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EFFECT OF POLYAMINE ON MICROSOMAL CYTOCHROME P-450 STIMULATION OF RATE AND IMPROVED COUPLING OF NADPH OXIDATION TO HYDROXYLATION

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Summary. Low concentration of polyamine (50 µM spermine) significantly stimulated (3 to 4 folds) hydroxylation of benzphetamine and 7-ethoxycoumarin by liver microsomes and a reconstituted P-450 system. Level and concentration dependence of this activation depended both on the type of P-450 used in the reconstituted system (LM_2 or LM_4) and on the substrate. NADPH oxidation and substrate hydroxylation were not stimulated to the same extent. Polyamines are therefore coupling or uncoupling factors in hydroxylation reactions depending on the experimental conditions.

Polyamines such as spermine, spermidine and putrescine have been postulated to play an essential role in cell regulation (1) (2). Many reports describe their effects on nucleic acid and protein synthesis (3) (4) (5), as well as on a number of other enzyme reactions (6-9). In this respect it was reported (10) that hydroxylation of drugs and xenobiotics catalyzed by liver microsomes could be significantly stimulated by polyamines. This stimulation was interpreted as the consequence of a stabilizing effect of polyamine on the microsomal membrane.

More recently, procedures for preparing highly purified homogeneous cytochrome P-450 and NADPH cytochrome P-450 reductase from rabbit liver have become available (11) (12). It appeared therefore of interest to reinvestigate the effect of polyamines on hydroxylation reactions catalyzed by the reconstituted highly purified enzyme system.

MATERIALS AND METHODS

Hepatic microsomes from uninduced and phenobarbital, treated rabbits, and NADPH cytochrome P-450 reductase were prepared according to van der Hoeven and Coon (11). Cytochrome P-450LM_2 and LM_4 were prepared according to Haugen and Coon (12). P-450 preparations migrated on SDS polyacrylamide gels as single polypeptide and their specific heme content ranged from 12 to 16 nmoles per mg protein. Cytochrome c reductase activity of NADPH cytochrome P-450 reductase (1 unit = 1 nmole cytochrome *c* reduced per min per mg protein) was determined according to Master *et al.* (13).

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Hydroxylation was carried out at 37°C in 50 mM Tris-HCl pH 7.4 (pH 7.9 at 20°C) and initiated by addition of NADPH final concentration 0.2 mM. In experiments with the reconstituted system, appropriate aliquots of stock solutions of P-450, reductase and dilauroylglyceryl 3-phosphorylcholine (di-12-GPC) were preincubated 3 min at room temperature prior to addition of the other constituents of the assay medium, which was then incubated 3 min at 37°C before initiation of the reaction. Polyamines, $MgCl_2$ and KCl, when used, were added to the assay medium by appropriate dilution of concentrated solutions, whose pH was 7.4.

Benzphetamine hydroxylation was determined according to the method of Nash (14), microsomes being precipitated by addition of 100 mM $CaCl_2$ to the assay medium. In experiments with microsomes the following conditions were used : microsomes 0.3 mg protein /ml and 0.5 mM benzphetamine ; with the reconstituted system the concentration of P-450, reductase, di-12-GPC, and substrate were respectively : 0.09 µM, 140 units/ml, 40 µM and 0.5 mM in the case of LM_2, and 0.16 µM, 280 units/ml, 40 µM and 0.5 mM in the case of LM_4.

7-Ethoxycoumarin hydroxylation was determined fluorimetrically with an Aminco-Keirs spectrofluorimeter according to the method of Ullrich and Weber (15) using 7-hydroxycoumarin as a standard. In assays with microsomes, the following conditions were used : microsomes 0.3 mg protein/ml and 0.2 mM 7-ethoxycoumarin ; with the reconstituted system the concentrations of P-450, reductase, di-12-GPC and substrate were respectively : 0.22 µM, 340 units/ml, 40 µM and 0.2 mM in the case of LM_2, and 0.1 µM, 340 units/ml, 40 µM and 0.2 mM in the case of LM_4.

NADPH consumption during hydroxylation of both benzphetamine and 7-ethoxycoumarin by the reconstituted system was determined spectrophotometrically (at 340 nm using $\varepsilon = 6.2\ mM^{-1}$) on a Beckman Acta III spectrophotometer under the experimental conditions previously mentionned for each substrate.

All rate determinations were made at least in duplicate, under conditions of linearity.

Benzphetamine (hydrochloride) was a gift from Dr. H. Glady, Upjohn Laboratories. 7-Ethoxycoumarin was synthetized in our laboratory from hydroxycoumarin (Merck, Darmstat), according to the method of Ullrich and Weber (15). Polyamines as hydrochlorides were from Sigma, St Louis, Mo. Other chemicals were of highest purity available.

