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INHIBITION OF HEPATIC MICROSOMAL DRUG METABOLISM BY THE CALCIUM CHANNEL BLOCKERS DILTIAZEM AND VERAPAMIL*

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Abstract—Diltiazem and verapamil were found to be inhibitors of the cytochrome P-450-dependent biotransformation of drugs. Diltiazem and verapamil competitively inhibited the N-demethylation of aminopyrine in hepatic microsomes with K_i values of 100 and 140 μM respectively. Both diltiazem and verapamil were N-demethylated themselves by hepatic microsomes with K_m values of 62 and 145 μM respectively. Both drugs also interacted directly with cytochrome P-450 as measured by difference spectra. Diltiazem caused a type I spectral change and verapamil caused a reverse type I spectral change. No metabolic intermediate complexes could be demonstrated for either drug. Inhibition also occurred *in vivo* as both drugs could prolong pentobarbital-induced sleeping times in mice at doses comparable to those used in man. These results suggest that diltiazem and verapamil may have the potential to cause drug interactions involving inhibition of drug biotransformation.

Diltiazem and verapamil are two of a new group of drugs known as calcium channel blockers that have become available for the treatment of angina pectoris, cardiac arrhythmias and a variety of other cardiovascular disorders [1, 2]. Both diltiazem and verapamil contain a number of chemical groups which have the potential to be oxidized by cytochrome P-450-dependent mixed function oxidase in the liver (e.g. O- and N-demethylation; ring hydroxylation) [3]. Indeed it has been demonstrated that diltiazem and verapamil are metabolized extensively in the liver of both animals and man, and that O- and N-demethylated species are major metabolites [4, 5]. The many potential sites for oxidation by cytochrome P-450 might also lead to the speculation that diltiazem and verapamil could compete with the metabolism of other drugs by the mixed function oxidase system in the liver. Such an inhibition could result in a decrease in clearance and an increase in the plasma levels of other drugs used concomitantly and result in a toxic drug interaction.

Only a few drug interactions involving diltiazem and verapamil have been reported in the clinical trials carried out to date, and none of these resulted from the inhibition of the metabolism of other drugs [6, 7]. In this study diltiazem and verapamil were found to be potent inhibitors of cytochrome P-450-dependent mixed function oxidase in the mouse liver. This inhibition has the potential to cause drug interactions when the calcium channel blockers are used in combination with other drugs in the treatment of cardiovascular disease.

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MATERIALS AND METHODS

Materials. Diltiazem was obtained from Nordic Laboratories Inc., Laval, Quebec, and verapamil HCl from Searle & Co. Canada Ltd., Oakville, Ontario.

Animals. Male Swiss strain mice obtained from Jackson Laboratories (Bar Harbor, ME) were used throughout these studies. Animals were kept on clay chip bedding and allowed to acclimatize in our facility for at least 1 week before use.

Microsomes. Hepatic microsomes were prepared as described by El Defrawy El Masry *et al.* [8] and were used on the day they were prepared. Microsomal protein levels were determined by the method of Lowry *et al.* [9], using bovine serum albumin as a standard. Cytochrome P-450 and cytochrome b_5 levels in microsomes were determined by the method of Omura and Sato [10].

N-Demethylation assays. The N-demethylation of aminopyrine was determined by assessing the amount of $H^{14}CHO$ produced from the incubation of [*methyl*-^{14}C]aminopyrine with microsomes as described by Poland and Nebert [11]. This method was utilized in order to measure HCHO produced specifically from aminopyrine without interference from the HCHO produced from the calcium channel blockers. The incubation mixtures used contained diltiazem or verapamil in the concentration indicated in Results. The O- and N-demethylation of diltiazem and verapamil was determined by measuring the amount of formaldehyde produced from these drugs using the colorimetric procedure based on the Hantzsch reaction [12, 13].

