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SHORT COMMUNICATIONS

Identification of a class of low affinity binding sites for verapamil on liver plasma membranes

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Hepatocytes possess a plasma membrane receptor-activated Ca^{2+} inflow system (RACIS*) which is stimulated by a variety of agonists, including α_1-adrenergic agonists, vasopressin and angiotensin II (reviewed in Ref. 1). This Ca^{2+} inflow system is inhibited by the Ca^{2+} antagonists verapamil and nifedipine, although at much higher concentrations of the drugs than those which inhibit L-type VOCCs [2–5] (but see Ref. 6). Verapamil was found to be a more effective inhibitor than nifedipine [2]. Other Ca^{2+} antagonists which are potent inhibitors of L-type VOCCs have little effect on the hepatocyte RACIS [2, 7]. It is been proposed that the effects of verapamil and nifedipine on agonist-stimulated Ca^{2+} inflow in hepatocytes reflect the interaction of these drugs with a putative Ca^{2+} channel [2]. In the sarcolemma of cardiac and skeletal muscle, high affinity binding sites for dihydropyridines and other Ca^{2+} antagonists have been detected (reviewed in Ref. 8), and used to isolate the L-type VOCC protein complexes (reviewed in Ref. 9). However, few studies of the binding of Ca^{2+} antagonists to hepatocyte plasma membranes have been reported. Although high affinity binding sites for nitrendipine, a dihydropyridine Ca^{2+} antagonist, have been detected on rat liver microsomes [10], dihydropyridines are not very effective inhibitors of Ca^{2+} inflow in hepatocytes [2, 4, 7].

The aims of the present experiments were to determine whether the interaction of verapamil with the rat hepatocyte plasma membrane can be detected directly by measuring the binding of verapamil to a purified hepatocyte plasma membrane preparation and to characterize the nature of this binding with a view to using verapamil in the isolation of the putative hepatocyte RACIS. Since low concentrations of verapamil inhibit the binding of adrenaline and noradrenaline to α_1-adrenergic receptors in hepatocyte plasma membrane vesicles [6] and Zn^{2+} is a potent inhibitor of the hepatocyte RACIS [7], the effects of adrenaline and Zn^{2+} on the binding of verapamil were also tested.

Materials and Methods

Rat liver plasma membranes were prepared by the method of Prpic *et al.* [11] which involves centrifugation of a crude membrane preparation through a 12% (v/v) Percoll gradient. The final plasma membrane pellet was resuspended at approximately 2 mg protein/mL in 50 mM Tris–Cl, pH 7.4, containing 1 mM EGTA, and stored as 1 mL aliquots at −70°. The amount of protein present in plasma membrane preparations was determined by the procedure of Lowry *et al.* [12]. In other studies, the purity of the plasma membrane vesicles has been assessed by electron microscopy and measurement of the marker enzyme 5′-nucleotidase. Examination of the membrane preparation by transmission electron microscopy revealed membrane vesicles which were essentially free of mitochondria. The degree of enrichment of the specific activity of 5′-nucleotidase was 11-fold with respect to that in the liver homogenate (Hurst, K., Hughes, B. P. and Barritt, G. J., unpublished observations).

The binding of [^{3}H]verapamil to rat liver plasma membranes was measured in the following way. The incubation mixtures (1.8 or 2.5 mL total volume), which were prepared in glass tubes, contained 50 mM Tris–Cl (pH 7.4), 1 mM EGTA, 2 mg of plasma membrane protein and [^{3}H]verapamil (2000–4000 dpm/nmol) at the concentrations indicated. The reaction mixtures were incubated at room temperature (22–25°) for 30 min in order to allow binding to reach equilibrium. Ice-cold 50 mM Tris–Cl, pH 7.4 containing 1 mM EGTA and 0.1% (w/v) bovine serum albumin (Tris–EGTA–albumin) (5 mL) was then added to each tube, the suspension mixed and immediately filtered through a pre-treated Whatman GF/C glass filter disc. The filter discs were rapidly washed twice with 5 mL of ice-cold Tris–EGTA–albumin. The glass filter discs were pre-treated by soaking them for 30 min in Tris–EGTA–albumin. This was found to be essential in order to inhibit the binding of [^{3}H]verapamil to the filter discs. Blank incubations were also prepared in order to assess the amount of [^{3}H]verapamil which bound to the filters in the absence of plasma membrane vesicles. The blank reactions contained an equivalent volume of 50 mM Tris–Cl, pH 7.4 and 1 mM EGTA in the place of plasma membranes.

