The effect of substrates and inhibitors of cytochrome P-450 on the NADPH inhibition of the ATP-dependent, hepatic, microsomal calcium pump

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In hepatic microsomes one or more isozymes of cytochrome P-450 inhibits the ATP-dependent Ca^{2+} pump. This inhibition is reversible by GSH and appears to be due to a direct oxidation of the pump proteins by the oxygenated cytochrome. To determine which isozyme mediates this inhibiton, we have examined the effect of various substrates and inhibitors on the NADPH inhibition of Ca^{2+} uptake. We find that aminopyrine, benzphetamine and SKF-525A reverse this inhibition while a number of other substrates do not. This pattern suggests that a previously unreported isozyme of cytochrome P-450 mediates the Ca^{2+} pump inhibition.

Cytochrome P-450 inhibition; Microsomal Ca^{2+} pump

1. INTRODUCTION

NADPH inhibits the ATP-dependent Ca^{2+} pump of hepatic microsomes from untreated male rats by 20–50% [1–3]. This inhibition is blocked by nitrogen and partially by CO/O_2, suggesting that it is mediated through a cytochrome P-450 pathway. Further, it is reserved by GSH through a reparative process [1–3]. Since it is known that the sulfhydryls of the Ca^{2+} pump are readily oxidized with a loss of Ca^{2+} uptake [4], these data suggest that NADPH serves as a cofactor for the cytochrome P-450 catalyzed production of reactive oxygen which reversibly oxidizes the pump [1–3]. The reactive oxygen is not released into the medium, since reactive oxygen scavengers have no effect on the inhibition [3]. Hence a cytochrome P-450-feroxyl species is apparently in close proximity to the pump and directly oxidizes it [2].

Since cytochrome P-450 is a family of isoforms [5], the question arises as to whether all or only a few isozymes oxidize the Ca^{2+} pump. To assess the role of the various forms in this inhibition, we have examined the effect of a number of substrates and inhibitors on the NADPH effect. Since various agents show marked differences in metabolism with different forms of cytochrome P-450, this approach has proven to be a powerful technique, both in vivo and in vitro, to determine which cytochrome P-450 isoform catalyzes a hydroxylation reaction. Testosterone [8] and warfarin [9] have been the most widely employed agents in these studies.

In the current study we have utilized this technique to determine which cytochrome P-450 isoform(s) catalyzes the NADPH inhibition of the Ca^{2+} pump by examining whether various substrates for and inhibitors of the cytochrome P-450-dependent mixed-function oxidases block the NADPH inhibition of Ca^{2+} uptake. These studies are based on the assumption that if a substrate is metabolized by the cytochrome P-450 isoform which also mediates the NADPH inhibition, then the addition of the substrate should compete for the activated oxygen and reverse the NADPH inhibition of the pump. Although there are exceptions to this concept, it is probably generally valid. A similar result should be seen if an inhibitor is added to the incubations, whether it acts as a competitive substrate for the oxygenated cytochrome or by blocking the formation of the oxygenated cytochrome P-450. We have not used either testosterone or warfarin in these studies since they are both metabolized by a large number of isozymes and would therefore not delineate which form inhibits the pump. We have, instead, examined the effect of substrates with only one or two major metabolic reactions. We find that the NADPH inhibition of the Ca^{2+} pump appears to be catalyzed by a cytochrome P-450 isoform(s) which catalyzes the metabolism of aminopyrine and benzphetamine, is inhibited by SKF-525A and imidazole, and is stimulated by metyrapone.

2. MATERIALS AND METHODS

Glucose 6-phosphate, $NADP^+$, ATP, and glucose 6-phosphate

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dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO); aminopyrine from J.T. Baker (Phillipsburg, NJ); ethylmorphine from Merck (Rahway, NJ); hexobarbital from Winthrop Laboratories (New York, NY); aniline and 7-ethoxycoumarin from Aldrich Chemical Co. (Milwaukee, WI); and $^{45}CaCl_2$ (NEZ-013) from New England Nuclear (Boston, MA). Benzphetamine was a gift of UpJohn Laboratories (Kalamazoo, MI); zoxazolamine of NcNeil Laboratories (Fort Washington, PA); and SKF-525A of Smith Kline Laboratories (Philadelphia, PA).

Microsomes were prepared from male, Sprague-Dawley rats (140–160 g) and Ca^{2+} uptake was determined as previously described [2]. Incubations were run in triplicate with drug concentrations from 10 μM to 2 mM in the presence of 1 mM $NADP^+$ or NADPH. The coefficient of variation was 5% for both the basal Ca^{2+} uptakes and the percentages of the NADPH inhibition for incubations performed on a single preparation. All studies were replicated 3 or more times and gave the same pattern on replication. Protein was determined by the method of Lowry et al. [8].

