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The role of protein kinase C in nicotinic responses of bovine chromaffin cells

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Abstract

The effects of the protein kinase C inhibitor CGP 41251 (31-benzoyl-staurosporine) on nicotinic responses of cultured bovine adrenal chromaffin cells have been investigated. CGP 41251 inhibited tyrosine hydroxylase activation by phorbol 12,13-dibutyrate, with an IC_{50} of < 0.3 μM and complete inhibition at 1 μM. In contrast, it had little effect on nicotine-stimulated tyrosine hydroxylase activity up to 1 μM, and did not fully inhibit it even at 10 μM. From 1 to 10 μM, CGP 41251 caused a similar concentration-dependent inhibition of tyrosine hydroxylase activity stimulated by nicotine, K^+, forskolin and 8-Br-cyclic AMP. CGP 42700 (19,31-dibenzoyl-staurosporine), a structural analogue of CGP 41251 that lacks activity as a protein kinase C inhibitor, had no effect on tyrosine hydroxylase activity stimulated by any of the agonists. CGP 41251 had no effect on catecholamine secretion induced by nicotine. The results suggest phorbol ester-sensitive protein kinase C isozymes do not play a major role in nicotinic stimulation of tyrosine hydroxylase activity or catecholamine secretion in chromaffin cells.

Keywords: Protein kinase C; CGP 41251; Chromaffin cell; Tyrosine hydroxylase; Catecholamine secretion; Nicotinic receptor

1. Introduction

Tyrosine hydroxylase (L-tyrosine, tetrahydropterine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2) is the rate-limiting enzyme for catecholamine biosynthesis (Zigmond et al., 1989). Its activity is regulated acutely by changes in the phosphorylation state of four serine residues, serines 8, 19, 31 and 40, near the N-terminal of the enzyme (Campbell et al., 1986; Haycock, 1990, 1993; Haycock and Wakade, 1992). In vitro, Ca^{2+}/calmodulin-dependent protein kinase II can phosphorylate Ser^{19} and Ser^{40}, while protein kinase C and protein kinase A can each phosphorylate Ser^{40} (Campbell et al., 1986; George et al., 1989; Funakoshi et al., 1991; see Haycock, 1993). Ser^{31} can be phosphorylated by two mitogen activated protein kinases (MAP-kinases), MAP-kinases 1 and 2 (Haycock et al., 1992). In addition, two kinases phosphorylated and activated by MAP-kinases can also phosphorylate tyrosine hydroxylase, MAP-kinase-activated protein kinase 1 on Ser^{40} and MAP-kinase-activated protein kinase 2 on both Ser^{19} and Ser^{40} (Sutherland et al., 1993). Phophorylation of Ser^{8} does not appear to be regulated in non-neoplastic cells, but can be mediated by a proline-directed protein kinase in vitro (Vulliet et al., 1989).

The contributions of these different kinases to tyrosine hydroxylase phosphorylation and activation in situ in intact cells, however, has been difficult to establish. There are three reasons for this. Firstly, the correlation between the phosphorylation of specific serines and the activity of tyrosine hydroxylase is not simple. For example, in vitro, tyrosine hydroxylase is phosphorylated on Ser^{40} by protein kinase A, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II, while tyrosine hydroxylase activity is increased after phosphorylation by protein kinase A but not following phosphorylation by protein kinase C or Ca^{2+}/calmodulin-dependent protein kinase II (Funakoshi et al., 1991). Secondly, the serines that get phosphorylated during activation of kinases in intact cells are different from those in vitro. For example, in vitro, protein kinase C phosphorylates Ser^{40} while in intact cells protein kinase C activation with phorbol esters also increases Ser^{31} phosphorylation, probably because protein kinase C activates MAP-kinases in the intact cells which can then phosphory-

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late Ser^{31} (Funakoshi et al., 1991; Haycock et al., 1992; Haycock, 1993). Finally, predicting the degree of tyrosine hydroxylase activation in intact cells from in vitro results is complicated. Phosphorylation of tyrosine hydroxylase changes its K_m for tyrosine and tetrahydrobiopterin, its V_{max}, its pH optimum and the K_i for inhibition of tyrosine hydroxylase by catecholamines (Zigmond et al., 1989), and the concentrations of tyrosine, tetrahydrobiopterin, protons and catecholamines in situ in intact cells is not accurately known.

