may be useful as a model system for studying not only the direct effect of extracellular ATP but also the heterologous regulation coupled to calcium signalling of renal tubular cell function.

The present study has demonstrated that there are specific receptors for extracellular ATP and ADP in renal epithelial cells, and that the binding of ATP induced the biphasic increase in $[Ca^{2+}]_i$, which consists of an initial and a second phase. The transient increase in $[Ca^{2+}]_i$ is considered to be caused by the release of calcium from the intracellular Ca^{2+} pool and the sustained increase may be induced by the influx from the extracellular buffer.

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Glucuronidation of imipramine in rabbit and human liver microsomes: assay conditions and interaction with other tertiary amine drugs

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Direct conjugation of nitrogen atoms in drugs with the C-1 of glucuronic acid has been observed for a number of different amines [1]. Of particular interest is the formation and urinary excretion of quaternary ammonium glucuronides from tertiary amine drugs such as cyproheptadine [2, 3], tripelennamine [4], cyclobenzaprine [5] and chlorpromazine [6]. This pathway of excretion was originally thought to be unique to the higher primates (e.g. man, chimpanzees), with most laboratory animals apparently unable to excrete these conjugates [3, 5], however more recent reports have demonstrated the excretion of quaternary ammonium glucuronides of the imidazole antifungal agents tioconazole and croconazole and the anti-anaphylactic ketotifen in the rabbit [7–9]. In addition to *in vivo* studies, it has been shown that both human and rabbit (but not rat) liver can form *N*-glucuronides *in vitro* [8, 10–12]. However, the analytical methods generally associated with the investigation of these reactions have until now been cumbersome and time consuming, requiring specialist analysis such as mass spectrometry (e.g. Refs 10 and 11), thereby severely limiting the investigation of the uridine diphosphoglucuronosyltransferase(s) (UDPGT*) [13] involved in these reactions.

We have therefore developed a radiometric assay procedure for determining the microsomal glucuronidation of the tricyclic antidepressant imipramine which offers considerable advantages over other procedures used to measure the formation of quaternary ammonium glucuronides, such as HPLC and mass spectrometry.

* Abbreviations: UDPGT, uridine diphosphoglucuronosyltransferase; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid.

Materials and Methods

Imipramine hydrochloride cyproheptadine hydrochloride, ketotifen (fumarate salt), cyclizine hydrochloride,

amitritpyline hydrochloride, chlorpromazine hydrochloride, promethazine hydrochloride, carbamazepine, (±)-chlorpheniramine maleate, uridine diphosphoglucuronic acid (sodium salt) and Lubrol PX were purchased from the Sigma Chemical Co. (Poole, U.K.). Imipramine hydrochloride, [*benzene ring*-^{3}H(N)] (48.7 mCi/mmol) was purchased from Du Pont (U.K.) Ltd (Stevenage, U.K.) and dichloroethane (HPLC grade) was from Rathburn Chemicals (Lanarkshire, U.K.). All other chemicals were obtained from commonly used local suppliers, and were of analytical grade or better.

Adult female New Zealand White Rabbits (approx. 2500 g) and adult male Wistar rats (approx. 200 g) were obtained from the Medical School animal facility. Human liver samples (50–500 g) were obtained from kidney donors or from patients undergoing surgery for liver cancer (histologically normal tissue), and were immediately frozen in liquid nitrogen and stored at −70°. Microsomes were prepared by differential centrifugation from 10% homogenates prepared in 0.25 M sucrose, 5 mM HEPES, pH 7.4. Homogenates were centrifuged at 10,000 *g* for 10 min and the resulting supernatants centrifuged for 1 hr at 105,000 *g*. Microsomal pellets were resuspended in sucrose/HEPES buffer to a protein concentration of approximately 20 mg/mL, aliquoted and stored at −70° until use. All microsomal samples were used within 2 months of preparation, and in order to maintain the intactness of the microsomal membrane, were thawed only once before assay [13]. The protein content of microsomal suspensions was estimated using the method of Lowry *et al.* [14], with bovine serum albumin as standard, on samples which had been thawed only once.

