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Digitonin-solubilized histamine H_1-receptors bind to polyethylenimine-treated glass-fibre filters

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Abstract—The binding of [^{3}H]mepyramine to histamine H_1-receptors solublized from guinea-pig cerebellum by 1% digitonin could be assayed by adsorption of the receptor-bound [^{3}H]ligand to glass-fibre filters pretreated with 0·3% polyethylenimine (PEI). Non-specific binding was higher than in parallel experiments in which the bound and free [^{3}H]ligand was separated by gel-filtration on Sephadex G-25 columns, but the parameters characterizing the inhibition curves were otherwise similar. The PEI-treated filter method could also be used to assay [^{3}H]-(+)-*N*-methyl-4-methyldiphenhydramine ([^{3}H]QMDP) binding to solubilized H_1-receptors, but the level of non-specific binding was higher and was only satisfactorily defined by icotidine or temelastine. A particular utility of the PEI-treated filter assay will be in measurements of the kinetics of H_1-receptor-ligand interactions.

Progress in the isolation of the histamine H_1-receptor in pure form has been disappointing. H_1-Receptors can be solubilized with 0·5–1·0% digitonin (Gavish et al 1979; Toll & Snyder 1982; Garbarg et al 1985), but only limited studies have been made of the binding properties of the solubilized receptor. In part this reflects the lack of a rapid and simple method for the separation of the bound and free [^{3}H]ligand. Previous studies have used either charcoal to remove the free ligand or polyethylene glycol/ammonium sulphate precipitation of the protein, but these methods have limitations of speed and convenience of operation. A much more promising technique, which has been applied to a number of solubilised receptors, involves the adsorption of the receptor-ligand complex to polyethylenimine (PEI)-treated glass-fibre filters (Bruns et al 1983). We have examined whether the binding of [^{3}H]mepyramine and [^{3}H]-(+)-*N*-methyl-4-methyldiphenhydramine ([^{3}H]QMDP) to digitonin-solubilized histamine H_1-receptors can also be assayed in this way and we have compared the results with measurements in which gel-filtration was used to separate bound and free [^{3}H]ligand.

Methods

Solubilization of H_1-receptors. Cerebella from male Dunkin-Hartley strain guinea-pigs (300–600 g; Tucks, Battlebridge, Essex) were homogenised in 15 volumes of either ice-cold 100 mM Na phosphate buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4), pH 7·4, containing 320 mM sucrose or in 50 mM Na-K phosphate buffer (37·8 mM Na_2HPO_4, 12·2 mM KH_2PO_4), pH 7·5, using a Polytron blender (3 times for 20 s at setting 5) followed by a final dispersion in a Teflon-glass homogenizer with a motor-driven Teflon pestle. The suspension was centrifuged at 50 000 g for 15 min and the pellet then resuspended in the original volume of buffer. After a further centrifugation at 50 000 *g* for 15 min the resulting pellet was suspended in the same volume containing 1% (w/v) digitonin (see below) and incubated in a shaking water bath at 4°C for 40 min. The suspension was then centrifuged at 100 000 *g* for 1 h and the supernatant, containing the solubilized receptor material (1·08–1·55 mg protein mL^{-1}), either used immediately or stored for up to a week at −20°C. Protein was determined essentially as described by Lowry et al (1951).

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Preparation of water-soluble digitonin. The water solubility of commercially available samples was greatly increased by treatment essentially as described by Janski et al (1980). Digitonin (Sigma) was dissolved in three volumes of dimethylsulphoxide and then precipitated out by addition of 10 volumes of peroxide-free diethylether with vigorous mixing. The precipitate was separated by centrifugation at 2000 *g* for 10 min and then washed by shaking first with six volumes of chloroform and then with seven volumes of diethylether–chloroform (10:6, v/v). After a final centrifugation, solvent remaining in the pellet was removed under vacuum. The yield was 80% (calculated from the weight of the starting material).