Nicotinic stimulation of intact bovine adrenal chromaffin cells causes a Ca^{2+}-dependent activation of tyrosine hydroxylase and a concurrent Ca^{2+}-dependent increase in the phosphorylation of three of the serines, Ser^{19}, Ser^{31} and Ser^{40} (Haycock et al., 1982; Pocotte et al., 1986: Waymire et al., 1988; Haycock, 1993). Activation of protein kinase C in these cells also activates tyrosine hydroxylase (Houchi et al., 1985; Marley et al., 1996), and this activation is accompanied by increased phosphorylation of two of the same serines affected by nicotinic stimulation, Ser^{31} and Ser^{40} (Pocotte and Holz, 1986; Tachikawa et al., 1987; Haycock, 1993). Furthermore, nicotinic stimulation causes Ca^{2+}-dependent increases in diacylglycerol levels and in membrane-associated protein kinase C in chromaffin cells (Frye and Holz, 1984; TerBush and Holz, 1986), suggesting nicotinic receptors can activate protein kinase C in these cells. Depolarisation of chromaffin cells with K^+ mimics the effects of nicotinic stimulation on tyrosine hydroxylase phosphorylation and activation (Haycock et al., 1982; Haycock, 1993), and, in cells rendered deficient in protein kinase C by chronic phorbol ester treatment, tyrosine hydroxylase phosphorylation by K^+ is impaired (Cahill et al., 1989). Taken together, these findings suggest that protein kinase C may play a role in the nicotinic stimulation of tyrosine hydroxylase activity in intact adrenal chromaffin cells.

In the present study, we have used a membrane-permeant, potent, selective inhibitor of protein kinase C, CGP 41251 (31-benzoyl-staurosporine), to investigate further the possible role of protein kinase C in tyrosine hydroxylase activation in cultured bovine adrenal chromaffin cells. The indolocarbazole compound CGP 41251 is a derivative of the potent but non-selective protein kinase inhibitor staurosporine. CGP 41251 inhibits protein kinase C competitively with respect to ATP with an IC_{50} of about 50 nM against purified porcine brain protein kinase C (Meyer et al., 1989). It has varying potency for different protein kinase C isozymes, having IC_{50} values of 17–60 nM for the classical and some of the novel isozymes, but being inactive against protein kinase Cξ (Marte et al., 1994). It has little selectivity for protein kinase C over phosphorylase kinase, but is some 20–40-fold selective for protein kinase C over protein kinase A and even more selective over S6 kinase and the tyrosine kinase activity of the epidermal growth factor receptor (Meyer et al., 1989). Using this compound, we have investigated the role of protein kinase C in the nicotinic activation of tyrosine hydroxylase. We have also assessed the possible involvement of protein kinase C in nicotinic stimulation of catecholamine secretion, since phorbol esters have been reported to enhance Ca^{2+}-dependent exocytosis in these cells (Knight and Baker, 1983; Burgoyne et al., 1988; Bittner and Holz, 1990).

2. Materials and methods

2.1. Measurement of tyrosine hydroxylase activity in intact cultured bovine chromaffin cells

Bovine adrenal chromaffin cells were isolated by collagenase digestion and cultured at a density of 0.5×10^6/well in rat tail collagen-coated 24-well tissue culture plates as described by Livett et al. (1987b). Cells were used 2–4 days after plating. Tyrosine hydroxylase activity was measured at 37°C in intact cells still attached to the culture plates using a modification (Cheah et al., 1995) of methods described for cell suspensions by Meligeni et al. (1982) and Marley et al. (1995b). The method measures the production of $^{14}CO_2$ from the hydroxylation and rapid decarboxylation of labelled tyrosine offered to the cells.

Culture medium was aspirated from each well and the cells received two 10 min washes in 400 μl Hepes-buffered saline (HBS) solution (composition in mM: NaCl 150, Hepes 15, D-glucose 5.5, K_2HPO_4 3.8, $MgSO_4$ 1, $CaCl_2$ 1.5, sodium ascorbate 0.5, supplemented with 0.5% BSA, pH 7.4). After aspiration of the second wash solution, cells were exposed for 10 min to agonists in 200 μl HBS containing 10 μM L-[carboxyl-^{14}C]tyrosine (radioactive concentration either 0.59 or 0.3 μCi/ml). In experiments using elevated K^+ concentrations to stimulate the cells, the stated concentration of KCl was used to replace an equimolar amount of NaCl in the HBS solution. Each well was immediately fitted with a 60×15.9 mm acrylic cylinder smeared around the base with Vaseline to form an airtight seal to the well. Each acrylic cylinder was capped with a rubber stopper below which was suspended a plastic cup containing 200 μl of 1 M NaOH to absorb the $^{14}CO_2$ produced by the cells. After 10 min exposure to agonists, the reaction was terminated by injecting 200 μl of ice-cold 10% trichloroacetic acid through the rubber stopper to lyse the cells. The plates with the acrylic tubes and rubber stoppers still fitted were then left for > 2 h to allow the NaOH to absorb the emitted $^{14}CO_2$. An aliquot of the NaOH was then removed from each plastic cup for determination of [^{14}C] by scintillation counting in Emulsifier-SAFE scintillant (Packard).