After filtration, the discs were dried, placed in 10 mL of ACSII scintillation cocktail (Amersham Australia, Sydney, Australia) and the amount of radioactivity present determined using a Beckman LS-5000TD scintillation counter (Beckman Instruments, Inc., Irvine, CA, U.S.A.). The amount of [^{3}H]verapamil bound to the filters in the absence of membranes was subtracted and the results expressed as nmol verapamil bound per mg membrane protein. Binding equations were fitted to the experimental data by non-linear regression analysis using the EBDA and LIGAND programs (Biosoft, NJ, U.S.A.). When measured at 100 μM verapamil, plots of the amount of [^{3}H]verapamil bound as a function of the time elapsed after the addition of [^{3}H]verapamil to the membranes reached a plateau at 30 min. This result indicates that, under the conditions chosen for the measurement of the binding of [^{3}H]-verapamil, the interaction of verapamil with the plasma membranes has reached equilibrium. The binding of verapamil was shown to be a linear function of the protein concentration in the range 0.5 to 2.0 mg at 400 μM verapamil (data not shown).

[*N-methyl*-^{3}H]Verapamil (81.1 Ci/mol) was purchased from Dupont Ltd, Melbourne, Australia; verapamil hydrochloride from the Sigma Chemical Co., St Louis, MO, U.S.A.; Percoll from Pharmacia, North Ryde, Australia; and Whatman GF/C filters from Selby Scientific, Adelaide, Australia. All other reagents were obtained from the sources described previously. Verapamil was dissolved in dimethyl sulphoxide and subsequently diluted in

* Abbreviations: RACIS, receptor-activated Ca^{2+} inflow system; VOCC, voltage-operated Ca^{2+} channel; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate.

50 mM Tris–Cl, pH 7.4, containing 1 mM EGTA. The concentration of dimethyl sulphoxide in the binding assays did not exceed 1% (v/v).

Results and Discussion

Data for the binding of [^{3}H]verapamil to liver plasma membranes are shown in Fig. 1a. The amount of [^{3}H]-verapamil bound approached a plateau as the concentration of verapamil was increased (Fig. 1a). Saturation was not observed since it was not possible to employ verapamil at a concentration greater than 700 μM because the drug has a limited solubility in water. The simplest equation which gave a good fit to the data when tested by non-linear regression analysis was a rectangular hyperbola. A fit of this equation to the data yielded a value of 460 ± 150 μM for the apparent dissociation constant, K_d, and 4.7 ± 1.2 nmol verapamil bound/mg protein for the maximum binding, B_{max} (mean ± SEM). A plot of the data in the Scatchard form [13] is shown in Fig. 1b. The simplest equation consistent with the data was a straight line. Zn^{2+} (1.5 mM) and adrenaline (1 mM) had no effect on the binding of [^{3}H]verapamil to plasma membranes when tested at verapamil concentrations of 1, 5, 100, 200 and 400 μM (results not shown).

The results provide evidence for the existence on the liver cell plasma membrane of a single class of low affinity binding sites for verapamil. Since it was not possible to measure the binding of verapamil at high concentrations of the drug, the K_d is not well defined. The properties of the verapamil binding sites on hepatocyte plasma membranes are clearly very different from the high affinity low density sites on heart and skeletal muscle sarcolemma [14, 15]. Since the K_d is in the same range as the concentration of verapamil which inhibits the hepatocyte RACIS [2], the low affinity sites detected on hepatocyte plasma membranes in the present study may include those on the RACIS. However, the number of verapamil binding sites on the hepatocyte plasma membrane is very much larger than the number of verapamil binding sites (in the range of 1 to 50 pmol/mg protein) on skeletal or heart muscle sarcolemma [14, 15]. This suggests that the observed binding of verapamil to hepatocyte plasma membranes includes the interaction of the Ca^{2+} antagonist with other sites, such as those which mediate the effects of verapamil on phalloidin transport [16] and the internalization of low density lipoproteins [17], as well as sites on the RACIS.