RESULTS AND DISCUSSION

Spermine activates the hydroxylation of benzphetamine and 7-ethoxycoumarin by microsomes from liver of untreated and phenobarbital induced rabbits as shown in Figure 1 (a). The magnitude and concentration dependence of this activation depended both on treatment of animals and the type of substrate, the latter point being in agreement with the work of Chapman (10). Significant stimulation was achieved with relatively low spermine concentrations : 7-ethoxycoumarin hydroxylation was activated by a factor of 3.2 with only 50 µM in spermine.

Figure 1b and 2a and 2b show that polyamines were also potent activators of the reconstituted system in which membrane structure is known to be absent. Of special interest was the finding that although the concentration dependence of the stimulation differed from that of microsomes, a similar activation level was reached with the reconstituted system. It is therefore clear that the polya-

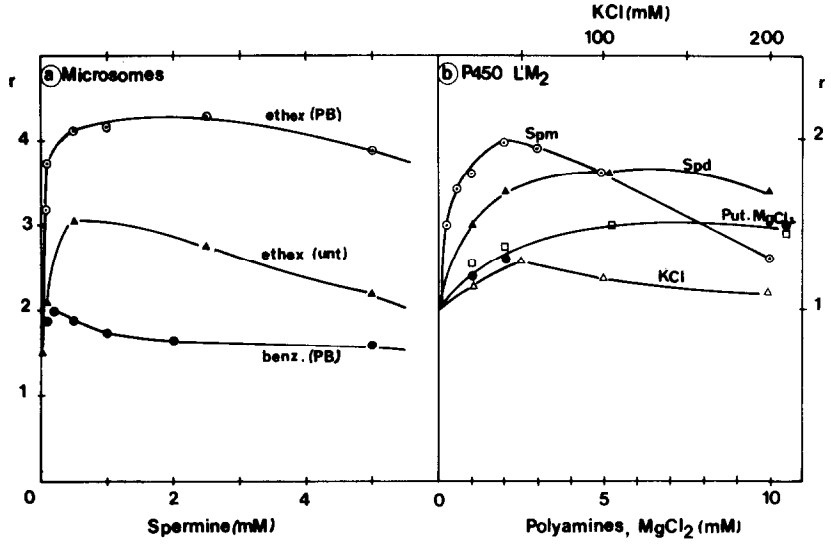

Figure 1. Effect of polyamines, $MgCl_2$ and KCl on hydroxylation of benzphetamine and ethoxycoumarin by hepatic microsomes from untreated-and phenobarbital-induced rabbit, and P-450LM_2.

a) Microsomes : activation ratio r (r = turnover in the presence of spermine/turnover of control) as a function of spermine concentration. —●— benzphetamine hydroxylation by microsomes from phenobarbital induced rabbits, TN (control) = 4.12 nmoles/min/mg proteins. —▲— ethoxycoumarin hydration by microsomes of untreated rabbits, TN (control) = 0.83 nmole/min/protein . —○— ethoxycoumarin hydroxylation by microsomes of phenobarbital induced rabbits, TN (control) = 2.50 nmole/min/mg protein .

b) P-450LM_2 system : activation ratio r as a function of polyamine , $MgCl_2$ and KCl concentration for benzphetamine hydroxylation. —○— spermine ; —▲— spermidine ; —●— putrescine ; —□— $MgCl_2$; —△— KCl (note the different concentration scale). TN (control) = 20.7 min^{-1}.

mine induced stimulation was not mediated through membrane structural change. The concentration of polyamine giving maximal activation of benzphetamine hydroxylation by P-450LM_2 increased and the magnitude of maximal activation decreased as the charge of the polyamine decreased (spermine, spermidine and putrescine bear respectively 4, 3 and 2 charges). The response to Mg^{++} was similar to that of putrescine and K^+ induced only a slight activation (25 %) at relatively high concentration (25 mM). The high stimulation brought about by spermine is therefore not a pure ionic strength effect. Kinetic measurements, showed that spermine only affects V_{max}, whereas $K_{m(app)}$ was not modified for 7-ethoxycoumarin and slightly increased (10 %) for benzphetamine. On the other hand cytochrome c-reductase activity of NADPH cytochrome P-450 reductase was not affected by spermine, in agreement with Chapman (10).

Some important aspects of this activation are shown in Figure 2 and Table I.