Measurement of [^{3}H]ligand binding. Equilibrium measurements of the inhibition of [^{3}H]ligand binding to the solubilized preparation were made in either 320 mM sucrose/100 mM phosphate buffer or 50 mM Na-K phosphate buffer, pH 7·4 (final volume 1 mL) containing solublized receptor, 0·06–0·98 mg protein, 0·35–0·88 nM [^{3}H]mepyramine or 0·39–1·16 nM [^{3}H]QMDP, and various concentrations of unlabelled antagonists. In some experiments the level of non-H_1-receptor binding was determined by inclusion of incubations containing 2 μM promethazine (for [^{3}H]mepyramine) or 1 μM temelastine (for [^{3}H]QMDP). Equilibration was for 60 min at 30°C. The receptor-bound [^{3}H]ligand was separated in two ways. (a) Gel filtration. Incubations were cooled to 0°C for 10 min and a 0·5 mL aliquot applied to a column of Sephadex G-25M (PD-10 column, 9 mL; Pharmacia), pre-equilibrated at 6°C with the incubation buffer, and then eluted with the same buffer. Samples (0·5 mL) were collected for determination of the tritium by scintillation counting. The receptor-bound [^{3}H]ligand was eluted in the void volume in samples 4–8 (2–4 mL). Significant amounts of the free [^{3}H]ligand appeared in sample 10 onwards. At 6 C dissociation of the receptor-bound complex is slow (see below). (b) Filtration. Equilibration was terminated by addition of 4 mL ice-cold incubation buffer (in most experiments 50 mM Na-K phosphate) and the whole volume filtered under vacuum through Whatman GF/B glass-fibre filters which had been soaked in 0·3% PEI (v/v) for at least 5 h. The filters were washed three times with 4 mL ice-cold buffer and then transferred to scintillation vials. Scintillator (Quickszint 212 (Zinnser)/water, 95:5, v/v) (10 mL) was added and the vials allowed to stand overnight before measurement of tritium by liquid scintillation counting. In some early experiments the incubation mixture was cooled on ice for 10 min before filtration to make the conditions more closely analogous to those used in the gel filtration experiments (cf (a) above), but in all later experiments this step was omitted.

Preliminary measurements of the rate of formation of the [^{3}H]mepyramine-H_1-receptor complex indicated that at 30°C with 0·56 nM [^{3}H]mepyramine the equilibration was complete within 20 min. Similarly with 0·48 nM [^{3}H]QMDP complex formation was complete within 40–50 min.

Measurements of the rate of dissociation of the complex between [^{3}H]mepyramine and the solubilized receptor were made by incubating 0·35–0·50 nM [^{3}H]mepyramine and solubilized receptor (0·07–0·14 mg protein mL^{-1}) in 50 mM Na-K

phosphate buffer, pH 7·4 (total volume 55 mL) for 60 min at 30°C before addition of 1 μM non-radioactive mepyramine to initiate dissociation. At given times after addition of mepyramine 1 mL aliquots were filtered through PEI-treated filters, which were then washed three times with 4 mL ice-cold buffer. Changes in the non-receptor binding of [^{3}H]mepyramine were monitored in parallel incubations containing 2 μM promethazine. In experiments in which dissociation was measured at 6°C, [^{3}H]mepyramine and solubilized receptor were first incubated at 30°C for 60 min as above and the mixture then cooled to 6°C (over ca 15 min) before addition of 1 μM mepyramine. The rate of dissociation of [^{3}H]mepyramine from membrane-bound cerebellar H_1-receptors depends only on the temperature at which dissociation is initiated and is independent of the temperature of the preliminary incubation (Wallace & Young 1983).

Data analysis. Inhibition curves were fitted by non-linear regression analysis to the equation: % of uninhibited binding of [^{3}H]ligand = $(100 - NS)/((A/IC50)^n + 1) + NS$, where NS is the level of inhibitor-insensitive binding of the [^{3}H]ligand, A is the concentration of inhibitor, IC50 is the concentration of inhibitor required for 50% inhibition of the inhibitor-sensitive binding and n is the Hill coefficient. All points were weighted according to the reciprocal of the variance associated with them. The Harwell library routine VB01A was used to obtain the best-fit parameters ± estimated s.e. Affinity constants, K_a, were obtained using the relationship: $K_a = ([^3H\text{-ligand}] \cdot K_L + 1)/IC50$, where K_L is the affinity constant for the binding of the [^{3}H]ligand. In the special case of mepyramine inhibition of [^{3}H] binding the expression simplifies to: $K_a = 1/(IC50\text{-}[^3H\text{-ligand}])$. Dissociation rate constants, k_{-1}, were obtained by fitting the equation: $B = B_0 \cdot \exp(-k_{-1} \cdot t)$, where B is the amount of promethazine-sensitive binding of [^{3}H]mepyramine at time t and B_0 is the mepyramine amount at time zero.