For experiments with kinase inhibitors, a pre-incubation step was used between the two washes and the agonist treatment. After aspirating the second wash solution, 200 μl of kinase inhibitor or appropriate solvent were added to each well in HBS. After 15 min, the preincubation solution

was aspirated off the cells and replaced with 200 μl HBS containing agonist, 10 μM [^{14}C]tyrosine and kinase inhibitor or solvent, as appropriate. Wells were fitted with acrylic cylinders for trapping emitted $^{14}CO_2$ as described above.

2.2. Catecholamine release

Catecholamine secretion was measured at 37°C over a 10 min period. Cells cultured in 24-well culture plates were given two 10 min washes in 400 μl HBS solution. They then received a 15 min preincubation with 400 μl HBS containing kinase inhibitors or solvent. After aspiration of the preincubation solution, they were treated for 10 min with 400 μl of HBS containing agonist together with kinase inhibitor or solvent. After a 10 min stimulation period, the incubation solution was collected and acidified to 0.4 M with perchloric acid, and the remaining cellular catecholamines extracted into 0.4 M perchloric acid. Endogenous adrenaline and noradrenaline were measured by high pressure liquid chromatography with electrochemical detection, essentially as described by Livett et al. (1987a).

2.3. Measurement of tyrosine hydroxylase activity in vitro with recombinant enzyme

The direct effect of inhibitors on tyrosine hydroxylation by tyrosine hydroxylase was measured essentially as described by Reinhard et al. (1986). The reaction volume was 100 μl and contained: 0.054 mg/ml catalase, 16.6 μM β-mercaptoethanol, 3.33 mM potassium phosphate buffer pH 7.0, 20 μM L-[3,5-^{3}H]tyrosine (radioactive concentration 0.83 μCi/ml), 40 μM $FeSO_4$, 100 μM tetrahydrobiopterin ((6*R*)-5,6,7,8-tetrahydro-L-biopterin dihydrochloride), 92.5 μg/ml rat full-length recombinant tyrosine hydroxylase expressed in *E. coli* (approximately 95% pure; Quinsey et al., 1996), and 1% dimethylsulphoxide containing the inhibitors. The tyrosine hydroxylase enzyme was preincubated in the above reaction mixture without tetrahydrobiopterin for 6 min at room temperature before the reaction was started by the addition of tetrahydrobiopterin and transfer of the tubes to a 37°C water bath. After 6 min, the reaction was terminated by adding 700 μl of a 7.5% w/v suspension of activated charcoal in 1 M HCl and vortexing. Samples were then spun (5 min, $9000 \times g$), the supernatant removed and respun, and an aliquot of the final supernatant taken for determination of ^3H_2O by scintillation counting in EcoLITE scintillant (ICN Biomedicals).

2.4. Data presentation and statistics

Tyrosine hydroxylase activity in intact chromaffin cells was determined as cpm $^{14}CO_2$ produced/10^6 cells/10 min, since determination of the counting efficiency for gaseous $^{14}CO_2$ trapped in aqueous NaOH is technically demanding. Basal tyrosine hydroxylase activity varied greatly between different cell preparations, in the range 1244–12754 cpm/10^6 cells/10 min (5526 ± 314 cpm/10^6cells/10 min, mean ± S.E.M. for 81 experiments from 25 cell preparations). Consequently, the tyrosine hydroxylase activity for each experiment has been expressed as a percentage of the control basal activity for that experiment with the basal activity given in the figure legends. Results for the in vitro tyrosine hydroxylase assay with recombinant tyrosine hydroxylase were expressed as pmol tyrosine hydroxylated/9.25 μg tyrosine hydroxylase enzyme/6 min. Catecholamine release is presented as percentage cell content released/10 min. All results are means ± S.E.M. for the stated number of observations from a single preparation of cells and are representative of similar results from the stated number of cell preparations. Multiple comparisons were made using either Tukey-Kramer or Dunnett's tests protected by a one-way analysis of variance (ANOVA). A P value of < 0.05 was taken as indicating statistical significance. P values are shown in the figures only for selected comparisons.