Binding spectra. The binding spectra of diltiazem and verapamil with cytochrome P-450 were measured in a suspension of microsomes containing 1 mg/

Table 1. Kinetic constants for the inhibition of aminopyrine N-demethylase by diltiazem and verapamil*

Treatment	K_m (μM)	V_{max} (nmoles HCHO/mg protein/hr)
Control 1	121 ± 19	128.1 ± 6.4
Diltiazem (0.5 mM)	1057 ± 105	106.0 ± 6.4
Control 2	91 ± 16	138.9 ± 7.0
Verapamil (0.5 mM)	537 ± 57	105.7 ± 5.5

* Values were obtained using aminopyrine concentrations ranging from 0.02 to 1 mM. The kinetic constants were calculated from a direct fit of rate of reaction versus substrate concentration values to a hyperbola as described in Methods. Each value is expressed as the calculated constant ± error of the estimate. A different preparation of microsomes was used for each of the calcium channel blocking drugs.

ml microsomal protein in 0.1 M phosphate buffer (pH 7.4) [14]. Various amounts of the drugs were added in 10 μl water to 2 ml of microsomal suspension in the test cuvette and compared to 2 ml of microsomal suspension in the reference cuvette containing 10 μl water. Binding spectra were recorded from 350 to 500 nm in a Pye–Unicam double beam spectrophotometer.

Metabolic intermediate complexes. The possible formation of metabolic intermediate complexes was examined by incubating diltiazem or verapamil (1 mM) with microsomes (1 mg protein/ml) and NADPH (0.5 mM) for 20 min at 37° as described by Franklin [15]. The difference spectra between 400 and 500 nm were obtained by comparing this incubation mixture to identical mixtures incubated for only 15 sec.

Pentobarbital sleep time. The length of sleeping time for an 80 mg/kg dose of pentobarbital (i.p.) was determined in mice pretreated for 2 hr with saline or various doses of diltiazem or verapamil. Sleeping time was defined as the length of time between the loss of righting reflex to the time this reflex was regained.

Statistics. An unpaired Student's *t*-test was used to compare two variables. In experiments with more than two variables, various groups were compared using the Student–Newman–Keuls test for multiple comparison [16]. Enzyme kinetic data using Michaelis–Menten rate equations were fitted to a hyperbola using the method and computer program described by Barlow [17]. The same method of analysis was used to estimate the binding constants for the interaction of diltiazem and verapamil with cytochrome P-450.

RESULTS

Inhibition of hepatic microsomal aminopyrine N-demethylase by diltiazem and verapamil. The N-demethylation of aminopyrine in hepatic microsomes was inhibited significantly by diltiazem and verapamil. Diltiazem and verapamil induced changes in the saturation curve and the Lineweaver–Burk double-reciprocal plot for the N-demethylation of various concentrations of aminopyrine which were characteristic of competitive inhibition. As shown in Table 1, the K_m for aminopyrine N-demethylase was increased 8.7-fold by 0.5 mM diltiazem and 5.9-fold by 0.5 mM verapamil. The changes in V_{max} were very small in the presence of the two inhibitors. Dixon plots for the inhibition of the N-demethylation of three different concentrations of aminopyrine by various amounts of diltiazem or verapamil are illustrated in Figs. 1 and 2. These data also indicate that the inhibition by both of these drugs was competitive in nature, yielding a series of lines converging to an intercept above the level of the abscissa. The K_i values for diltiazem and verapamil were 100 and 140 μM respectively.

N-Demethylation of diltiazem and verapamil by hepatic microsomes. The N-demethylation of various concentrations of diltiazem and verapamil is illustrated in Fig. 3. The maximum rate of N-demethylation was 36.5 ± 2.3 nmoles HCHO/mg protein/hr for diltiazem and 15.9 ± 2.0 nmoles HCHO/mg protein/hr for verapamil. The K_m values for diltiazem and verapamil were 62.4 ± 12.3 and 145 ± 45.2 μM respectively.

Binding spectra for the interaction of diltiazem and verapamil with cytochrome P-450 in hepatic microsomes. The difference spectra for the binding of diltiazem and verapamil to cytochrome P-450 in microsomes are illustrated in Fig. 4. The addition of diltiazem to microsomes caused a type I spectral change characterized by an absorbance maximum at 384 nm and an absorbance minimum at 416 nm. The addition of verapamil to microsomes caused a reverse type I spectral change characterized by an absorbance maximum at 420 nm and an absorbance minimum at 384. The absorbance changes for diltiazem ($A_{384-416}$) and verapamil ($A_{420-384}$) followed Michaelis–Menten kinetics and increased with increasing concentrations. Maximum absorbance change was 0.0115 ± for diltiazem and 0.0095 ± for verapamil. The spectral dissociation constants (K_d) calculated from a reciprocal plot were 225 ± 16 μM for diltiazem and 538 ± 156 μM for verapamil (Fig. 5).

