose response curve does not parallel that of histamine, but no more than 40% inhibition of the control response was achieved i.e. below the linear part of the curve. The drug was supplied as a solution for injection which did not permit higher concentrations to be used. Bearing in mind this problem, the relative position of the curves indicates that ametazole is at least 10 000 times less potent than histamine at inhibiting adherence.

Clonidine was also investigated in this model because it has been shown to stimulate H_2-receptors elsewhere in the body. It produced a graded inhibition of adherence, which in potency was between that produced by ametazole and by histamine (Fig. 1). The dose-response curve has a similar shape to that produced by histamine, and indicates that clonidine on a molar basis is about 50 times less potent than histamine.

The effect of a single concentration of clonidine (2×10^{-4} M), selected in a similar manner to the histamine concentration for the antagonism experiments, was inhibited in a graded manner by increasing concentrations of cimetidine, but was unaffected by increasing concentrations of mepyramine (Fig. 3). Although the clonidine was less potent than histamine at inhibiting adherence the ability of cimetidine at given molar concentrations to reverse both effects was very similar. Thus 10^{-6} M cimetidine produced 6% inhibition of clonidine and 17% inhibition of histamine, whilst 10^{-4} M cimetidine produced 89% inhibition of clonidine and 82% inhibition of histamine.

Discussion

Adherence of polymorphonuclear leucocytes to glass beads can be inhibited by a variety of agents which interfere with the metabolic state (e.g. theophylline) and functional integrity (e.g. vincristine, cytochalasin B) of the cells (Schneier et al 1977). None of these substances are found naturally in the body and the changes they invoke are strictly pharmacological. In the case of histamine the position is different, for histamine is released in inflammation and in allergic conditions in amounts which in this study have been shown to impair in vitro granulocyte adherence.

Bryant & Sutcliffe (1974) using leucocyte rich human plasma and measuring adherence by counting the number of cells adhering to glass capillary tubes made a similar observation. They found that 10^{-6} M histamine produced 65–78% inhibition of adherence which is in the same general range of effect as the present experiments. Higher concentrations of histamine were needed in results reported by Lackie & Smith (1980); 10^{-4} M histamine inhibiting

adhesion to serum coated glass by only 20% ($P < 0.005$) and to endothelial monolayers by 95% ($P < 0.05$). In neutrophil aggregation studies, which in other respects seem to respond to drugs in a similar manner to adherence studies, histamine was without effect on the aggregation of rabbit peritoneal leucocytes (Lackie 1977). Furthermore in experiments which attempted to measure adherence of leucocytes to endothelial walls in vivo by observing the rolling granulocyte count in the blood vessels of the hamster cheek pouch and mouse mesentery, histamine was again ineffective (Atherton & Born 1972). It is probably no coincidence that the in vitro experiments in which histamine demonstrated its greatest effect, used peripheral leucocytes rather than peritoneal leucocytes which may have already had their responsiveness affected by passage to the extravascular space and exposure to chemoattractant and chemokinetic agents. The in vivo experiments are probably too complex in their interactions to identify the action of histamine solely on neutrophil adhesiveness: other histamine-induced responses such as vasodilatation, increased permeability and changes in blood flow and shear stress on the walls of the vessel are all likely to affect the final picture and may mask the direct effect of histamine on the neutrophils.

The fact that granulocyte adherence is inhibited by both histamine and ametazole and that the histamine effect is antagonized by cimetidine but not by mepyramine implies that H_2-receptors are present on these cells. This agrees with other studies on secretion and chemotaxis (Busse & Sosman 1976; Radermecker & Maldague 1981), and reinforces the proposed hypothesis that histamine has a regulatory role in neutrophil function.

Bryant & Sutcliffe (1974) when studying adherence did not identify the receptor type involved in the histamine response, but because it was potentiated in the presence of theophylline (which was by itself inactive) and because dibutyryl cAMP produced a similar inhibitory effect, they concluded that the histamine response was probably mediated by a rise in intracellular cAMP level. Other workers (Lichtenstein & Gillespie 1973; Wadee et al 1980; Hill et al 1975) measuring different neutrophil functions have shown by direct measurement that inhibitory effects of histamine are accompanied by a rise in intracellular cAMP. Furthermore increased cAMP levels produced by a variety of agents (e.g. adrenaline, PGs of the E and A series, cholera toxin) were accompanied by inhibition of chemotaxis and spontaneous cell movement, although the correlation between degree of inhibition of cellular activity and rise in cAMP level was not always very good (Rivkin et al 1975).

Since histamine does not initiate the cellular activities described, it simply modifies a reaction which has been provoked by another stimulus, in all probability it does so by modifying an intracellular metabolic event and much of the evidence points to a rise in cAMP. In any event stimulation of one specific receptor type seems to modify a number of apparently different cellular functions, viz. adhesion, chemotaxis, secretion, which implies that a single common process is involved at some stage in all these events.

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