3. RESULTS AND DISCUSSION

Benzphetamine (750 μM) completely blocked the NADPH inhibition of Ca^{2+} uptake (Fig. 1). SKF-525A (5 μM) and imidazole (100 μM) showed similar patterns (Table I). Aminopyrine only partially blocked the NADPH inhibition with a half-maximal effect at 750 μM. On the basis of the reported metabolic activities of the rat cytochromes P-450 isoforms, these data indicate that cytochromes P-450a [9,10], P-450f [11], P-450g [11], P-450pcn1 [12], P-450 RLM 5b [13] and P-450 LAw [14] could not mediate the NADPH inhibition of the Ca^{2+} pump, since they do not metabolize either aminopyrine or benzphetamine.

Metyrapone, an inhibitor of only some isoforms of cytochrome P-450, had no effect on the NADPH inhibition of the Ca^{2+} pump in concentrations up to 500 μM, but at 1–2 mM it increased the NADPH inhibition to 53% compared to an inhibition of 36% seen in the absence of metyrapone (Table I). Hence cytochromes P-450b and P-450e are not likely candidates, even though they both actively metabolize benzphetamine [10], since both are also inhibited by metyrapone [15]. Further, cytochrome P-450b metabolizes hexobarbital, which had no effect (Table I). Even though cytochrome PB-1 is stimulated by metyrapone [15], it also has a high turnover number for *p*-nitroanisole [10j which had no effect here and therefore this isoform does not appear to catalyze the NADPH inhibition (Table I).

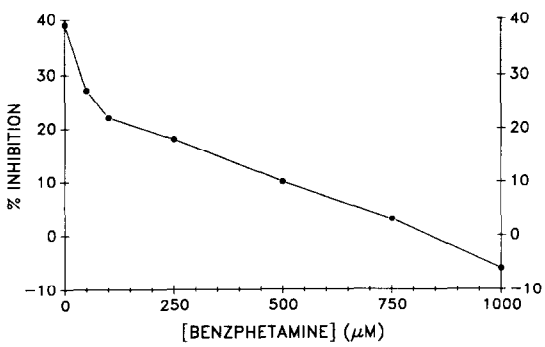

Fig. 1. The percentage of NADPH inhibition of the Ca^{2+} uptake at each concentration of benzphetamine.

Table I

The effect of substrates and inhibitors of the cytochrome P-450 dependent mixed-function oxidases on the NADPH induced inhibition of the hepatic ATP-dependent, microsomal Ca^{2+} pump

Block inhibition	Increase inhibition	No effect on inhibition
aminopyrine	metyrapone	aniline[a]
benzphetamine		ethylmorphine
SKF-525A		7-ethoxycoumarin
imidazole		hexobarbital
		morphine
		p-nitroanisole
		zoxazolamine

[a] The effect of all substrates was determined at 10 μM–2 mM in the presence of $NADP^+$ and NADPH. The NADPH inhibition was taken as blocked if at 1 mM the substrate or inhibitor showed more than 50% reversal of the NADPH inhibition when compared to the Ca^{2+} uptake seen with $NADP^+$ at the same concentration of substrate. Incubations at all concentrations of drug or inhibitor were run in triplicate

Aniline, ethylmorphine, 7-ethoxycoumarin, hexobarbital, morphine, *p*-nitroanisole and zoxazolamine had no effect on Ca^{2+} uptake in the presence of either $NADP^+$ or NADPH (Table I). Hence cytochromes P-450c or P-450d are not involved, since both readily metabolize 7-ethoxycoumarin and zoxazolamine [9]. Similarly cytochromes P-450h and C-M/F are not involved since they actively metabolize ethylmorphine [10,11,16]. Further, cytochrome P-450j probably does not catalyze this inhibition since it readily metabolizes aniline [17]. Cytochrome P-450 dbl is probably not involved since it *O*-dealkylates codeine and should therefore have been blocked by ethylmorphine [18].

In conclusion, these studies indicate that only a limited number of forms of cytochrome P-450 modulate the activity of the hepatic microsomal Ca^{2+} pump and suggest that none of the forms which have been purified to date catalyze this inhibition. Our current studies do suggest that immunoinhibition studies with antibodies to some of the purified forms might assist in further defining which gene family is involved. In particular, members of the CYPIIB or CIPIIIA families may play a role in this process, since the purified CYPIIB and microsomes from pregnenolone-16α-carbonitrile very actively metabolize benzphetamine, the most potent blocker of the substrates examined [10,12].

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