Formation of metabolic-intermediate complexes with cytochrome P-450. Diltiazem and verapamil (1 mM) were incubated with microsomes and 0.5 mM NADPH for periods up to 20 min. When the spectra of these microsomes were compared to similar mixtures which were not incubated, no difference spectra were apparent which would be indicative of the formation of metabolic intermediate complexes.

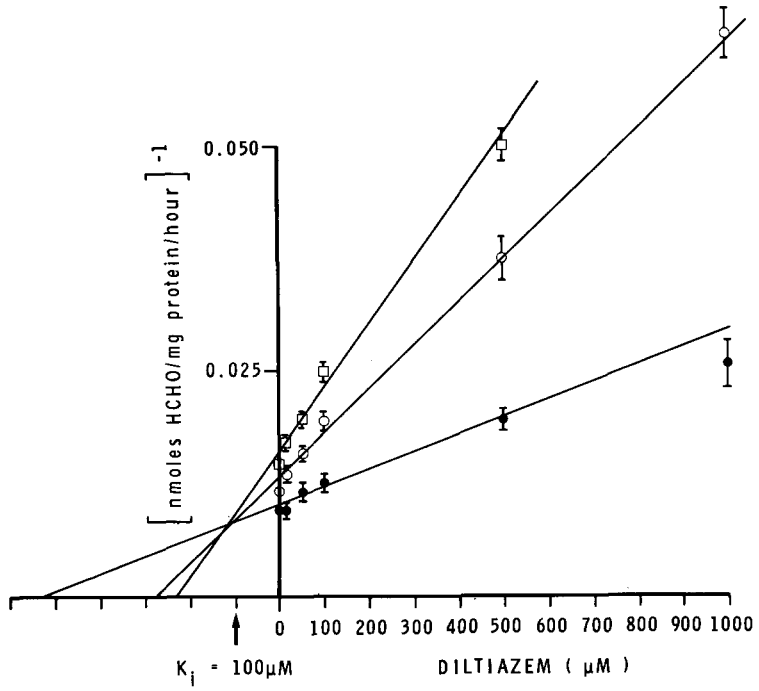

Fig. 1. Effect of diltiazem on the N-demethylation of aminopyrine by hepatic microsomes. The results are illustrated using a Dixon plot. The concentrations of aminopyrine used were 0.25 mM (□), 0.5 mM (○) and 1.0 mM (●). Each value is the mean ± S.E. for three individual incubation mixtures. The line of best fit was determined using the method of least squares.

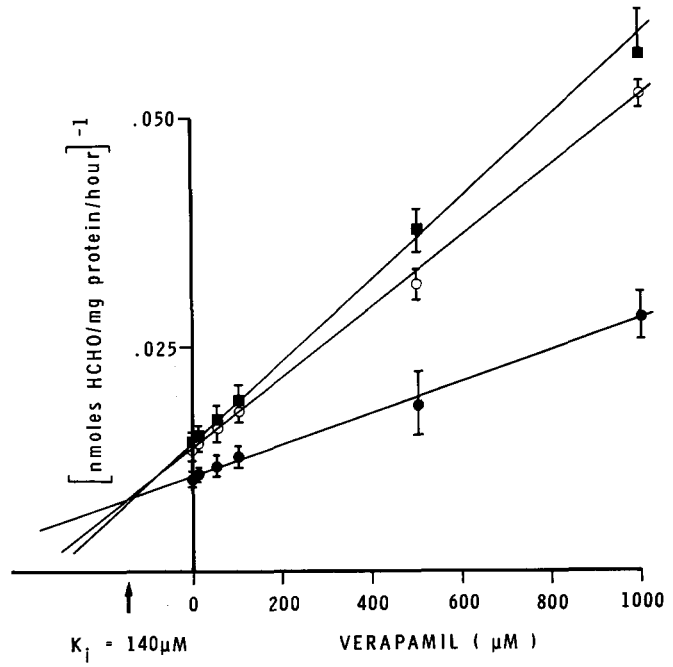

Fig. 2. Effect of verapamil on the N-demethylation of aminopyrine by hepatic microsomes. The results are illustrated using a Dixon plot. The concentrations of aminopyrine used were 0.25 mM (■), 0.5 mM (○) and 1.0 mM (●). Each value is the mean ± S.E. for three individual incubation mixtures. The line of best fit was determined using the method of least squares.