Chemicals. [^{3}H]Mepyramine, 26 Ci mmol^{-1}, was purchased from Amersham International. [^{3}H]-(+)-*N*-Methyl-4-methyldiphenhydramine ([^{3}H]QMDP), 83 Ci mmol^{-1}, was synthesized and purified as described previously (Treherne & Young 1988a). Mepyramine maleate and promethazine hydrochloride were obtained from Sigma and icotidine (SK&F 93319) and temelastine (SK&F 93944) were gifts from Smith, Kline & French Research Ltd. Unlabelled (+)-QMDP was kindly made available to us by Prof. H. Timmerman.

Results and discussion

Digitonin-solubilized H_1-receptors labelled with [^{3}H]mepyramine bound to PEI-treated glass-fibre filters. The results of two experiments in which mepyramine inhibition of [^{3}H]mepyramine binding was measured on the same soluble receptor preparation and in which the receptor-bound [^{3}H]ligand was separated by gel-filtration or adsorption to PEI-treated filters are shown in Fig. 1. The level of mepyramine-insensitive binding in the G-25M column experiment is strikingly low. In eight such experiments with 0·38–1·72 nM [^{3}H]mepyramine, the mean level of inhibitor-insensitive binding ± s.e. was 9 ± 1%, compared with 23 ± 2% (8) using the PEI-treated filter method. The characteristics of the curves of mepyramine inhibition of [^{3}H]mepyramine binding were similar for the two methods of assay (Fig. 1). A similar direct comparison between gel-filtration and PEI-treated filter adsorption for the assay of promethazine inhibition of [^{3}H]mepyramine binding (8 concentrations of promethazine) gave a similar result (K_a $8·7 \pm 0·8 \times 10^7$ and $1·1 \pm 0·2 \times 10^8$ M^{-1}, Hill coefficients 1·04 ± 0·10 and 1·37 ± 0·24, for column and filter methods, respectively). Initial measurements were made in 320 mM sucrose/100 mM Na phosphate buffer, the medium originally

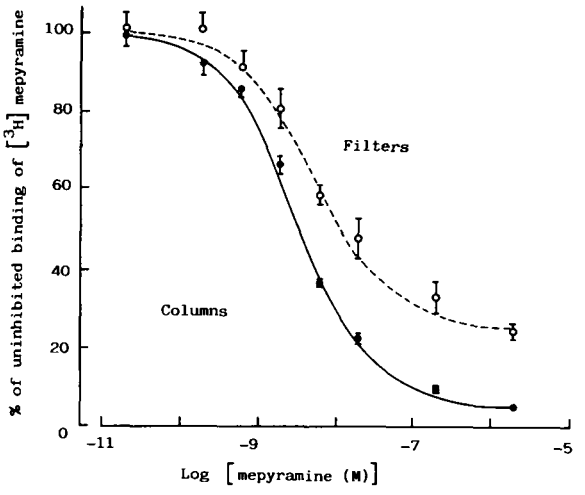

FIG. 1. Comparison of gel-filtration and filtration through PEI-treated filters for the assay of mepyramine inhibition of [^{3}H]mepyramine binding to digitonin-solubilized histamine H_1-receptors. The data are from 2 separate experiments on the same solubilized receptor preparation in 50 mM Na-K phosphate buffer, pH 7·4, containing 1·06 mg protein mL^{-1} and 0·69 or 0·88 nM [^{3}H]mepyramine. Each point is the mean ± approximate s.e. from 3 replicate determinations. Where no error bars are shown the error was within the size of the symbol. Separation of receptor-bound [^{3}H]ligand by: (●) Sephadex G-25M columns, (○) filtration through Whatman GF/B glass-fibre filters pre-soaked in 0·3% PEI. Best-fit parameters ± estimated s.e. from non-linear regression were: K_a $3·8 \pm 0·3 \times 10^8$ and $2·1 \pm 0·3 \times 10^8$ M^{-1}, Hill coefficient 0·87 ± 0·06 and 0·89 ± 0·13, % of mepyramine-insensitive binding 5 ± 1 and 26 ± 2%, for gel-filtration and PEI-treated filter experiments, respectively.

