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Effects of adrenergic agents, vasopressin and ionophore A23187, on the phosphorylation of, and flux through, phenylalanine hydroxylase in rat liver cells

Michael J. FISHER, M. Angelica SANTANA and Christopher I. POGSON
Department of Biochemistry, University of Manchester, Oxford Road, Manchester M13 9PL, U.K.

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1. The adrenergic amines noradrenaline and adrenaline increased flux through phenylalanine hydroxylase by approx. 50%. 2. This effect, which appears to be mediated by an α-adrenergic mechanism, was accompanied by a rapid increase in the phosphorylation of phenylalanine hydroxylase. 3. Although ionophore A23187 mimicked the effects of the adrenergic amines, vasopressin was completely without effect on either phenylalanine hydroxylation or enzyme phosphorylation. 4. Flux through phenylalanine hydroxylase in young rats (80g) was insensitive to α-adrenergic, but sensitive to β-adrenergic, agents. 5. Consistent with previous observations [Fisher & Pogson (1984) *Biochem. J.* **219**, 79–85] the present data indicate a close correlation between phosphorylation state and flux rate (i.e. enzyme activity).

Phenylalanine hydroxylase [L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating); EC 1.14.16.1] catalyses the first, and physiologically irreversible, step in phenylalanine catabolism:

$$\text{L-Phenylalanine} + \text{tetrahydrobiopterin} + O_2 \rightarrow \text{L-tyrosine} + \text{dihydrobiopterin} + H_2O$$

Enzyme activity is controlled in the short term by a phosphorylation–dephosphorylation cycle (Abita *et al.*, 1976; Donlon & Kaufman, 1980), and in the longer term by changes in total enzyme activity, presumably reflecting altered protein concentration (Carr & Pogson, 1981; Donlon & Beirne, 1982).

Phosphorylation by the cyclic AMP-dependent protein kinase is well characterized for the purified protein (Abita *et al.*, 1976), *in vivo* (Donlon & Kaufman, 1978), and in isolated liver cells (Fisher & Pogson, 1984). The involvement of other protein kinases has not, however, been clearly demonstrated. Wilgus & Kaufman (unpublished work, cited in Hasegawa & Kaufman, 1982) reported that adrenaline increases phosphorylation through the cyclic AMP-dependent system. Similarly, on the basis of activity measurements Abita *et al.* (1980) suggested that stimulation of phosphorylation by exposure of cells to noradrenaline was blocked by propranolol, a finding consistent with a 'β' mode of action of the hormone. To the contrary, Garrison has shown that phosphorylation is stimulated by α-adrenergic agents (Garrison, 1978; Garrison *et al.*, 1979; Garrison & Wagner, 1982; Garrison, 1983). Another possibility, raised by Bublitz (1971), is that adrenaline may act directly and reversibly to inhibit phenylalanine hydroxylase itself (Miller & Shiman, 1976).

More recent work has provided indications that vasopressin may act by a cyclic AMP-independent mechanism distinct from that for α-adrenergic effectors. Thus fatty acid synthesis is stimulated by vasopressin, but not by phenylephrine (Assimacopoulos-Jeannet *et al.*, 1981); there are also differences in the way in which vasopressin and α-adrenergic amines regulate gluconeogenesis (Kneer & Lardy, 1983). Additionally, hepatic protein-phosphorylation patterns are not identical for the two agents (Garrison, 1983).

The studies described in the present paper were undertaken in order to clarify the responsiveness of phenylalanine hydroxylase to a range of hormonal stimuli. Simultaneous measurements of flux through the hydroxylase in cells confirm that phosphorylation is associated with changes in catalytic activity under physiologically relevant conditions.

Materials and methods

Animals

Male Sprague–Dawley rats (University of Manchester breeding colony) were used throughout. Except where stated to the contrary, rats of 180–220g body wt. were used. Animals were fed *ad*

libitum (Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.).