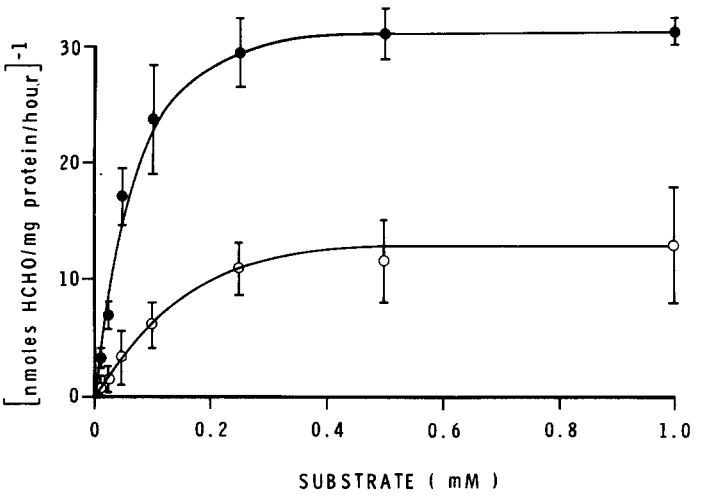

Fig. 3. N-Demethylation of various concentrations of diltiazem (●) and verapamil (○) by hepatic microsomes. Each value is expressed as the mean ± S.E. for four individual incubation mixtures. The K_m values for diltiazem and verapamil were 62.4 ± 12.3 and 145 ± 45.2 μM respectively.

Effects of diltiazem and verapamil on pentobarbital sleep time. The administration of diltiazem and verapamil for 2 hr significantly prolonged the hypnotic effect of pentobarbital (Table 2). Doses of diltiazem of 0.1 mg/kg, 1 mg/kg and 20 mg/kg increased pentobarbital sleep time by 41%, 48% and 154% respectively. Verapamil in doses of 0.1 mg/kg, 1 mg/kg and 20 mg/kg increased pentobarbital sleep time by 21%, 54% and 67% respectively.

DISCUSSION

The cytochrome P-450-dependent N-demethylation of aminopyrine N-demethylase was competitively inhibited by the calcium channel blocking drugs diltiazem and verapamil. The competitive nature of the inhibition is perhaps not surprising as both diltiazem and verapamil have multiple chemical groups which could be oxidised by the mixed function oxidase system and compete with aminopyrine for the active sites of the enzyme. Other reports indicate that both O- and N-demethylated metabolites are found in the urine of patients treated with these drugs [5, 18]. The K_m values for the N-demethylation of diltiazem and verapamil were similar to the K_i values determined for their competitive inhibition of aminopyrine N-demethylase. Although the maximum rate of N-demethylation of diltiazem and verapamil is much less than that for aminopyrine, their affinity for the active site of the enzyme is very similar

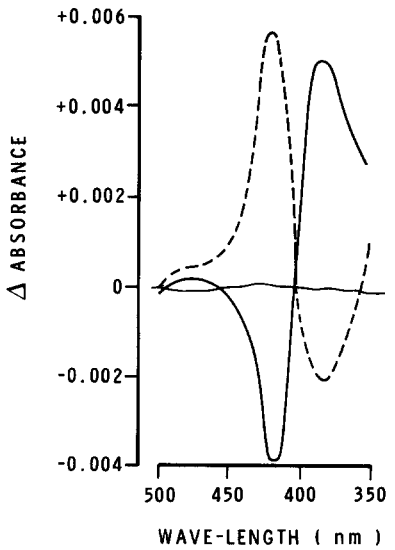

Fig. 4. Interaction of diltiazem (——) and verapamil (- - - - -) with cytochrome P-450 in hepatic microsomes. The concentration of drug used was 1 mM in both cases.

