INACTIVATION OF CYTOCHROME P450S 2B1, 2B4, 2B6, AND 2B11 BY ARYLALKYNES

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(Received March 20, 1997; accepted June 10, 1997)

ABSTRACT:

The time-dependent loss of the 7-ethoxy-4-trifluoromethylcoumarin (EFC) O-deethylase activity of rat P450 2B1, rabbit P450 2B4, or dog P450 2B11 by 1-ethynynaphthalene (1EN), 2-ethynynaphthalene (2EN), 2-(1-propynyl)naphthalene (2PN), 1-ethynylanthracene (1EA), 2-ethynylanthracene, 2-ethynylphenanthrene, 9-ethynylphenanthrene, 9-(1-propynyl)phenanthrene (9PPh), 9-(1-propynyl)phenanthrene (9EPPh), 4-ethynylpyrene (4EP), and 4-(1-propynyl) biphenyl (4PbP) was investigated. The rate constants for inactivation by arylalkynes in descending order of effectiveness for the top five compounds were 9EPPh > 9PPh > 1EN, 2EN, 2PN for 2B11, 9EPPh > 2EN > 4EP > 1EN, 1EA for 2B4, and 9EPPh > 1EA > 4EP, 9PPh > 2EN for 2B1. The size and the shape of the aryl ring system and the placement of the alkyne functional group were important for inactivation. The most effective inactivator with all the isozymes was 9EPPh. This compound also inactivated the EFC activity in microsomes from human lymphoblastoid cells expressing human P450 2B6. The specificity of 9EPPh for the inhibition or inactivation of different P450 activities in microsomes from rats treated with various inducing agents was determined by measuring lidocaine, testosterone, p-nitrophenol, or erythromycin metabolism. The greatest effect was observed with the 2B-specific products from lidocaine and testosterone, whereas no effect was seen with p-nitrophenol or erythromycin. When the covalent binding of [14C]2EN to microsomal protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, a radiolabeled protein band that corresponds to 2B1 was observed in the lanes containing microsomes from rats treated with phenobarbital and, to a lesser extent, pyridine and isosafrole after incubation with NADPH. When these microsomes were incubated with [14C]9EPPh or [14C]1EP, two NADPH-dependent bands were radio-labeled. One corresponded to 2B1/2 and the other to a protein of approximately 59 kDa, which was observed in the lanes from phenobarbital-treated male and female rats and pyridine-treated male rats. No radiolabeled bands were observed with [14C]4PbP with any of the microsomes.

The P4502 superfamily of enzymes is a group of heme proteins that oxidize an extensive series of compounds including drugs, carcinogens, steroids, and fatty acids (1). Many of the P450 isozymes have been investigated for their ability to oxidize acetylenic compounds, resulting in a time-dependent loss of the heme chromophore after an intermediate becomes covalently bound to a heme nitrogen (2). More recently, several aromatic acetylenes, including 2EN and 9EPPh, have been shown to act as reversible inhibitors or mechanism-based inactivators that alkylate the protein moiety of cytochrome P450 in microsomes or reconstituted systems from rats or rabbits (3–7). In addition, the structure-activity relationships for the inhibition and inactivation of 1A1-, 1A2-, and 2B1-dependent reactions in rat liver microsomes by a series of aryl acetylenes have been investigated (6, 8). It was determined that the size and the shape of the polycyclic aromatic ring system and the placement of the alkyne function on the ring system were critical for suicide inhibition (6, 8). In addition, there was selectivity for different P450 isozymes when the compound contained either an ethynyl or propynyl group. In microsomes, the substitution of a propyne group for the ethyne moiety enhanced the inhibition of P450 1A enzymes (6). However, ethynes were more effective suicide inhibitors of P450 2B-dependent reactions in microsomes than the corresponding propynes (6). In addition, 9EPPh and 2EN were also found to be mechanism-based inactivators of mouse 2b-10 in liver and lung microsomes (7).

The present study describes the structure-activity relationships for the inactivation of P450s 2B1, 2B4, or 2B11 in a reconstituted system by a series of arylalkynes (fig. 1). The most effective inactivator of these 2B enzymes, 9EPPh, was investigated for its potential as an inactivator of human 2B6 expressed in a human lymphoblastoid cell line. The ability of 9EPPh to inactivate 2B2 was investigated using lidocaine as a substrate monitoring the formation of the 2B2-specific product (9). To further investigate the specificity of 9EPPh for rat liver...
P450s, we monitored the effect on testosterone, p-nitrophenol, and erythromycin metabolism in liver microsomes from rats treated with inducers of different forms of P450. Finally, the NADPH-dependent binding of several radiolabeled arylalkynes to microsomal proteins were determined by SDS-PAGE and autoradiography.