Reagents

Reagents and radiochemicals were obtained from the sources given previously (Fisher & Pogson, 1984). The following hormones, agonists and antagonists were obtained from Sigma: phenylephrine, isoprenaline (isoproterenol), adrenaline (epinephrine), noradrenaline (arterenol), propranolol, vasopressin ([arginine]vasopressin; grade VI) and ionophore A23187. Phentolamine was a gift from Ciba Research Laboratories, Horsham, Sussex, U.K. All other chemicals were of the purest grade available from standard suppliers.

Preparation and incubation of hepatocytes

Cells were prepared as described previously (Elliott *et al.*, 1976) except that glucose (20mM) was added to the perfusion medium. In all experiments, metabolic integrity was assessed by measurement of ATP content (Dickson & Pogson, 1977).

Cells were preincubated for 30min with lactate/pyruvate (9:1, final concn. 10mM) and glucose (10mM). Incubations (volume 2ml) were as previously described (Smith & Pogson, 1980).

Measurement of phenylalanine hydroxylation flux

L-Phenylalanine was added to a final concentration of 0.05mM and a specific radioactivity of 1.2×10^5 c.p.m./ml. Effectors were added immediately before phenylalanine, except for glucagon, which was added 2min before substrate. All adrenergic agonists and antagonists were prepared immediately before use in Krebs–Henseleit (1932) buffer. A23187 was prepared (final concn. 250μM) in ethanol. Ethanol on its own had no significant effect on flux through phenylalanine hydroxylase (results not shown). All incubations were terminated with 0.2ml of 2M-$HClO_4$.

Flux through both phenylalanine hydroxylase and homogentisate oxidase was assessed by the ^3H-release assay described by Fisher & Pogson (1984). All flux data presented here are based on initial rates over 30min.

Determination of phenylalanine hydroxylase phosphorylation

Cells were incubated with [^{32}P]P_i for 60–75min before addition of effectors; specific immunoprecipitation of phenylalanine hydroxylase as described previously (Fisher & Pogson, 1984) allowed determination of phosphate content. The [γ-^{32}P]ATP specific radioactivity of cells incubated with [^{32}P]P_i was determined by the method of Hawkins *et al.* (1983); it was unaffected by hormones, agonists or antagonists (results not shown).

Results and discussion

Table 1 shows the effect of various agents on the phosphorylation of phenylalanine hydroxylase in incubations of liver cells from adult rats. Control cells under these conditions contain enzyme with approx. 1 molecule of phosphate/tetramer. Glucagon at maximally effective concentrations increases the phosphate content, as previously shown (Fisher & Pogson, 1984). Neither isoprenaline, a β-agonist (Levitzki, 1981), nor vasopressin alters the degree of phosphorylation. Exposure of cells to phenylephrine, an α-agonist (Levitzki, 1981), however, leads to a 50% increase in enzyme phosphory-

Table 1. *Effects of hormones and hormonal agonists on phosphorylation of phenylalanine hydroxylase and on flux through phenylalanine hydroxylase and homogentisate oxidase in isolated rat liver cells*

Materials and methods were as described in the text. The phenylalanine concentration in the medium was 50μM. Results are means ± S.E.M. for the numbers of independent observations in parentheses. The significance of differences between means was assessed by the paired *t* test. *P* (versus control values): * < 0.05, ** < 0.01; all other differences were not significant ($P > 0.05$).