The standard assay for imipramine UDPGT activity was performed in a final volume of 0.2 mL containing: 25 mM potassium phosphate buffer, pH 7.0, 7 mM magnesium chloride, 0.5 mM [^{3}H]imipramine hydrochloride (0.05 μCi), 0.5 mg rabbit liver microsomal protein and Lubrol PX at a ratio of 0.20 mg detergent/mg microsomal protein. The microsomal protein and detergent were mixed and allowed to sit on ice at +1° for 20 min prior to addition to the assay in order to fully disrupt the microsomal membrane and abolish the latency of UDPGT [13]. Reaction mixtures were preincubated at 37° for 2 min prior to the addition of UDP-glucuronic acid (in 50 μL distilled water) to a final concentration of 5 mM. Control incubations ("blanks") received 50 μL of distilled water in place of the UDP-glucuronic acid. Following incubation at 37° for 45 min reactions were terminated by the addition of 3 mL of water-saturated dichloroethane. A volume of 0.25 mL of 0.25 M Tris–HCl, pH 8.7 was added, and the unreacted imipramine extracted by shaking vigorously for 2 min. Following brief centrifugation (3 min) at approx. 1000 *g* to separate the phases, 0.2 mL of the aqueous phase was mixed with 3 mL of scintillation fluid (Emulsifier Safe, Canberra Packard, Pangbourne, U.K.) and subjected to liquid scintillation spectrometry.

Recovery of [^{3}H]imipramine in the organic phase was routinely >99%, yielding "blank" values of between 200 and 400 cpm (i.e. in the 0.2 mL counted), with at least three times this value in the samples. For maximally

