Characterization of the sites phosphorylated on tyrosine hydroxylase by Ca^{2+} and phospholipid-dependent protein kinase, calmodulin-dependent multiprotein kinase and cyclic AMP-dependent protein kinase

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Tyrosine hydroxylase purified from rat pheochromocytoma is phosphorylated rapidly by the Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) purified from rat or sheep brain. Phosphorylation was stimulated 14-fold by Ca^{2+} and phosphatidylserine and occurred at a rate comparable with that of the phosphorylation of histone H1. The phospholipid-dependent protein kinase phosphorylates a single site which is identical to that phosphorylated by cyclic AMP-dependent protein kinase and to the secondary site of phosphorylation by the calmodulin-dependent multiprotein kinase. The implications of these results with respect to the regulation of catecholamine biosynthesis in adrenal medulla are discussed.

Tyrosine hydroxylase *Phosphorylation* *Cyclic AMP-depdent protein kinase*
Phospholipid-dependent protein kinase *Calmodulin-dependent protein kinase* *Acetylcholine*

1. INTRODUCTION

Treatment of dissociated adrenal chromaffin cells with either acetylcholine or cyclic AMP analogues leads to stimulation of catecholamine biosynthesis and activation of the rate-limiting enzyme in this pathway, tyrosine hydroxylase [1–3]. Since purified tyrosine hydroxylase is phosphorylated and activated in vitro by cyclic AMP-dependent protein kinase [4–6], it has been suggested that cyclic AMP is involved in mediating the effects of acetylcholine [2,3]. However, while acetylcholine has been reported to increase cyclic AMP levels in chromaffin cells [7], this is not always observed [1]. In addition, stimulation of catecholamine biosynthesis by acetylcholine, and by the depolarizing agents K^+ and veratridine, is dependent on the presence of Ca^{2+} in the external medium, and the pathway is also activated by the Ca^{2+} ionophore A23187. These observations suggest that the effects of acetylcholine on chromaffin cells may be mediated by Ca^{2+} rather than cyclic AMP.

Recently, tyrosine hydroxylase purified from a catecholamine secreting adrenal tumour has been shown to be phosphorylated by the calmodulin-dependent multiprotein kinase from rabbit skeletal muscle [8]. Here, we report that another Ca^{2+}-dependent protein kinase, the Ca^{2+} and phospholipid-dependent protein kinase (protein kinase C), also phosphorylates tyrosine hydroxylase. The sites that are phosphorylated by the two

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Abbreviation: HPLC, high performance liquid chromatography

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Ca^{2+}-dependent protein kinases and cyclic AMP-dependent protein kinase have been compared by HPLC peptide mapping and by Edman degradation of phosphorylated peptides, as a preliminary approach to determine which, if any of these protein kinases, mediates the effect of acetylcholine in vivo.

2. MATERIALS AND METHODS

2.1. *Materials*

Phosphatidylserine, mixed histone (Type IIA) and lysine-rich histone (Type III-S) were from Sigma (Dorset, UK); trypsin (TPCK-treated) from Worthington/Millipore (NJ, USA); bovine serum albumin (fraction V) from BDH Chemicals (Dorset, UK), and [γ-^{32}P]ATP from Amersham International (Bucks, UK).

Tyrosine hydroxylase was purified from rat pheochromocytoma [8]; the catalytic subunit of cyclic AMP-dependent protein kinase [9] and the calmodulin-dependent multiprotein kinase [10] from rabbit skeletal muscle. The Ca^{2+} and phospholipid-dependent protein kinase was purified from rat brain ([11]; DEAE-trisacryl method), or sheep brain (as in [12]; final phenyl-Sepharose step omitted). Calmodulin was purified from sheep brain [13], and histone-H1 from commercial mixed histone (type IIA) as in [14].