The observation that adrenaline did not inhibit the binding of verapamil indicates that the experiments did not detect the binding of verapamil to α_1-adrenergic receptors [6]. Moreover, the lack of inhibition by Zn^{2+} suggests either that Zn^{2+} (acting in place of Ca^{2+}) can displace verapamil from its low affinity binding sites on the hepatocyte RACIS but the contribution of the RACIS verapamil binding sites to the total number of low affinity verapamil binding sites is small, or that Zn^{2+} cannot displace verapamil from the RACIS binding sites.

This study has identified a class of low affinity binding sites for verapamil on hepatocyte plasma membranes.

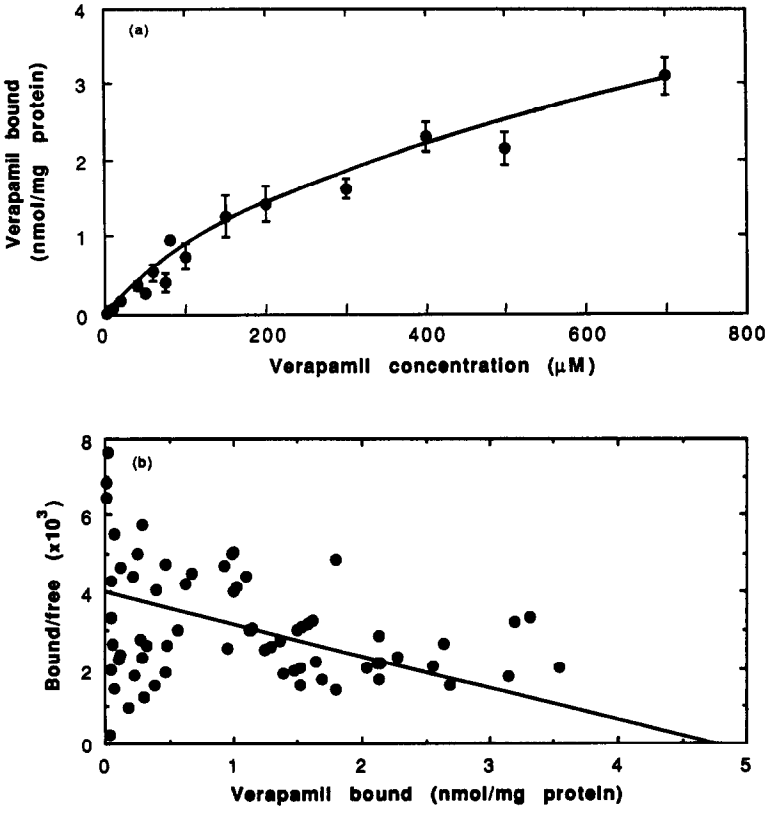

Fig. 1. Concentration dependence curve (Panel a) and Scatchard plot (Panel b) for the binding of verapamil to liver plasma membranes. Incubations were carried out as described in Materials and Methods. Data in Panel a represent means ± SEM of at least three measurements for two different plasma membrane preparations. Individual data points are represented in Panel b. The solid lines in (a) and (b) are the lines of best fit drawn using the values of the constants obtained from a fit of a rectangular hyperbola (a) or the Scatchard equation [13] (b) to the experimental data.

These may include sites on the receptor-activated Ca^{2+} inflow system as well as sites through which verapamil exerts other effects on hepatocytes.

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Impairment of bunitrolol 4-hydroxylase activity in liver microsomes of Dark Agouti rats

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Bunitrolol (BTL*), a β-adrenoceptor blocking agent, is predominantly biotransformed to 4-OH BTL in rat liver microsomes [1, 2]. We have shown that this microsomal oxidation reaction is mediated by the mixed function oxidase system including P450 in a previous study using various enzyme inhibitors [3]. Genetic polymorphisms in the oxidation of many drugs represented by DB and mephenytoin in man have been described [4]. Poor metabolism of DB is closely associated with various degrees of impairment in the oxidations of other, but not all, drugs

* Abbreviations: BTL, bunitrolol; 4-OH BTL, 4-hydroxybunitrolol; P450, cytochrome P450; DB, debrisoquine; DA, Dark Agouti; G-6-P, glucose-6-phosphate.