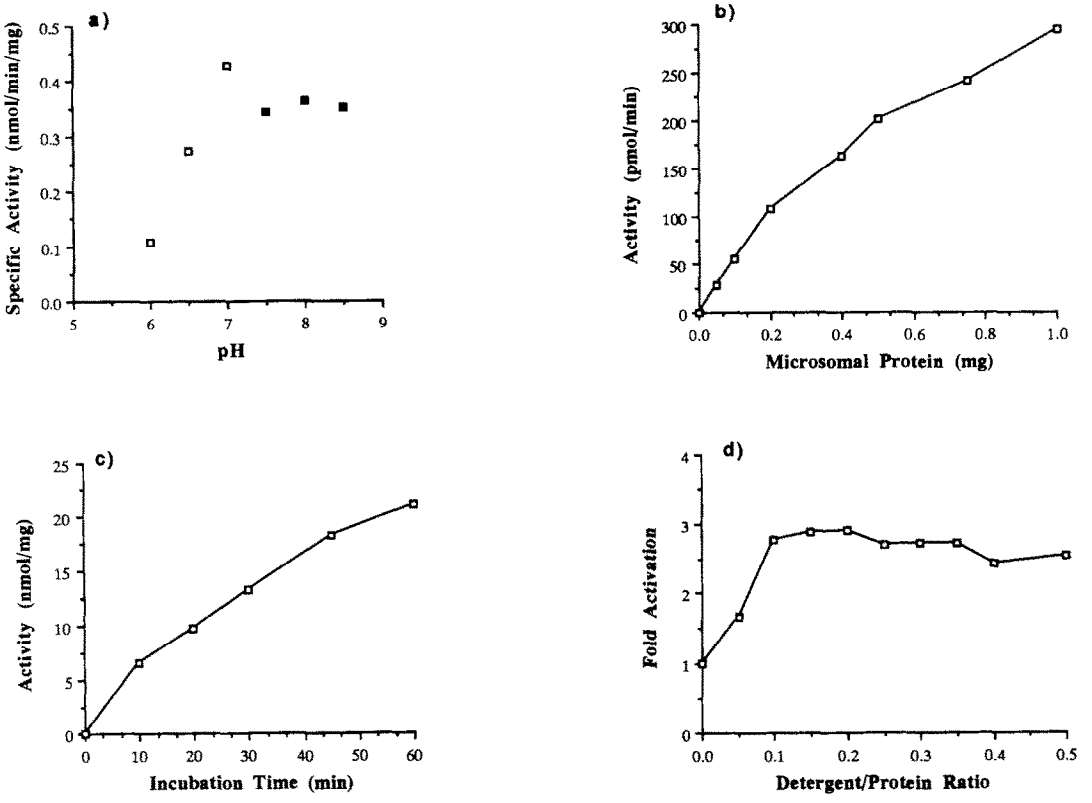

Fig. 1. Effects of pH, protein concentration, incubation time and detergent on rabbit liver microsomal imipramine UDPGT activity. (a) Potassium phosphate buffer (□), Tris–HCl buffer (■); (b) incubation time 45 min; (c) microsomal protein content was 500 μg per assay; (d) 500 μg protein per assay, incubation time was 45 min, activity in native microsomes was 0.157 nmol/min/mg protein. In panels (a), (b) and (c) microsomes which had been optimally activated with Lubrol PX were used. All data points represent duplicate determinations performed on a representative rabbit liver microsomal sample.

activated rabbit liver microsomes, 15,000–20,000 cpm/mL of reaction mixture counted were routinely obtained.

Results

The effects of pH, microsomal protein, incubation time and detergent on the imipramine UDPGT activity in rabbit liver microsomes are shown in Fig. 1. Optimum activity was achieved by incubating at pH 7.0 with potassium phosphate buffer for 45 min, with 0.5 mg microsomal protein at a detergent to protein ratio for 0.20 mg Lubrol PX/mg microsomal protein. The K_m for imipramine was determined to be approximately 450 μM (data not shown). We used a value of 500 μM imipramine for the standard assay since it increases the sensitivity of the assay; using a concentration 2- or 3-fold higher than the K_m would result in a substantial reduction in the amount of radioactivity incorporated into the product. For human liver imipramine UDPGT, different assay conditions were required. These were: 35 mM Tris–HCl, pH 8.0, 7 mM magnesium chloride, 0.4 mg microsomal protein incubated for 25 min in the presence of 0.15 mM imipramine. The concentration of detergent required to reveal maximum activity varied greatly between samples, and should always be determined in pilot experiments for each liver sample. For human liver microsomal imipramine UDPGT, the K_m for imipramine was considerably lower than for rabbit liver at approximately 100 μM (data not shown), and as described above for the rabbit liver enzyme assay, a value of 150 μM was used in order to obtain sufficient incorporation of radioactivity into the reaction product to obtain maximum sensitivity. The enzyme activities in rabbit, human and rat liver microsomes are given in Table 1, showing that the activity is highest in rabbit liver, and that rat liver microsomes are almost completely devoid of the enzyme activity, the activities recorded being at the very limits of resolution of the assay.

Table 1. Imipramine UDP-glucuronosyltransferase activities in hepatic microsomes prepared from different species

Species	Specific activity (nmol/min/mg)
Rabbit	0.554 ± 0.094
Human	0.167 ± 0.013
Rat	0.042 ± 0.022

Enzyme activity determinations were performed on microsomal samples which had been maximally activated by the detergent Lubrol PX. Data are presented as mean ± SEM for determinations on three (rabbit and rat) or 11 (human) liver samples.

In order to predict if other tertiary amines were metabolized by the same UDPGT as that conjugating imipramine, we performed inhibition experiments whereby a range of other related compounds containing a tertiary amine nitrogen, many of which have been demonstrated to form quaternary ammonium glucuronides *in vivo* (e.g. Refs 3, 6, 9 and 10), were incubated with rabbit liver microsomes in the presence of [^{3}H]imipramine (Fig. 2). Additionally, we also used carbamazepine, which is structurally very similar to imipramine, but has an amide side chain instead of a dimethylaminopropyl group as in