2.2. *Methods*

Assay procedures and units of activity for cyclic AMP-dependent protein kinase [15], calmodulin-dependent protein kinase [10] and phospholipid-dependent protein kinase [12] have been defined previously, except that in the present paper units are μmol phosphate incorporated per min rather than nmol per min. Tyrosine hydroxylase was phosphorylated in incubations (50 μl) containing tyrosine hydroxylase (0.11 mg/ml), [γ-^{32}P]ATP (0.2 mM; 4×10^5 cpm/nmol) and magnesium acetate (5 mM) in 25 mM sodium-Hepes buffer (pH 7.0) containing 0.5 mM EDTA and 0.5 mM dithiothreitol. Further additions were: (1) catalytic subunit of cyclic AMP-dependent protein kinase plus EGTA (0.1 mM); (2) calmodulin-dependent protein kinase plus $CaCl_2$ (1 mM) and calmodulin (10 μg/ml); or (3) phospholipid-dependent protein kinase plus $CaCl_2$ (1 mM) and phosphatidylserine (50 μg/ml). Amounts of protein kinase added are detailed in figure legends. Reactions were started by the addition of ATP, and incorporation of phosphate into protein was determined as in [16].

Tryptic peptides were prepared and separated by reversed-phase HPLC as in [8] except that an Altex Ultrasphere ODS column (25 × 0.4 cm) was used, and radioactivity in the column effluent was monitored continuously with a Reeve Analytical Monitor using the Cerenkov effect. For isoelectric focussing, fractions were dried down in vacuo and analysed as in [17]. Automated Edman degradation was carried out on HPLC fractions using a Beckman 890C sequencer as in [18]. The butylchloride extract from each cycle was dried, mixed with 5 ml optiphase MP (Fisons Fine Chemicals, Loughborough, UK) and counted.

3. RESULTS

3.1. *Phosphorylation of tyrosine hydroxylase by Ca^{2+} and phospholipid-dependent protein kinase*

As reported previously, tyrosine hydroxylase

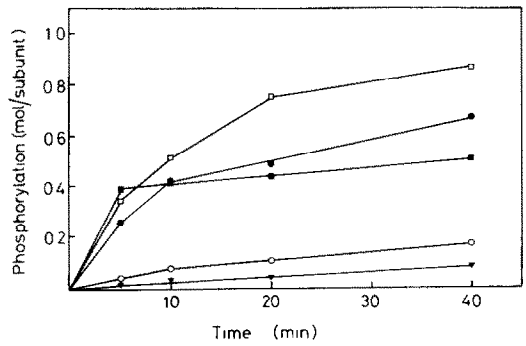

Fig.1. Time course of phosphorylation of purified tyrosine hydroxylase by purified protein kinases. Tyrosine hydroxylase was incubated with Mg-[γ-^{32}P]ATP and the following additions: none (▼); 2 mU/ml catalytic subunit of cyclic AMP-dependent protein kinase (■); 2 mU/ml calmodulin-dependent multiprotein kinase plus Ca^{2+} and calmodulin (□); 0.5 mU/ml rat brain phospholipid-dependent protein kinase with (●) or without (○) Ca^{2+} and phosphatidylserine. Phosphorylation in the absence of added protein kinase was not affected by addition of Ca^{2+} plus calmodulin or phosphatidylserine. Phosphorylation by calmodulin-dependent multiprotein kinase was completely dependent on Ca^{2+} and calmodulin (not shown).

from rat pheochromocytoma was phosphorylated by cAMP-dependent protein kinase and calmodulin-dependent multiprotein kinase. Tyrosine hydroxylase was also a good substrate for Ca^{2+} and phospholipid-dependent protein kinase purified from rat or sheep brain (fig.1). Phosphorylation was stimulated 14-fold by Ca^{2+} and phosphatidylserine and the final stoichiometry of phosphorylation approached 0.7 mol/mol of the 60-kDa subunit. With two different preparations of tyrosine hydroxylase, initial rates of phosphorylation at 0.1 mg/ml tyrosine hydroxylase were 95–110% of that of histone-H1 at 0.2 mg/ml (not shown). Since histone-H1 is one of the best substrates for Ca^{2+} and phospholipid-dependent protein kinase, tyrosine hydroxylase would also appear to be an excellent substrate for this protein kinase.