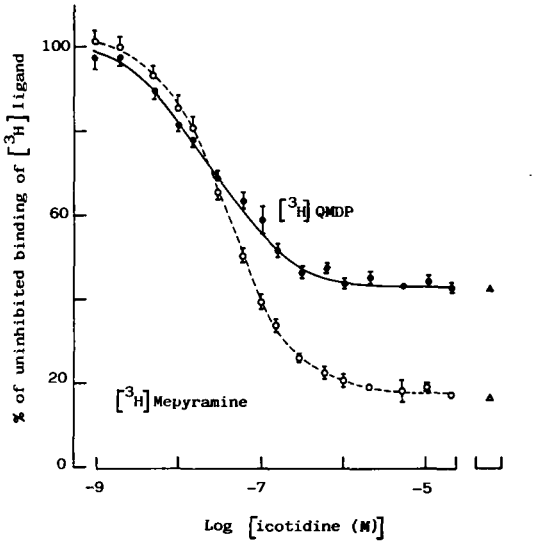

FIG. 2. Inhibition by icotidine of the binding of [^{3}H]mepyramine and [^{3}H]QMDP to digitonin-solublized H_1-receptors. The data are from 2 separate experiments with the same solubilized receptor preparation in 50 mM Na-K phosphate buffer, pH 7·4, containing 0·28 nM [^{3}H]mepyramine (○) or 0·39 nM [^{3}H]QMDP (●) and 0·14 mg protein mL^{-1}. Receptor-bound and free [^{3}H]ligand were separated by filtration through PEI-treated filters. Each point is the mean ± approximate s.e. from 5 replicate determinations. Where no error bars are shown the error was within the size of the symbol. Best-fit parameters: K_a $2·7 \pm 0·1 \times 10^7$ and $4·2 \pm 0·3 \times 10^7$ M^{-1}, Hill coefficient 1·12 ± 0·03 and 0·94 ± 0·05, % of icotidine-insensitive binding 18 ± 1 and 44 ± 1%, for [^{3}H]mepyramine and [^{3}H]QMDP, respectively. The level of binding in the presence of 1 μM temelastine (5 replicates), measured in the same experiments, is indicated by: (△) [^{3}H]mepyramine, (▲) [^{3}H]QMDP.

used by Gavish et al (1979), but closely similar results were obtained in 50 mM Na-K phosphate buffer, which was then used routinely. The values of K_a obtained for mepyramine and promethazine, $2{\cdot}9 \pm 0{\cdot}2 \times 10^8$ M^{-1} (3 measurements) and $1{\cdot}0 \pm 0{\cdot}1 \times 10^8$ M^{-1} (4), respectively, are lower than the values reported by Gavish et al (1979), $8{\cdot}3 \times 10^8$ and $3{\cdot}5 \times 10^8$ M^{-1}, but are closer to those, $5{\cdot}8 \times 10^8$ and $8{\cdot}8 \times 10^7$ M^{-1}, measured by Wallace (1983). All values are lower than those determined for membrane-bound receptors in guinea-pig cerebellar membranes (see e.g. Wallace & Young 1983).

The capacity of GF/B filters, pre-soaked in 0·3% PEI, to bind the soluble receptor was limited. In an experiment in which 0·06–0·98 mg protein mL^{-1} of the digitonin-solubilized receptor was incubated with 0·39 nM [^{3}H]mepyramine for 1 h at 30°C before filtration, the amount of promethazine-sensitive [^{3}H]-mepyramine binding trapped on the PEI-treated filters was maximal by 0·12 mg protein mL^{-1}. The level of binding insensitive to inhibition by 2 μM promethazine did not change significantly as the amount of protein was increased from 0·12 to 0·98 mg mL^{-1} (26 ± 1 to 28 ± 1%). The low capacity of the PEI-treated filters limits the number of counts min^{-1} trapped, but with an adequate number of replicate determinations an acceptable accuracy can be achieved (Fig. 2).