2.5. Materials

CGP 41251 (31-benzoyl-stauropsorine) and CGP 42700 (19,31-dibenzoyl-staurosporine) were generous gifts from Frau A. Sedlacek (Ciba Ceigy AG, Switzerland). Nicotine, phobol 12,13-dibutryrate (PDB), 8-Br-cyclic AMP (sodium salt) and tyrosine (di-sodium salt) were from Sigma Chemical Co., USA. Forskolin was from Calbiochem-Novobiochem Pty, Australia. L-[Carboxyl-^{14}C]tyrosine (specific activity 59 mCi/mmol, 2.18 GBq/mmol) and L-[3,5-^{3}H]tyrosine (specific activity 51 Ci/mmol, 1.88 TBq/mmol) were from Amersham Life Sciences, Australia. Biosynthetic recombinant rat full-length tyrosine hydroxylase enzyme was a generous gift from Dr Phillip Dickson and Noellene Quinsey (Department of Biochemistry and Molecular Biology, University of Melbourne). It was synthesised in *E. coli* using a PET3A plasmid and purified by heparin sepharose and Q sepharose column (Quinsey et al., 1996). (6*R*)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride was a generous gift from Dr B. Schrichs (Switzerland).

Forskolin, PDB, CGP 41251 and CGP 42700 were dissolved in dimethylsulphoxide and then diluted in HBS as required. Appropriate solvent (vehicle) controls were performed in every experiment.

3. Results

3.1. Effect of CGP 41251 on phorbol ester activation of tyrosine hydroxylase

Phorbol 12,13-dibutyrate (PDB) produced a concentration-dependent increase in tyrosine hydroxylase activity

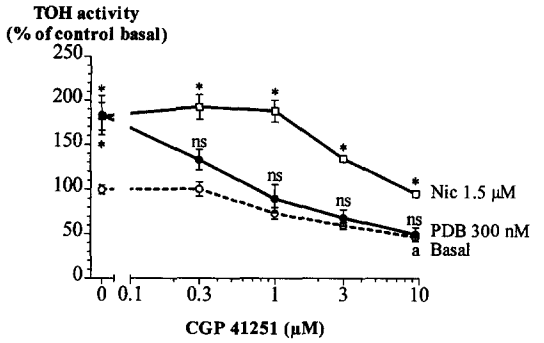

Fig. 1. Effect of CGP 41251 on basal tyrosine hydroxylase activity or on TOH activity stimulated by 300 nM phorbol 12,13-dibutyrate (PDB:) or 1.5 μM nicotine (Nic). Open circles and dotted line: basal. Solid circles and line: PDB. Open squares and solid line: nicotine. Results are means ± S.E.M. for $n = 3$–5 from a single preparation of cells and are representative of similar data from 3 cell preparations. Control basal TOH activity was 2831 (±143) cpm $^{14}CO_2$ produced/10 min/10^6 cells. ^{a} $P < 0.01$ compared with control basal in absence of CGP 41251; * $P < 0.001$, ^{ns} $P > 0.05$ compared with corresponding basal TOH activity in absence of agonist – Tukey-Kramer multiple comparison test.

with maximal stimulation at 3 μM and an EC_{50} of about 300 nM (data not shown). PDB was used at a concentration of 300 nM for all further experiments. CGP 41251 produced a concentration-dependent inhibition of PDB-stimulated tyrosine hydroxylase activity with 0.3 μM causing more than 50% inhibition (Fig. 1). In the presence of 1 μM CGP 41251, PDB failed to stimulate tyrosine hydroxylase activity in any of three experiments.

At concentrations > 1 μM, CGP 41251 also inhibited basal tyrosine hydroxylase activity (see Fig. 1 and Fig. 2). At 1 μM, CGP 41251 had no significant effect on basal tyrosine hydroxylase activity in 11 of 15 cell preparations, but at 10 μM, basal tyrosine hydroxylase was inhibited by 56.1 ± 3.6% (mean ± S.E.M. from 12 cell preparations).

3.2. Effect of CGP 41251 on nicotinic activation of tyrosine hydroxylase and catecholamine secretion

Nicotine also produced a concentration-dependent increase in tyrosine hydroxylase activity with a maximal response at 10 μM and an EC_{50} of about 2 μM (data not shown). Nicotine was used in subsequent experiments at either 2 or 1.5 μM, which caused 67.5 ± 13.0% ($n = 3$ cell preparations) or 56.6 ± 11.0% ($n = 4$ cell preparations) of the response to 10 μM nicotine, respectively. CGP 41251 caused a concentration-dependent inhibition of the tyrosine hydroxylase activation produced by nicotine (Fig. 1), however this differed from the inhibition of the PDB response. The nicotinic response was unaffected or only weakly inhibited by 0.3 or 1 μM CGP 41251, and nicotine still significantly stimulated tyrosine hydroxylase activity even in the presence of 3 μM CGP 41251 in each of three experiments. At 3 μM CGP 41251, the nicotinic response was reduced by about 50% and at 10 μM by about 70%.