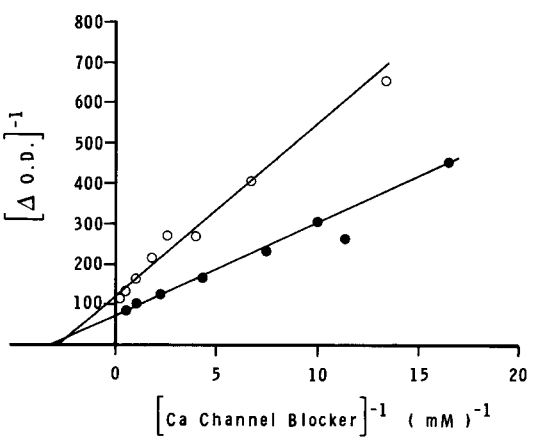

Fig. 5. Reciprocal plot of the changes in absorbance produced by the addition of various concentrations of diltiazem (●—●) or verapamil (○—○). The changes in absorbance used were $A_{384-416}$ nm for diltiazem and $A_{420-384}$ for verapamil. The lines of best fit were determined by the method of least squares. The spectral dissociation constant derived from a fit of the data to a hyperbola was 225 ± 16 μM for diltiazem and 538 ± 156 μM for verapamil.

Table 2. Effects of diltiazem and verapamil on the duration of pentobarbital-induced sleep

Treatment	Sleep time (min)
Control	64.3 ± 3.5
Diltiazem, 0.1 mg/kg	91.0 ± 7.7*†
Diltiazem, 1.0 mg/kg	95.2 ± 6.4*†
Diltiazem, 20.0 mg/kg	163.7 ± 10.5*
Control	83.4 ± 26.3
Verapamil, 0.1 mg/kg	101.3 ± 7.6
Verapamil, 1.0 mg/kg	128.7 ± 18.3*
Verapamil, 20 mg/kg	139.6 ± 14.3*

* Significantly different from control ($P < 0.1$), N = 9 or 10 in each group.

† Two animals died in these groups and were omitted from analysis.

to aminopyrine. This suggests that the inhibition caused by the calcium channel blocking drugs results from direct competition for the same routes of biotransformation by cytochrome P-450.

Both diltiazem and verapamil interacted with cytochrome P-450 in microsomes, but the nature of the binding was different for the two drugs. Diltiazem caused a type I change in the difference spectra and verapamil caused a reverse type I change in the difference spectra [14], indicating that the two drugs bind to cytochrome P-450 at different sites in the hemoprotein molecule. Diltiazem with a type I spectrum ($K_D = 225\ \mu M$) likely interacts with a hydrophobic region of the molecule [19], and verapamil with a reverse type I spectrum ($K_d = 538\ \mu M$) likely interacts with the more hydrophilic site of the heme iron [20]. These two drugs are also reported to bind to two separate membrane-located binding sites for their action in altering calcium channel transport [2]. Although the K_d values for these two drugs appear to be slightly higher than their K_i values for inhibition or their K_m values for N-demethylation, the binding of the two drugs to cytochrome P-450 does indicate that they can act directly with cytochrome P-450 and therefore can prevent the binding and subsequent metabolism of other drug substrates. The inhibition of aminopyrine N-demethylase by calcium channel blocking drugs is clearly not due to the formation of metabolic-intermediate complexes [15] as no spectral evidence for such complexes could be found when diltiazem or verapamil was incubated with microsomes and NADPH.

The K_i value obtained in mice for the two calcium channel blockers is well below the serum concentrations which are found during the clinical use of these drugs in man [2]. However, the tissue concentrations of these drugs have been found to be considerably higher than serum levels in animal experiments. For example, the concentrations of verapamil in the lung and liver are approximately 100 times higher than the plasma verapamil concentrations 1 hr following the administration of a single dose of 30 mg/kg, i.p., to rats [21]. It is therefore very difficult to compare the K_i value determined *in vitro* in microsomes to the tissue concentrations possible in the intact animal and to make predictions on the extent of the interaction in man. In support of a possible interaction at clinically relevant doses, an increase in pentobarbital sleeping time occurred at doses of diltiazem as low as 0.1 mg/kg. This dose is well below the usual recommended amount used in humans of 30 mg four times daily which can be increased to a total of 360 mg/day [1]. Although it is difficult to compare results in different species, the data obtained in mice suggest that both diltiazem and verapamil are capable of inhibiting drug biotransformation *in vivo* at doses comparable to those used in man.

In summary, these studies demonstrated that diltiazem and verapamil were competitive inhibitors of hepatic drug biotransformation in isolated mouse microsomes. This inhibition also occurred *in vivo* in the mouse at doses comparable to those used in humans. Although our results in mice cannot be transposed directly to man, they strongly suggest that drug interactions involving inhibition of drug biotransformation may be a complication during the use of diltiazem and verapamil for cardiovascular disease.

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