Materials and Methods

Materials. EFC was from Molecular Probes, Inc. (Eugene, OR), and HFC was from Enzyme Systems Products (Dublin, CA). 7-Ethoxycoumarin, 7-hydroxycoumarin, and testosterone were from Aldrich Chemical Co. (Milwaukee, WI). The specific activities were determined on an SLM-Aminco model SPF-500C spectrofluorometer with excitation at 360 nm and emission at 440 nm.

Enzymes. The microsomes were prepared from the livers of either male or female 10-week-old Fisher 344 rats (Charles River Labs, Portage, MI) or the Gentest Corp. (Woburn, MA). The reaction mixture, which was stirred with a magnetic stir bar and maintained at 20°C under an N₂ atmosphere for 4 hr and then allowed to warm to room temperature over a 0.5-hr period. The reaction was quenched with water, and the product was extracted with CH₂Cl₂. The CH₂Cl₂ extract was dried with Na₂SO₄, evaporated to dryness in vacuo, and the crude 1-acetylpyrene product obtained was purified by chromatography on silica gel with CH₂Cl₂ elution. The fractions containing 1-acetylpyrene were identified by thin layer chromatography on silica gel plates (CH₂Cl₂ elution), pooled, and evaporated to dryness to yield 88 mg (0.36 mmol, 72%) of [4,5,9,10-²H]1-acetylpyrene.

The [4,5,9,10-²H]1-acetylpyrene was dried in vacuo overnight, dissolved in 5.0 ml of dry tetrahydrofuran (THF), freshly distilled from benzophenone diion under N₂ atmosphere, cooled to –78°C under N₂ atmosphere, and treated with 2.5 equivalents (0.9 mmol) of base prepared by treating 152 μl of 2.2,6,6-tetramethylpiperidine (Aldrich) in 15 ml of dry THF with 0.56 ml of 1.6 M butyllithium solution in hexanes (Aldrich) for 0.5 hr. The combined solution was stirred with a magnetic stir bar at –78°C under N₂ atmosphere for 1 hr, and then 1.5 equivalents (78 μl, 93 mg) of diethyl chlorophosphate (Aldrich) were added. After 2 hr at –78°C, the reaction mixture was allowed to warm to room temperature and was then transferred with N₂ pressure into a second freshly prepared solution of 2.5 equivalents of 2,2,6,6-tetramethylpiperidine and butyllithium in THF at –78°C. After an additional 0.5 hr at –78°C, the reaction was allowed to warm slowly to room temperature and was then cooled again to –78°C and quenched with 5.0 ml of dilute sodium bicarbonate solution. The product was extracted with CH₂Cl₂, and the CH₂Cl₂ extract was washed twice with cold diluted HCl and then H₂O and then dried over Na₂SO₄. The CH₂Cl₂ was evaporated in vacuo, and the crude [²H]1EP product was purified by flash chromatography on silica gel with petroleum ether as the eluting solvent. The yield of [4,5,9,10-²H]1EP was 32 mg (0.14 mmol, 36%); radiochemical analysis performed with a Beckman LS 7000 liquid scintillation system with internal standardization established that the specific activity was 61.2 Ci/mole. The use of fresh, anhydrous reagents and carefully dried glassware is critical to the success of this synthetic conversion of aryl methyl ketones to alkynes on the small scale.

4-[(L-propynyl)Biphenyl ([²H,³H]4PbP), 4-Acetylbiphenyl] was labeled by catalytic exchange with tritium gas by Chemsyn Science Laboratories (Lenexa, KS). The tritiated sample was diluted with nonradioactive 4-acylbiphenyl (Aldrich) to a total of 225 mg (1.15 mmol), dissolved in dry THF, and reacted with diethyl chlorophosphate (Aldrich) at –78°C under N₂ atmosphere and described for 1EP. The reaction mixture generated by adding 2.5 additional equivalents of lithium diisopropylamide (Aldrich) at –78°C was then treated with 1.1 equivalents of [¹⁴C]CH₂Cl₂ (Amersham Corp., Arlington Heights, IL) and the reaction mixture was allowed to warm to room temperature overnight. The reaction, still under N₂ atmosphere, was then cooled to –78°C, 5.0 ml of dilute sodium bicarbonate solution was added, and the solution was allowed to warm to room temperature. The reaction product was extracted and purified by flash chromatography on silica gel as described above for [²H]1EP. The yield of [²H,³H]4PbP was 80 mg (36%). The specific activities were determined with a Beckman LS 7000 liquid scintillation system with internal standardization using a program that counts ¹⁴C and ³H in separate channels. The specific activities were 31 Ci/mole for ¹⁴C and 0.32 Ci/mole for ³H.