Expt. no.	Conditions	Phosphorylation of phenylalanine hydroxylase (mol of P/mol of subunit)	Increase (%)	Flux through phenylalanine hydroxylase (nmol/h per mg)	Increase (%)	Flux through homogentisate oxidase (nmol/h per mg)
I	Control	0.25 ± 0.02 (3)	–	3.33 ± 0.06 (4)	–	0.94 ± 0.05 (4)
	Glucagon (0.1 μM)	0.56 ± 0.09** (6)	93 ± 14	5.98 ± 0.10** (4)	80 ± 6	1.85 ± 0.24** (4)
	Isoprenaline (1 μM)	0.27 ± 0.01 (3)	7 ± 8	3.50 ± 0.21 (4)	5 ± 5	0.94 ± 0.05 (4)
	Phenylephrine (20 μM)	0.39 ± 0.04* (3)	50 ± 16	3.93 ± 0.04* (3)	18 ± 4	0.98 ± 0.13 (3)
	Vasopressin (0.1 μM)	0.26 ± 0.06 (3)	11 ± 3	3.43 ± 0.09 (3)	2 ± 2	0.93 ± 0.07 (3)
II	Control	0.25 ± 0.02 (3)	–	2.93 ± 0.41 (3)	–	1.07 ± 0.22 (3)
	Adrenaline (10 μM)	0.37 ± 0.01** (3)	45 ± 8	3.82 ± 0.48* (3)	39 ± 7	1.42 ± 0.24* (3)
	Noradrenaline (10 μM)	0.35 ± 0.02* (3)	39 ± 10	4.08 ± 0.74* (3)	47 ± 7	1.27 ± 0.26* (3)
	A23187 (2.5 μM)	0.36 ± 0.02* (3)	43 ± 4	3.52 ± 0.62* (3)	19 ± 4	1.10 ± 0.25 (3)

lation after 5min. Both adrenaline and noradrenaline elicit similar responses. Consistent with the view that α-adrenergic agents may act through changes in cytosolic Ca^{2+} concentrations, these effects are mimicked by the calcium ionophore A23187.

The maximum phosphorylation with α-adrenergic stimulation is always considerably less than that with glucagon. One explanation of this could be that the relative rates of phosphorylation differ against a background of a constant rate of dephosphorylation. However, examination of the time course of phosphorylation after addition of adrenaline or noradrenaline shows that the changes are maximal by 2min and remain constant for at least 10min (results not shown).

Simultaneous measurements of the changes in flux through the reaction catalysed by the hydroxylase demonstrate a general parallelism with changes in the phosphorylation state (Table 1). It is therefore reasonable to suppose that the phosphorylation changes are directly reflected in altered enzyme activity under physiological conditions.

The reasons for the poor correlations seen with phenylphrine and A23187 are not clear, but may be related to the fact that the time course of flux experiments is somewhat longer than that for phosphorylation studies; A23187 at least is known to exert a number of less specific side effects on liver cell metabolism, and these will be more apparent with increased incubation time (Reed & Lardy, 1972). Effects on the flux through homogentisate oxidase, further along the catabolic pathway, mirror those on hydroxylase flux, a not-unexpected finding in view of the rate-determining role of hydroxylase.

The similarity of the effects on phosphorylation of A23187 and the adrenergic agents suggested that the latter might be acting at the α-receptor. The results shown in Table 2 confirm this; the increase in hydroxylation rate with low concentrations of adrenaline or noradrenaline is blocked by the α-antagonist phentolamine, but not by the β-antagonist propranolol.

As noted above, vasopressin (at 0.1 μM) had no discernible effect on either phosphorylation or phenylalanine hydroxylation. We investigated the possibility that our cells might be 'desensitized' to vasopressin. Parallel measurements showed, however, that both vasopressin and glucagon stimulated glycogen breakdown in incubations of cells from fed animals, whereas glucagon alone increased the extent of hydroxylase phosphorylation (results not shown). Differences between the actions of vasopressin and α-adrenergic agents have been reported (see the introduction).

Some resolution of the discrepancies regarding the 'α' or 'β' mode of action of adrenaline can be achieved on the basis of studies demonstrating that age and sex are important factors; Blair *et al.* (1979) showed that β-effects occur in young rats, but are lost with growth, and Studer & Borle (1982) found that, although adult females exhibit β responses, adult males do not. (The β-adrenergic response reappears when hepatocytes isolated from adult male rats are maintained in primary culture; Nakamura *et al.*, 1983.)