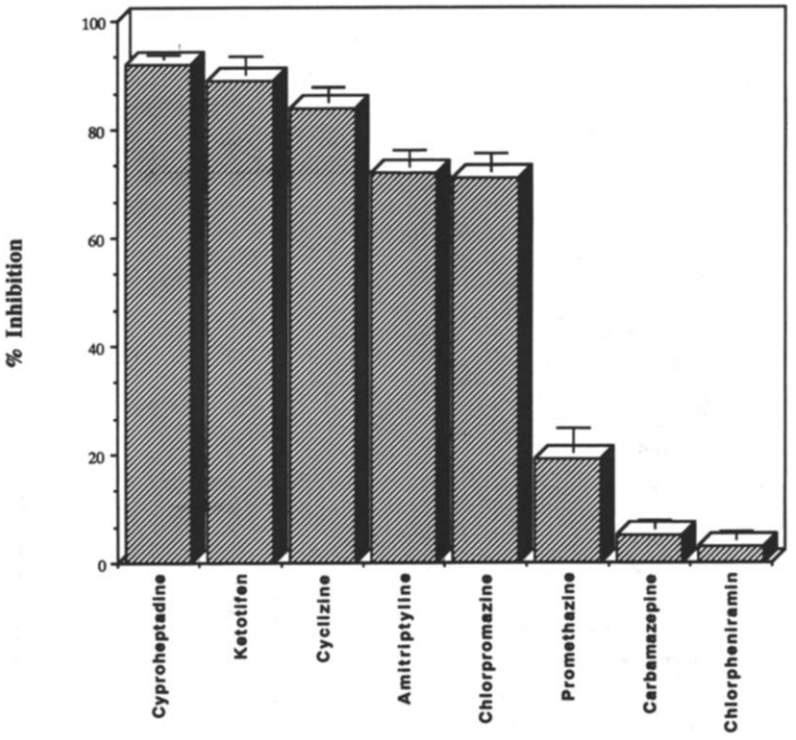

Fig. 2. Inhibition of rabbit liver microsomal imipramine UDPGT activity by various related drugs. All drugs were included in the incubation at 500 μM i.e. equimolar with substrate. Incubations were performed on duplicate samples of microsomes prepared from three different rabbit livers, and data are given as means of these determinations with the error bars representing the SEM. The values for enzyme activities in the absence of inhibitors were as follows (data expressed as nmol/min/mg microsomal protein ± SEM, N = 3): 0.553 ± 0.114 (promethazine, amitriptyline, chlorpromazine and chlorpheniramine—dissolved in water); 0.490 ± 0.118 (cyproheptadine and carbamazepine—dissolved in methanol) and 0.573 ± 0.104 nmol/min/mg (ketotifen and cyclizine—dissolved in 10% aqueous methanol, v/v).

imipramine. The structures of the compounds used in these experiments are given in Fig. 3. Inhibition was highest with compounds possessing a rigid three-ring structure, with a tertiary amine nitrogen, the only exception being cyclizine which has only a two benzene ring structure, but does possess the apparently important methylpiperazine side chain. On the basis of this analysis, compounds with the methylpiperazine side chain (e.g. cyproheptadine) appeared to be better inhibitors than those with the dimethylaminopropyl group (e.g. chlorpromazine) that imipramine possesses. Inhibition was virtually abolished with carbamazepine (an amide) and with (±)-chlorpheniramine which, although possessing a tertiary amine nitrogen, lacks the rigid three-ring structure of the other compounds. When kinetic analysis of the inhibition of imipramine UDPGT by cyproheptadine was performed, the inhibition was found to be competitive, with a K_i of approximately 6 μM (not shown).

We did not perform physical analysis of the reaction product formed in this assay procedure, however the following lines of evidence support the assumption that it is indeed imipramine glucuronide: (a) the activity was wholly dependent on the presence of UDP-glucuronic acid, (b) the enzyme activity in rabbit liver microsomes was strongly inhibited by an anti-rat liver UDPGT antibody [15] (data not shown) kindly provided by Professor B. Burchell and (c) cyproheptadine competitively inhibited the imipramine UDPGT enzyme activity, and other structurally closed related compounds also strongly inhibited.

Discussion

We have developed a rapid, simple and inexpensive assay for the glucuronidation of imipramine in rabbit and human liver microsomes. Data from the inhibition of this enzyme activity by other closely related drugs also