Inactivation of P450 Enzymes by Arylalkynes. For inactivation experiments of purified P450 in the reconstituted system with various aryl alkynes (table 1), a single time point EFC O-deethylase assay (15) was employed to determine the enzyme activity remaining after incubation with the arylalkynes. The primary incubations contained 0.25 μM P450, 0.5 μM reductase, 15 μg of DLPc, 125 units of catalase, 7.5 μM inactivator, or DMSO in control incubations and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.12 ml. The mixtures were preincubated at 30°C for 3 min before the addition of 0.83 mM NADPH to start the reaction. Aliquots containing 5 pmol of P450 were removed at various time points and added to 1.0 ml secondary reaction mixtures containing 0.2 mM NADPH, 100 μM EFC, 40 μg BSA, and 50 mM potassium phosphate buffer, pH 7.4. Secondary incubations were allowed to proceed for 5 min before the reactions were quenched with 0.3 ml of cold acetonitrile. For incubations with P450 2B4 or 2B11, the amount of reductase in the primary incubation was 0.75 μM. The fluorescent product, HFC, was measured on an SLM-Aminco model SPF-500C spectrophotometer with excitation at 410 (silt width, 5 nm) and emission at 510 nm (silt width, 5 nm). For the determination of the maximal rate constant of inactivation and the catalytic exchange with tritium gas as described above for [²H]1EP. The yields of [²H,³H]4PbP was 80 mg (36%). The specific activities were determined with a Beckman LS 7000 liquid scintillation system with internal standardization using a program that counts ¹⁴C and ³H in separate channels. The specific activities were 31 Ci/mole for ¹⁴C and 0.32 Ci/mole for ³H.
Experiments were carried out as described under Materials and Methods. Rate constants of inactivation were derived by linear regression analysis of the natural logarithm of the residual activity as a function of time. Values represent the average from two independent experiments that did not differ by more than 5%. The time 0 deethylase activities for P450 2B1, 2B4, and 2B11 were 6.17, 1.04, and 3.82 nmol HFC formed/min/nmol P450, respectively. Control values with no inhibitor were <0.010 min⁻¹. ND, values not different from control values.

<table>
<thead>
<tr>
<th>Arylalkyne</th>
<th>P450 2B1</th>
<th>P450 2B4</th>
<th>P450 2B11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1EN</td>
<td>0.16</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>2EN</td>
<td>0.14</td>
<td>0.34</td>
<td>0.11</td>
</tr>
<tr>
<td>2PN</td>
<td>0.12</td>
<td>0.04</td>
<td>ND</td>
</tr>
<tr>
<td>1EA</td>
<td>0.07</td>
<td>0.10</td>
<td>0.23</td>
</tr>
<tr>
<td>2EA</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3EpH</td>
<td>ND</td>
<td>ND</td>
<td>0.05</td>
</tr>
<tr>
<td>9EpH</td>
<td>0.03</td>
<td>0.03</td>
<td>ND</td>
</tr>
<tr>
<td>9PhP</td>
<td>0.88</td>
<td>0.43</td>
<td>0.49</td>
</tr>
<tr>
<td>4EP</td>
<td>0.45</td>
<td>ND</td>
<td>0.17</td>
</tr>
<tr>
<td>4PbP</td>
<td>0.02</td>
<td>0.18</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.08</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Maximal rate constant of inactivation.

b Concentration of inactivator that gives half-maximal inactivation.

d mmol EFC and NADPH, for those reactions where NADPH was not previously included.

The kinetic values were obtained as described previously (14).