A limited number of measurements were made of the binding of [^{3}H]QMDP to digitonin-solubilized H_1-receptors, using the PEI-treated filter method to separate the bound ligand. However, the affinity of [^{3}H]QMDP for the H_1-receptor, as determined from (+)-QMDP inhibition of the binding of [^{3}H]mepyramine, $1{\cdot}7 \pm 0{\cdot}1 \times 10^8$ M^{-1}, is also a factor of 4–5 lower than that measured in analogous experiments on membrane-bound receptors (Treherne & Young 1988a), whereas the affinity for a medium-affinity, non-receptor site appears not to be reduced. Thus inhibition of [^{3}H]QMDP binding by mepyramine, which at concentrations $\geqslant 1$ μM inhibits the binding to secondary sites in membrane preparations (Treherne & Young 1988a), resulted in an apparently monophasic inhibition curve over a 100 000-fold concentration range of mepyramine (0·2–20 000 nM) (Hill coefficient $0{\cdot}49 \pm 0{\cdot}06$) and in almost complete inhibition of [^{3}H]QMDP binding. This apparent lack of a large difference in affinity for H_1-receptors and secondary sites makes mepyramine unsuitable for use in defining the level of non-H_1-receptor binding of [^{3}H]QMDP to digitonin-solubilized preparations. However, icotidine and temelastine, H_1-antagonists structurally unrelated to QMDP or mepyramine (Brown et al 1986), gave hyperbolic inhibition curves with a well-defined maximum level of inhibition. Comparative measurements on the same solubilized receptor preparation of the inhibition of the binding of [^{3}H]mepyramine and [^{3}H]QMDP by icotidine are shown in Fig. 2. Receptor-bound [^{3}H]QMDP can clearly be assayed by the PEI-treated filter method, but the much higher level of non-specific binding is apparent. The levels of non-specific binding indicated by 1 μM temelastine, a concentration at the foot of the inhibition curve, measured in the same experiments, were not significantly different from those given by icotidine (Fig. 2). In two experiments in which 1 μM temelastine was present in every incubation to block H_1-receptor binding, curves of (+)-QMDP inhibition of [^{3}H]QMDP binding showed a major secondary site with an estimated K_a $2{\cdot}8 \pm 0{\cdot}4 \times 10^7$ M^{-1} (weighted mean ± s.e. from 2 measurements). This is similar to the estimated K_a for this site in membrane preparations, $1{\cdot}3 \pm 0{\cdot}1 \times 10^7$ M^{-1} (Treherne & Young 1988a).

The use of PEI-treated filters to trap soluble receptor-[^{3}H]ligand complexes was pioneered by Bruns et al (1983) and shown to be effective for several classes of receptor. The observations presented here indicate that the technique can be used for the assay of binding to solubilized H_1-receptors. A particular utility of a method for the rapid and reproducible separation of H_1-receptor-bound and free ligand, which can be applied readily to large numbers of samples, is illustrated by the measurements of the rate of dissociation of the [^{3}H]mepyramine-soluble receptor complex at 30°C and 6°C shown in Fig. 3. Clearly the marked temperature-dependence of the rate constant for dissociation of [^{3}H]mepyramine, k_{-1}, observed in membrane preparations (Wallace & Young 1983; Treherne & Young 1988b) is maintained in the solubilized state, although the magnitude of k_{-1} at 6°C seems to be increased significantly by solubilization. The availability of a suitable assay will enable this and related features of the kinetics of H_1-ligand binding to be investigated in detail.

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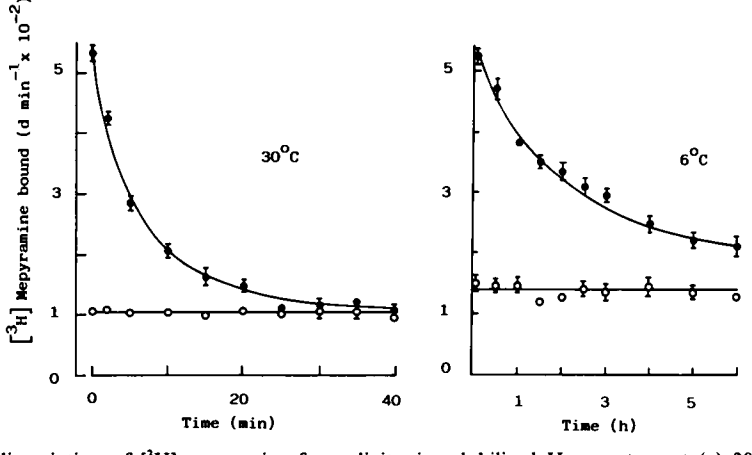

FIG. 3. Rate of dissociation of [^{3}H]mepyramine from digitonin-solubilized H_1-receptors at (a) 30°C and (b) 6°C. Measurements were made as described under Methods and dissociation terminated by filtration through PEI-treated filters. Incubations contained: (●) no additions, (○) 2 μM promethazine. Each point is the mean ± s.e. from 5 replicate determinations. Where no error bars are shown the error was within the size of the symbol. Best-fit values of k_{-1} determined from fitting the change in the promethazine-sensitive binding with time to an exponential function (see Methods) were $1{\cdot}5 \pm 0{\cdot}1 \times 10^{-1}$ min^{-1} at 30°C ($t_{\frac{1}{2}}$ 5 min) and $4{\cdot}8 \pm 0{\cdot}4 \times 10^{-3}$ min^{-1} at 6°C ($t_{\frac{1}{2}}$ 145 min).