3.2. *Comparison of sites phosphorylated on tyrosine hydroxylase by phospholipid-dependent, calmodulin-dependent and cyclic AMP-dependent protein kinases*

Tyrosine hydroxylase was phosphorylated by each of the three protein kinases in the presence of Mg-[γ-^{32}P]ATP. The protein was digested with trypsin and ^{32}P-labelled peptides analysed by reversed-phase HPLC. Cyclic AMP-dependent and phospholipid-dependent protein kinase both phosphorylated a major ^{32}P-labelled peptide (A), which comigrated on HPLC (fig.2). In contrast, calmodulin-dependent protein kinase phosphorylated two major ^{32}P-labelled peptides, C and A, the latter having an identical retention time to the peptide labelled by the cyclic AMP- and phospholipid-dependent protein kinases. Peptide A represents a secondary site of phosphorylation by the calmodulin-dependent protein kinase, since peptide C predominates when phosphorylation is performed with lower concentrations of protein kinase ([8] and D.G.H., unpublished).

Tyrosine hydroxylase was phosphorylated by the three protein kinases either individually or in combination, digested with trypsin, and peptide A purified by HPLC. Isoelectric focussing on thin-layer polyacrylamide showed a single ^{32}P-labelled peptide of p$I \simeq 4$ in every case (not shown). Since isoelectric focussing would resolve mono- and di-phosphorylated forms of the same peptide (see e.g. [19]), the results obtained using combinations of two protein kinases suggested that the three protein kinases phosphorylated the same residue in peptide A. This was confirmed by sequencer analysis of peptide A. A 'burst' of radioactivity occurred after the third cycle of Edman degradation, whether tyrosine hydroxylase was phosphorylated by cyclic AMP-dependent, calmodulin-dependent or phospholipid-dependent protein kinase (fig.3).

Isoelectric focussing was also carried out on peptide C labelled by the calmodulin-dependent protein kinase, revealing that it was more acidic than peptide A, with a pI of about 3. Sequencer analysis of peptide C also resulted in a 'burst' of ^{32}P-radioactivity after three cycles of Edman degradation, while thin-layer electrophoresis at pH 1.9 after partial acid hydrolysis revealed that serine was the only phosphorylated amino acid (not shown).

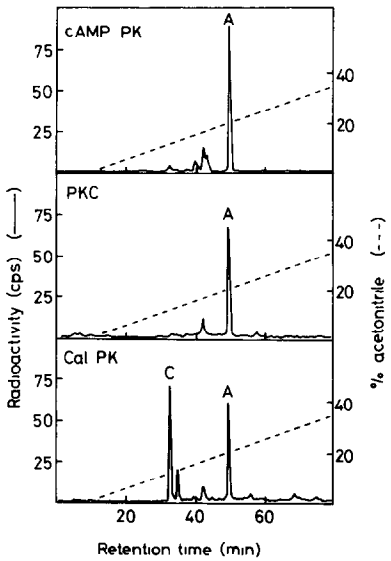

Fig.2. Reversed-phase HPLC analysis of tryptic peptides derived from ^{32}P-labelled tyrosine hydroxylase. cAMP PK, enzyme labelled to 1.3 mol/subunit using cyclic AMP-dependent protein kinase (10 mU/ml). PKC, enzyme labelled to 0.7 mol/subunit using sheep brain Ca^{2+} and phospholipid-dependent protein kinase (4 mU/ml). Cal PK, enzyme labelled to 1.3 mol/subunit using the calmodulin-dependent multiprotein kinase (20 mU/ml). The small peak eluting at 42 min in each run was also observed when this preparation of tyrosine hydroxylase was incubated in the presence of [γ-^{32}P]ATP without added protein kinase and appears to be due to trace contamination of the enzyme preparation with an unidentified protein kinase.

4. DISCUSSION

These results show that tyrosine hydroxylase from an adrenal tumour is a good substrate for the Ca^{2+} and phospholipid-dependent protein kinase in vitro, with near-stoichiometric phosphorylation of the identical residue phosphorylated by cyclic AMP-dependent protein kinase. Since phosphorylation of peptide A by cyclic AMP-dependent protein kinase increases tyrosine hydroxylase activity [8], phosphorylation by phospholipid-dependent protein kinase should be accompanied by a similar activation. This is supported by the recent work of Albert et al. [20], who have independently found that tyrosine hydroxylase can be phosphorylated and activated by the phospholipid-dependent protein kinase. They also reported that the phosphorylation occurs on a serine residue in the same peptide labelled by cyclic AMP-dependent protein kinase.