125 units of catalase, 0.05, 0.1, 0.25, 1.0, or 2.0 μM 9EpH, or DMSO in control incubations and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.15 ml. The mixtures were preincubated for 3.0 min at 30°C before the addition of 1.0 mM NADPH to start the reaction. Aliquots containing 10 pmol of P450 were removed at various time points and added to 1.0-m1 secondary reaction mixtures containing 1.0 mM NADPH, 300 μM 7-ethoxycoumarin, 40 μg of BSA, and 50 mM potassium phosphate buffer, pH 7.4. Secondary incubations were allowed to proceed for 10 min before the reactions were quenched with 0.1 ml of 2 N HCl. The reaction mixtures were extracted with 3.0 ml of CH2 Cl2, and the organic phase was back-extracted with 2.0 ml of 30% CH3 CN and 95% 0.1M potassium phosphate, pH 3.0, at a flow rate of 1.7 ml/min. Detection was at 214 nm.

**Testosterone Metabolism.** The metabolism of testosterone by liver microsomes from untreated or PB-treated rats was determined as described (17, 18). The mixtures contained 1.0 mg of protein, 250 μM testosterone, 20 μM 9EpH (when included), and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 1.0 ml. The mixtures were preincubated at 37°C for 3 min before the addition of 1.0 mM NADPH. After a 10-min incubation, the reactions were quenched with 5.0 ml of CH3Cl2. An internal standard (14α-hydroxytestosterone) was added, and the mixtures were extracted. The organic layer was dried down, and the residue was dissolved in methanol, filtered, and injected onto a C18 reversed phase column. The solvent system consisted of mixture A (MeOH:H2O:CH3CN, 39:60:1) and mixture B (MeOH:H2O:CH3CN, 80:18:2). The metabolites were eluted with an electrolyte gradient (curve #7 using a Waters automated gradient controller) from 11% to 85% mixture B over 23 min with analysis of the eluate at 254 nm.

**SDS-PAGE Analysis.** The incubation mixtures contained 0.25 mg of microsomal protein, 1.0 mM NADPH, 30 μM radiolabeled arylalkyne, and 50 mM potassium phosphate buffer, pH 7.4, in 0.2 ml. The mixtures were incubated at 30°C for 15 min, and the reactions were quenched with an equal volume of sample loading buffer. After heating for 5.0 min at 90°C, the samples were analyzed by SDS-PAGE on a 7.5% polyacrylamide gel using the Laemmli buffer system (19). The gels were stained with Coomassie Blue, destained, and then treated with Entensify (NEN Research Products, Boston, MA) before drying. The gels were exposed to Hyperfilm-MP (Amersham Corp., Arlington Heights, IL) for 12–16 days before developing.

**Results**

The ethynyl-substituted compounds 1EN, 2EN, 1EA, 2EA, 2EP, 3EP, 9EpH, and 4EP and the propynyl-substituted compounds 2PN, 9PhP, and 4PbP (fig. 1) at a concentration of 7.5 μM were each investigated for their ability to cause a time-dependent loss of 2B1, 2B4, or 2B11-dependent EFC O-deethylase activity in a reconstituted system containing rat reductase and lipid. Fig. 2 shows representative graphs when 2B1, 2B4, or 2B11 was incubated with substituted phenanthrylalkynes, and the activity remaining at various time points was determined by removing an aliquot and assaying for the EFC O-deethylase activity. The 50-fold dilution into the secondary reaction mixture was necessary to minimize the reversible inhibition described previously with rat liver microsomes (8). Incubation with 9EpH resulted in a time-dependent loss of activity with all three isozymes. Rate constants of inactivation were determined from the slopes of the lines when the natural logarithm of the per cent activity remaining was plotted vs. time. Each of the isozymes was incubated with each of the 11 compounds, and the rate constants of inactivation were determined (table 1). With every isozyme, the most effective inactivator at a concentration of 7.5 μM was 9EpH. With P450 2B1, the order of effectiveness for the top five compounds was 9EpH > 9PhP > 1EN, 2EN, 2PN. The order of effectiveness with the rabbit isozyme, P450 2B4, was 9EpH > 2EN > 4EP > 1EN, 1EA. With 2B11, the rate constants of inactivation in descending order were 9EpH > 1EA > 4EP, 9PhP > 2EN. Similar experiments were performed to obtain the maximal rate constant of inactivation (k inactivation) and the 9EpH concentration required for half-maximal inactivation (K i ) of the 7-ethoxy- coumarin O-deethylase activity. There are two differences between the two sets of experiments described in tables 1 and 2. One is the
enzyme activity used to monitor the 2B activity remaining at various time points and the other is the temperature of the reactions. Although the assay used to measure the product of EFC metabolism in table 1 is convenient for screening a large number of compounds, the enzyme activity associated with several 2B isozymes is low. Therefore, 7EC was used to more accurately examine the characteristics of this mechanism-based inhibitors. The experiments described in table 1 were performed at 30°C, while those described in table 2 were performed at 20°C. It was easier to monitor the linear portion of the very rapid inactivation by 9EPh when the reaction was slowed by lowering the temperature. Various concentrations of 9EPh were added to primary incubations containing 2B1, 2B4, or 2B11 in reconstituted systems, and 7-ethoxycoumarin-0-deethylase activity was used as a marker enzyme in the secondary reaction mixture to monitor the loss of activity at various time points. First order inactivation constants were determined by linear regression analysis of the slopes of the lines. From the plot of the reciprocal of the initial rate constant of inactivation as a function of the reciprocal of the 9EPh concentration, the maximal rate constant of inactivation and the inactivator concentration required for half-maximal inactivation were determined (table 2) as previously described (14).