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Amelioration of glycerol-induced acute renal failure in the rat with 8-phenyltheophylline: timing of intervention

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Abstract—The importance of timing and duration of 8-phenyltheophylline (8-PT) administration in determining its beneficial action in glycerol-induced acute renal failure (ARF) was investigated by examining the effects of a single dose of 8-PT given immediately following (0 h) glycerol injection and at 1 and 3 h after glycerol injection. 8-PT when given at 0 h significantly lowered plasma urea and creatinine concentrations and significantly increased inulin clearance when compared both to untreated animals and those that received the vehicle for the drug. By contrast, 8-PT when administered at 1 h afforded no protective effect on renal function and, when injected at 3 h, the only significant effect was lowered plasma creatinine levels when compared to untreated rats; at this latter time it did not lower plasma urea levels or improve inulin clearance. None of the 8-PT injections attenuated the increase in kidney weight associated with ARF or reduced the kidney damage as assessed by histological examination. The results show that a single administration of 8-PT made immediately following glycerol injection can ameliorate the biochemical and functional indices of impaired renal function, but does not produce an improvement in kidney morphology.

Recent studies in rats have demonstrated that administration of the alkylxanthines theophylline or 8-phenyltheophylline (8-PT) can reduce the severity of acute renal failure (ARF) produced by intramuscular glycerol injection (Bidani & Churchill 1983; Bowmer et al 1986). This beneficial effect has been attributed to the antagonistic action of these two xanthines at adenosine receptors, particularly those which mediate vasoconstriction of the afferent renal arterioles (Churchill & Bidani 1982). Although both theophylline and 8-PT possess diuretic activity (Brater et al 1983; Collis et al 1986), the salutary action of these compounds in ARF is unlikely to be dependent on a diuretic effect mediated via an action on the renal tubule since both the "tubular diuretics" frusemide and hydrochlorothiazide increase the severity of glycerol-induced ARF (Bidani et al 1987; Yates et al 1987).

During our recent study in which we compared the effects of hydrochlorothiazide and 8-PT in ARF (Yates et al 1987), it was noted that a single injection of 8-PT given 24 h after the initiation of glycerol-induced ARF did not alter plasma urea or creatinine levels. The protective effects observed with 8-PT in ARF have occurred when this drug was given twice daily for 2 days with the initial injection made immediately after glycerol administration (Bowmer et al 1986; Yates et al, 1987). This suggests that the timing and/or duration of administration of 8-PT is important in determining its effect in ARF. We have investigated this by examining the effects of a single dose of 8-PT or its vehicle, injected at various intervals after the induction of ARF, on biochemical, functional and morphological indices of renal function.

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Materials and methods

Materials

8-PT, inulin and polyethylene glycol 400 were obtained from Sigma Chemical Co. [G-^{3}H]Inulin (180mCi g^{-1}) of stated radioactive purity >98% was obtained from New England Nuclear Ltd and was used without further purification. Reagents for the assay of creatinine and urea were purchased from Pierce and Warriner and BDH Ltd, respectively.

Methods

Induction of acute renal failure. The method for production of ARF has been previously described in detail (Bowmer et al 1982). Male Wistar albino rats (250–300 g) were deprived of water for 24 h and ARF was produced by i.m. injection of 50% v/v glycerol in sterile saline (0·9% w/v NaCl), 10 mL kg^{-1}.

Experimental protocol. Glycerol-injected rats received either no treatment or a single injection of 8-PT (10 mg kg^{-1} i.p.) or vehicle (1·0 mL kg^{-1} i.p. of 50% v/v polyethylene glycol 400 in 0.1 M NaOH) immediately after (0 h) glycerol injection. Further groups of rats received a single injection of 8-PT (10 mg kg^{-1} i.p.) at 1 h or 3 h after glycerol adminstration.

Blood samples (about 0·7 mL) were taken from the tail vein before (0 h) and 24 h after glycerol administration. Forty eight hours after glycerol injection rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and cannulae placed in the left jugular vein and right carotid artery. The single-injection method of Hall et al (1977) was then used to measure the clearance of [^{3}H]inulin (100 mg kg^{-1}; 20 μCi kg^{-1} i.v.) from plasma (C_{IN}). At the end of the experiment a blood sample was taken from the carotid artery (for the determination of creatinine and urea) and the kidneys were removed, weighed, bisected longitudinally and placed in formal-saline (BDH Ltd).

Plasma creatinine and urea. Standard spectrophotometric assays