We previously reported that the secondary site phosphorylated by the calmodulin-dependent protein kinase comigrated with peptide A on HPLC. However, phosphorylation did not appear to be accompanied by activation of tyrosine hydroxylase, and it was suggested that the calmodulin-dependent protein kinase must phosphorylate a different residue within peptide A. The present work has shown this conclusion to be incorrect. The site in peptide A phosphorylated by calmodulin-dependent protein kinase is clearly identical to that phosphorylated by the cyclic AMP-dependent and phospholipid-dependent protein kinases. A likely explanation for our failure to detect activation of tyrosine hydroxylase by calmodulin-dependent protein kinase is suggested by the recent work of Albert et al. [20]. They showed that activation of tyrosine hydroxylase by the phospholipid-dependent protein kinase was transient. The more active phosphorylated form of tyrosine hydroxylase appears to be unstable, so that activation is rapidly followed by inhibition of the enzyme [21,22]. Perhaps, this inactivation is accentuated in the presence of Ca^{2+} and/or calmodulin. Yamauchi et al. [23] have reported that the calmodulin-dependent multiprotein kinase from rat brain, which is closely related to the skeletal muscle enzyme [24], can activate tyrosine hydroxylase from adrenal medulla, provided that an additional 'activator protein' is present in the incubations. It seems possible that the 'activator protein' is functioning in their system by stabilising the phosphorylated form of tyrosine hydroxylase.

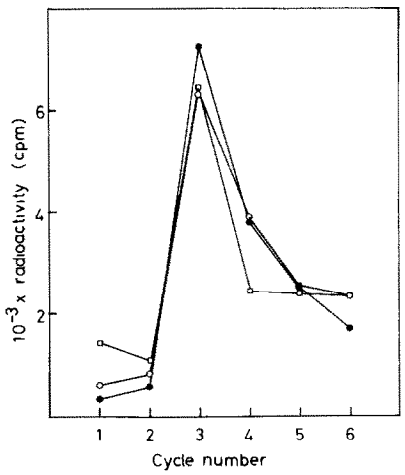

Fig.3. Release of ^{32}P radioactivity during successive cycles of Edman degradation of peptide A labelled using: cyclic AMP-dependent protein kinase (○); sheep brain phospholipid-dependent protein kinase (●); and the calmodulin-dependent protein kinase (□). Amounts added to the spinning cup were 123000 cpm, 76266 cpm, and 50385 cpm, respectively. The low recovery of radioactivity is due to extensive breakdown of phosphoserine during the cleavage step to free phosphate, which is only partially extracted from the cup by butylchloride. Similar yields (1–5% in the cycle containing the phosphoamino acid) are obtained when peptides of defined sequences are analysed.

Treatment of adrenal medullary cells with acetylcholine stimulates phosphorylation of two tryptic peptides in tyrosine hydroxylase [6]. However, only the peptide with the least anodic migration at pH 6.0 or 7.4 was stimulated by 8-bromo-cyclic AMP. It is tempting to equate peptide A (isoelectric point ≃ 4) with the less acidic peptide phosphorylated in response to both acetylcholine and 8-bromo-cyclic AMP, and peptide C (isoelectric point ≃ 3) with the more acidic peptide phosphorylated only in response to acetylcholine. While further work is required to confirm that the peptides phosphorylated in intact cells are identical with those phosphorylated on the purified enzyme, it is clear that activation of the calmodulin-dependent protein kinase could account for the increased phosphorylation of both peptides in response to acetylcholine, whereas ac-

tivation of the cyclic AMP-dependent or phospholipid-dependent protein kinases could not.

Since peptides A and C are generated by tryptic digestion, the primary structure N-terminal to these sites should be lys/arg-x-x-ser(P), which is typical of sites phosphorylated by cyclic AMP-dependent protein kinase [25] and the calmodulin-dependent multiprotein kinase [26]. Furthermore, it is known that both sites reside in the same 37 kDa cyanogen bromide peptide [8]. This information should aid identification of the phosphorylation sites in the primary structure, when the cDNA sequence [27] has been completed.

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