9EPh was further investigated for its ability to inactivate human 2B6 expressed in human lymphoblastoid cells. The 2B6-dependent activity was followed by measuring the O-deethylation of EFC. Microsomes from cells that did not express 2B6 showed no measurable EFC deethylase activity. When microsomes from the cells expressing 2B6 showed no measurable activity, and 7-ethoxycoumarin was rapidly inactivated by 9EPh when the reaction was slowed by preincubation for 5 min at 30°C before the addition of NADPH to a final concentration of 0.2 mM or water to the mixtures without NADPH. The deethylase activity was measured spectrophotometrically as described under Materials and Methods. The experiments described in table 1 were used to more accurately examine the characteristics of these enzymes. The assay used to measure the product of EFC metabolism in table 1 was convenient for screening a large number of compounds, the enzyme activity used to monitor the 2B activity remaining at various time points and the other is the temperature of the reactions. Although the assay used to measure the product of EFC metabolism in table 1 is convenient for screening a large number of compounds, the enzyme activity associated with several 2B isozymes is low. Therefore, 7EC was used to more accurately examine the characteristics of this mechanism-based inhibitors. The experiments described in table 1 were performed at 30°C, while those described in table 2 were performed at 20°C. It was easier to monitor the linear portion of the very rapid inactivation by 9EPh when the reaction was slowed by lowering the temperature. Various concentrations of 9EPh were added to primary incubations containing 2B1, 2B4, or 2B11 in reconstituted systems, and 7-ethoxycoumarin-0-deethylase activity was used as a marker enzyme in the secondary reaction mixture to monitor the loss of activity at various time points. First order inactivation constants were determined by linear regression analysis of the slopes of the lines. From the plot of the reciprocal of the initial rate constant of inactivation as a function of the reciprocal of the 9EPh concentration, the maximal rate constant of inactivation and the inactivator concentration required for half-maximal inactivation were determined (table 2) as previously described (14).

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To investigate the specificity of the inhibition of several rat P450 isozymes by 9EPh, lidocaine, and testosterone metabolism in microsomes from untreated and PB-treated rats was determined in the presence or absence of 9EPh (tables 4 and 5). Many forms of P450 have the ability to N-deethylate lidocaine to form monoethylglycin-
**TABLE 4**

Effect of 2EN or 9EPh on lidocaine metabolism by microsomes from PB-treated rats

Incubation conditions and analysis of the products were as described under Materials and Methods. The values represent the average ± SD from three determinations. The numbers in parentheses represent the percentage of activity relative to the control incubation at time 0.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Me-OH lidocaine formation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MEGX formation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time 0 (nmol/min/mg)</td>
<td>Time 5 (nmol/min/mg)</td>
</tr>
<tr>
<td>None</td>
<td>8.1 ± 0.3 (100)</td>
<td>7.6 ± 0.7 (94)</td>
</tr>
<tr>
<td>2EN</td>
<td>4.5 ± 0.2 (56)</td>
<td>3.8 ± 0.3 (47)</td>
</tr>
<tr>
<td>9EPh</td>
<td>7.1 ± 0.3 (88)</td>
<td>2.3 ± 0.1 (28)</td>
</tr>
</tbody>
</table>

<sup>a</sup> 2B2-specific product.
<sup>b</sup> 2B1 and other isozymes contribute to this product.
<sup>c</sup> Time in the presence of 2EN or 9EPh before addition of lidocaine.