Table 3 details the results of experiments in which flux through the phenylalanine-catabolic pathway was measured in cells from 80g rats. Exposure to isoprenaline increased flux through the hydroxylase, and this was blocked substantially by the β-antagonist, propranolol. In a separate experiment it was shown that neither antagonist on its own affected flux, nor did noradrenaline in the presence of propranolol [control, 4.82 ± 0.24; propranolol (10 μM), 5.18 ± 0.32; phentolamine (10 μM), 4.45 ± 0.40; noradrenaline (10 μM) plus propranolol (10 μM), 4.86 ± 0.24; all nmol/h per mg dry wt. means ± S.D. from triplicate determinations]. Similar changes, although of smaller magnitude, was seen in homogentisate oxidation. These observations are consistent with earlier claims

Table 2. *Effect of adrenergic antagonists on flux through phenylalanine hydroxylase in isolated rat liver cells*
Materials and methods were as described in the text. Results are expressed as percentage increases above the control rate, as means ± S.E.M. from four independent observations. The significance of differences between means was assessed by the paired *t* test. Abbreviation: N.S., not significant.

	Agonist	Antagonist	Phenylalanine hydroxylation (% increase)	*P*
I	Adrenaline (1 μM)	–	21 ± 3	versus control, <0.01
II	Adrenaline (1 μM)	Propranolol (10 μM)	14 ± 4	versus control, <0.05; versus I, N.S.
III	Adrenaline (1 μM)	Phentolamine (10 μM)	7 ± 2	versus control, N.S.; versus I, <0.05
IV	Noradrenaline (1 μM)	–	27 ± 6	versus control, <0.05
V	Noradrenaline (1 μM)	Propranolol (10 μM)	24 ± 7	versus control, <0.05; versus IV, N.S.
VI	Noradrenaline (1 μM)	Phentolamine (10 μM)	1 ± 4	versus control, N.S.; versus IV, <0.05
VII	–	Propranolol (10 μM)	0 ± 3	versus control, N.S.
VIII	–	Phentolamine (10 μM)	0 ± 6	versus control, N.S.

Table 3. *Effect of the β-adrenergic agent isoprenaline on flux through phenylalanine hydroxylase and homogentisate oxidase in liver cells from young (80g) rats*

Materials and methods were as described in the text. The phenylalanine concentration in the medium was 50 μM. Results are means ± S.E.M. from three independent observations. The significance of differences between means was assessed by the paired *t* test. Abbreviation: N.S., not significant ($P > 0.05$).

	Conditions	Flux through phenylalanine hydroxylase (nmol/h per mg)	*P*	Flux through homogentisate oxidase (nmol/h per mg)	*P*
I	–	3.52 ± 0.31		0.93 ± 0.26	
II	Dibutyryl cyclic AMP (100 μM)	7.37 ± 0.79	versus I, <0.05	2.24 ± 0.50	versus I, <0.05
III	Isoprenaline (1 μM)	6.47 ± 0.68	versus I, <0.02	1.82 ± 0.56	versus I, N.S.
IV	Isoprenaline (1 μM) + propranolol (10 μM)	4.05 ± 0.33	versus I, N.S.; versus III, <0.05	1.22 ± 0.34	versus I, N.S.; versus III, N.S.

with regard to phenylalanine hydroxylase (Abita *et al.*, 1980; Hasegawa & Kaufman, 1982) and support the observations made by Blair *et al.* (1979).

The results of our studies further develop the hypothesis that phenylalanine hydroxylase activity in cells, and, by implication, *in vivo*, is modulated by protein phosphorylation, and that this phosphorylation may be catalysed by the cyclic AMP-dependent protein kinase and a Ca^{2+}-dependent kinase associated with α-receptors (Exton, 1981, 1982).