1/ With a particular substrate, the level and polyamine concentration dependence of

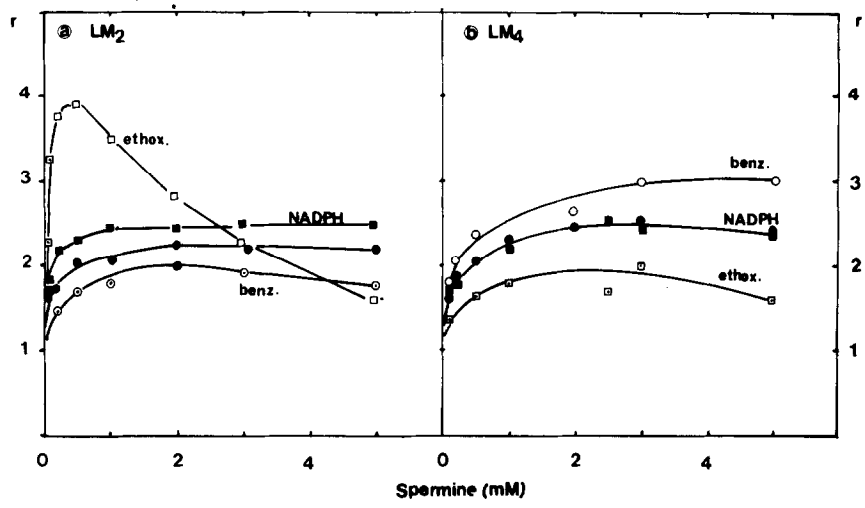

Figure 2. Effect of spermine on hydroxylation of benzphetamine and ethoxycoumarin, and on oxidation of NADPH by P-450LM$_2$ and LM$_4$.

a) P-450LM$_2$ system : activation ratio r as a function of spermine concentration. —o— benzphetamine hydroxylation ; —●— corresponding NADPH consumption, TN (control) = 20.7 and 40.9 min^{-1} respectively. —□— ethoxycoumarin hydroxylation ; —■— corresponding NADPH consumption. TN (control) = 0.41 and 8.6 min^{-1} respectively.

b) P-450LM$_4$ system : activation ratio r as a function of spermine concentration. —o— benzphetamine hydroxylation ; —●— corresponding NADPH consumption. TN (control) = 4.42 and 16.2 min^{-1} respectively. —□— ethoxycoumarin hydroxylation ; —■— corresponding NADPH consumption. TN (control) = 2.29 and 22.1 respectively.

activation depended on the type of cytochrome P-450 used. 2/ With a particular type of cytochrome P-450 the level and polyamine concentration dependence of activation depended on the substrate used. In particular with either P-450LM$_2$ or LM$_4$, greater stimulation was obtained with the non-specific substrate (benzphetamine and 7-ethoxycoumarin are specific substrates of P-450LM$_2$ or LM$_4$ respectively). 3/ Total NADPH consumption and substrate hydroxylation were not stimulated to the same ex-

Table I. Efficiency of hydroxylation of benzphetamine and ethoxycoumarin catalyzed by P-450LM$_2$ and LM$_4$, expressed as the ratio : rate of hydroxylation/ rate of NADPH oxidation. For experimental conditions see Materials and Methods and Figure 2.

Spm (mM)	0	.1	.5	1	2	3	5
LM$_2$ benz.	50.6	44.5	41.2	44.9	44.1	44.8	41.5
LM$_2$ ethox.	4.7	8.3	7.9	6.8	5.2	4.2	3.0
LM$_4$ benz.	27.3	30.9	31.3	27.8	29.2	32.1	34.4
LM$_4$ ethox.	10.4	8.5	7.1	8.8	8.7	8.6	7.2

tent : for instance with P-450LM_2 and 7-ethoxycoumarin, substrate hydroxylation was much more stimulated than NADPH consumption, especially at low polyamine level. Spermine appears therefore to affect the coupling of NADPH oxidation to substrate hydroxylation. With both types LM_2 and LM_4 the coupling was significantly increased when the non-specific substrate was used, while the reverse was found with specific substrate , Table I. Since according to the recent reports, the poor efficiency (uncoupling) of cytochrome P-450 catalyzed hydroxylations is thought to result from the unproductive autoxidation of the oxyferrous intermediate (16) (17), our observations suggest that polyamines may affect either the transfer of the second electron from reductase to cytochrome P-450 or the stability of the oxyferrous intermediate. Further experiments are currently being made to clarify this point. Finally, in liver, in contrast to magnesium concentration (18) the in vivo polyamines level is known to be subjected to large fluctuations (8). Moreover, polyamine concentration was shown to increase after treatment of animals with various drugs (phenobarbital, 3-methycholanthrene or 3-4 benzpyrene) which are known to induce a specific form of P-450 (19). The observations reported here showing an important effect of spermine in the 50-100 µM concentration range, is therefore of possible significance in vivo.

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