**TABLE 5**

Effect of 9EPh on testosterone metabolism in microsomes from untreated and PB-treated rats

Testosterone metabolism by liver microsomes from untreated or PB-treated rats in the presence or absence of 20 μM 9EPh. Analysis of metabolites was as described under Materials and Methods. The values represent the mean ± SD from three determinations. The numbers in parentheses represent the percentage of testosterone activity relative to the incubations without 9EPh. *p < 0.05, **p < 0.002, ***p < 0.001.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>9EPh</th>
<th>Testosterone metabolism (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6β/7α</td>
</tr>
<tr>
<td>None</td>
<td>–</td>
<td>2.11 ± 0.13</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>1.32 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(63)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PB</td>
<td>–</td>
<td>3.72 ± 0.09</td>
</tr>
<tr>
<td>PB</td>
<td>+</td>
<td>3.87 ± 0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(104)</td>
</tr>
</tbody>
</table>

<sup>a</sup> 17-Oxidation, androstenedione formation.
<sup>b</sup> ND, not detected.
<sup>c</sup> Only one determination.

 FIG. 3. SDS-PAGE and autographic analysis of rat liver microsomes after incubation with radiolabeled arylacetylenes and NADPH.

Lanes 1–4 are from rats treated with no inducer, PB, PYR, or βNF, respectively, with either [1<sup>3</sup>H]2EN (panel A) or [1<sup>3</sup>H]9EPh (panel B). Experimental procedures were as described under Materials and Methods.

treated with no inducer, PB, PYR, or βNF, the band corresponding to 2B1 and 2B2 in the lanes containing microsomes from PB- or PYR-treated rats showed radiolabel bound to protein. Only a portion of the gel is shown, but there was no radioactivity in other areas of the lanes. When [1<sup>3</sup>H]9EPh was incubated with these microsomes, there were two bands that were covalently modified by radiolabeled substrate (fig. 3B). One corresponds to that seen with [1<sup>3</sup>H]2EN and migrates with 2B1/2. The other band migrates at approximately 59 kDa and was seen in the lanes containing the microsomes from male PB- and PYR-treated rats. The same doublet pattern was observed with microsomes from PB-treated female rats (data not shown). No bands were seen in the lanes containing microsomes from the clofibrate-, PCN-, or βNF-treated rats with either [1<sup>3</sup>H]2EN or [1<sup>3</sup>H]9EPh (data not shown). The band corresponding to 2B1/2 was very faint in the lane containing microsomes from isosafrole-treated rats with either [1<sup>3</sup>H]2EN or [1<sup>3</sup>H]9EPh. Fig. 4 shows the results for microsomes from PB-induced male rats incubated with [1<sup>3</sup>H]9EPh, [1<sup>3</sup>H]2EN, [1<sup>3</sup>H]1EP, or [1<sup>3</sup>H,14C]4PbP. The doublet is seen after incubation with [1<sup>3</sup>H]9EPh and [1<sup>3</sup>H]1EP. No bands were observed when [1<sup>3</sup>H,14C]4PbP was incubated with any of the microsomal preparations (data not shown). [1<sup>3</sup>H]1EP showed the doublet only with microsomes from female and male PB-treated rats (data not shown). In microsomes from both male and female rats, [1<sup>3</sup>H]2EN covalently labeled only 2B1, while [1<sup>3</sup>H]9EPh covalently labeled 2B1/2 and another protein whose inactivation pattern followed that of 2B1.