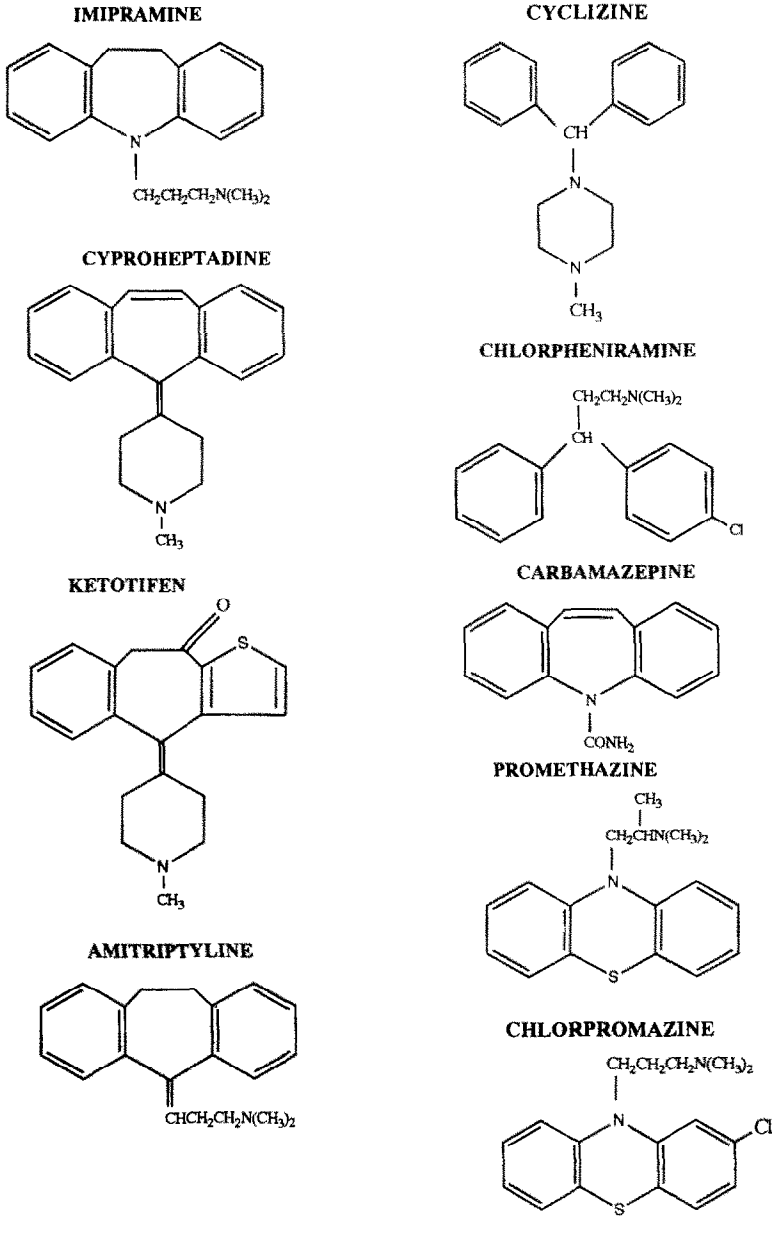

Fig. 3. Structures of the compounds used in these experiments.

containing tertiary amine nitrogen atoms suggests that the glucuronidation of these compounds may also reside with the same UDPGT isozyme. The observation that rat liver possessed only very low levels of imipramine UDPGT activity is in good agreement with *in vivo* data which suggests that the rat is not able to excrete quaternary ammonium glucuronides formed from tertiary amine drugs (e.g. Refs 5 and 9). Therefore, this assay is likely to prove a useful tool for studying tertiary amine UDPGT *in vitro*.

The importance of the N-glucuronidation of tertiary amines stems principally from the apparent restriction of this pathway of metabolism to man and higher primates and, for some drugs, rabbits [5, 9]. Safety evaluation and metabolism studies during the development stages of new therapeutic compounds, which usually involve non-primate species, particularly rats, may not predict accurately the metabolic fate of such drugs, and indeed may overlook potential toxicity and/or therapeutic benefits. In addition many of the drugs containing tertiary amino groups which are conjugated with glucuronic acid are also affected by the sparteine/debrisoquine cytochrome P450 polymorphism [16, 17], and therefore it is likely that N-glucuronidation will play a more important role in the metabolism of these drugs in the so-called "poor metabolizers".

In summary, the present work confirms that rabbit liver microsomes afford a good *in vitro* model for studying the glucuronidation of drugs containing tertiary amino groups. Furthermore, the availability of the assay procedure described above will allow us to understand further the basis for the inter-species differences in the metabolism of tertiary amines, and will eventually aid in our identification of the UDPGT isozyme(s) responsible for the conjugation of these compounds in man.

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