Garrison (1983), in his analysis of two dimensional gel electrophoretograms of hepatic phosphoproteins, noted relatively small effects of vasopressin on phenylalanine hydroxylase, and no effect at all of phorbol esters, which, he proposed, may act by mechanisms related to those of vasopressin and involving protein kinase C (Castagna *et al.*, 1982; Kiss & Mhina, 1982; Niedel *et al.*, 1983). Our present results are no more than consistent with this postulate, but do suggest that caution should be exercised in the interchangeable use of vasopressin and α-adrenergic agents.

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References

Abita, J.-P., Milstien, S., Chang, N. & Kaufman, S. (1976) *J. Biol. Chem.* **251**, 5310–5314

Abita, J.-P., Chamras, H., Rosselin, G. & Rey, F. (1980) *Biochem. Biophys. Res. Commun.* **92**, 912–918

Assimacopoulos-Jeannet, F., Denton, R. M. & Jeanrenaud, B. (1981) *Biochem. J.* **198**, 485–490

Blair, J. B., James, M. E. & Foster, J. L. (1979) *J. Biol. Chem.* **254**, 7579–7584

Bublitz, C. (1971) *Biochem. Pharmacol.* **20**, 2543–2553

Carr, F. P. A. & Pogson, C. I. (1981) *Biochem. J.* **198**, 655–660

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851

Dickson, A. J. & Pogson, C. I. (1977) *FEBS Lett.* **83**, 27–32

Donlon, J. & Bierne, E. (1982) *Biochem. Biophys. Res. Commun.* **108**, 746–751

Donlon, J. & Kaufman, S. (1978) *J. Biol. Chem.* **253**, 6657–6659

Donlon, J. & Kaufman, S. (1980) *J. Biol. Chem.* **255**, 2146–2152

Elliott, K. R. F., Ash, R., Pogson, C. I., Smith, S. A. & Crisp, D. M. (1976) in *Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies* (Tager, J. M., Söling, H.-D. & Williamson, J. R., eds.), pp. 139–143, North-Holland, Amsterdam

Exton, J. H. (1981) *Mol. Cell. Endocrinol.* **23**, 233–264

Exton, J. H. (1982) *Trends Pharmacol. Sci.* **3**, 111–115

Fisher, M. J. & Pogson, C. I. (1984). *Biochem. J.* **219**, 79–85

Garrison, J. C. (1978) *J. Biol. Chem.* **253**, 7091–7100

Garrison, J. C. (1983) in *Isolation, Characterization and Use of Hepatocytes* (Harris, R. A. & Cornell, N. W., eds.), pp. 551–559, Elsevier/North-Holland, Amsterdam

Garrison, J. C. & Wagner, J. D. (1982) *J. Biol. Chem.* **257**, 13135–13143

Garrison, J. C., Borland, M. K., Florio, V. A. & Twible, D. A. (1979) *J. Biol. Chem.* **254**, 7147–7156

Hasegawa, H. & Kaufman, S. (1982) *J. Biol. Chem.* **257**, 3084–3089

Hawkins, P. T., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **210**, 717–720

Kiss, Z. & Mhina, V. (1982) *FEBS Lett.* **148**, 131–134

Kneer, N. M. & Lardy, H. A. (1983) *Arch. Biochem. Biophys.* **225**, 187–195

Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66

Levitzki, A. (1981) *Top. Mol. Pharmacol.* **1**, 23–62

Miller, M. R. & Shiman, R. (1976) *J. Biol. Chem.* **251**, 3671–3676

Nakamura, T., Tomomura, A., Noda, C., Shimoji, M. & Ichihara, A. (1983) *J. Biol. Chem.* **258**, 9283–9289

Niedel, J. E., Kuhn, L. J. & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci.* U.S.A. **80**, 36–40

Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970–6977

Smith, S. A. & Pogson, C. I. (1980) *Biochem. J.* **186**, 977–986

Studer, R. K. & Borle, A. B. (1982) *J. Biol. Chem.* **257**, 7987–7993