Discussion

The results of these studies establish that the size and shape of the aromatic ring system and the placement of the acetylene group on the nine position correlates with the regiospecific metabolism of phenanthrene where 84% or 93% of the 2B6. The specific metabolism at the nine position is important determinants for the inactivation of P450s lipids. With each of the isozymes, the most effective inactivator at a concentration of 7.5 μM was 9EPh. The effectiveness of 9EPh as an inactivator of 2B1, 2B4, and 2B11 is further supported by the high inactivation values and low values. When the ethynyl group was placed at the 2 or 3 position of the phenanthryl backbone, little inactivation was seen. 9EPh was also found to inactivate human EME metabolism by microsomes from untreated or PB-treated rats in the presence or absence of 20 μM 9EPh. Analysis of metabolites was as described under Materials and Methods. The values represent the average ± SD from three determinations. The numbers in parentheses represent the percentage of activity relative to the control incubation at time 0.
lidocaine. In addition, 9EPh has been found to be an effective suicide inactivator of mouse 2b-10 in liver and lung microsomes from TCPOBOP-induced mice (7). MALDI-MS analysis of the cyanogen bromide-generated peptides from 9EPh-inactivated P450 2B1 confirmed the addition of a phenanthryl acetyl group to the peptide corresponding to residues 290–314. When the peptide was further digested with pepsin, MALDI-MS analysis confirmed the site of attachment to be in the segment F297 to L307 (14). When comparing the sequences of the proteins inactivated by 9EPh, P450s 2B1, 2B2, 2B4, 2B6, 2b-10, and 2B11 (fig. 5), they are nearly identical in this 15-amino acid peptide that includes the F297–L307 segment. These isozymes must bind 9EPh in such a way that optimizes the orientation of the reactive intermediate ketone so that it is in close proximity to a catalytically important amino acid at the active site.

The order of effectiveness of the top five compounds is different with each of the isozymes. With rat liver microsomes from PB-induced animals, the order of effectiveness for the inactivation of the pentoxysresorufin O-deethylase activity was 9EPh > 1EN > 2EN > 1EA (8) compared with the results with purified 2B1, 9EPh > 9PPh > 1EN > 2EN, 2PN. The order of effectiveness with 2B4 was 9EPh > 2EN > 4EP > 1EN, 1EA and with 2B11 was 9EPh > 1EA > 4EP, 9PPh > 2EN. 2B1 was inactivated by the propynyl compounds more so than the other isozymes. As previously seen with the rat liver microsomes, the pyrene compound seemed to be too large to bind efficiently with critical regions of the active site of 2B1 (8). It would seem that 2B4 and 2B11 have more open active sites because 4EP was an efficient inactivator of these enzymes. Ortiz de Montellano and co-workers (27, 28) investigated the active sites of P450s 2B1, 2B2, 2B4, and 2B1 using topographical analysis. The results of studies on the reaction of phenyl diazenes with these purified proteins resulted in the conclusion that the region above pyrrole ring B of the prosthetic heme group was masked by protein residues (the I helix in P450cam) in all of the proteins. This aligns with the region identified by Montellano and co-workers (27, 28) investigated the active sites of P450s 2B1, 2B2, 2B4, 2B6, 2b-10, and 2B11. P450s 2B4 and 2B11 seem to have more open active sites because all three of the remaining pyrrole rings are arylated by phenyldiazene, while only two of the pyrrole rings are arylated in 2B1 and 2B2 (27, 28).

The specificity of 9EPh as a selective inhibitor or inactivator of other P450 isozymes was also investigated. There was no effect on 2E1-dependent p-nitrophenol hydroxylase or 3A1/2-dependent erythromycin demethylase activity. As previously reported, 9EPh was not an inactivator of 1A1-dependent ethoxyresorufin O-deethylase activity in microsomes from βNF-treated rats (8). When the effect of 9EPh on testosterone metabolism was investigated, there was a marked effect only on the 2B1-dependent activities including 16-keto formation and 16α- and 16β-hydroxylase in microsomes from PB-treated rats.

There was a specific covalent labeling of 2B1/2 in microsomes from PB- and PYR-treated animals by [3H]2EN and [3H]9EPh. In addition, there was another band at approximately 59 kDa that was labeled by [3H]9EPh and [3H]1EP in the PB- and PYR-induced microsomes. The presence of this band followed the induction patterns observed for 2B1, strong induction by PB and a weaker induction by PYR (26). Given the specificity of 9EPh and the results from the induction studies, it is possible that this is an as yet uncharacterized member of the rat 2B subfamily.

In attempting to define the catalytic specificity of the cytochrome P450 enzymes, one approach that is used is selective inhibition of individual isozymes (29). These acetylenic compounds may prove themselves to be relatively specific inhibitors of cytochrome P450 enzymes. 9EPh, in particular, may prove to be a selective mechanism-based inactivator of 2B6 in human liver microsomes and thus aid in the identification of 2B6-dependent drug metabolism.

Acknowledgments. We thank David A. Putt for preparation of radioactive liver microsomes and the purification of 2B4 and Hsia-Lien Lin for the purification of reductase.

References