At concentrations up to 10 μM, CGP 41251 had no effect on basal adrenaline or noradrenaline secretion or on catecholamine secretion evoked by 1.5 μM nicotine: adrenaline secretion (% cell content/10 min, $n = 7$–8)): basal 0.26 (±0.05), 10 μM CGP 41251 0.23 (±0.05), nicotine 2.0 (±0.10), nicotine with 10 μM CGP 41251 2.0 (±0.05); noradrenaline secretion: basal 0.22 (±0.04),

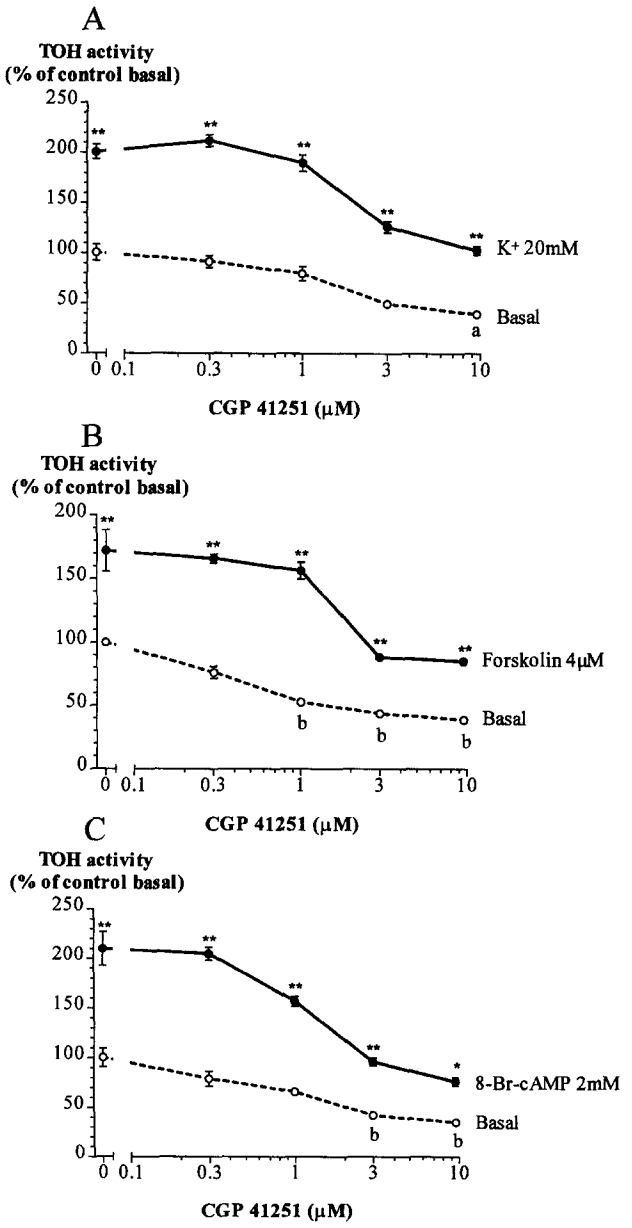

Fig. 2. Effect of CGP 41251 on basal TOH activity or on TOH activity stimulated by 20 mM extra K^+ (A), 4 μM foskolin (B) or 2 mM 8-Br-cyclic AMP (C). Open symbols and dotted lines: basal. Solid symbols and lines: in the presence of agonists. Results are means ± S.E.M. for $n = 3$–5 from a single cell preparation each, and are representative of similar data from 2 (A) or 3 (B and C) cell preparations. Control basal TOH activities were (cpm $^{14}CO_2$ produced/10 min/10^6 cells): (A) 7160 (±580), (B) 8026 (±47), and (C) 5565 (±516). ^{a} $P < 0.05$, ^{b} $P < 0.001$ compared with control basal in the absence of CGP 41251; * $P < 0.05$, ** $P < 0.001$ compared with corresponding basal activity without agonist – Tukey-Kramer multiple comparison test.

10 μM CGP 41251 0.21 (±0.03), nicotine 1.4 (±0.11), nicotine with 10 μM CGP 41251 1.2 (±0.04).

3.3. Effects of CGP 41251 on tyrosine hydroxylase activation by other agents

CGP 41251 was tested against other agonists which stimulate tyrosine hydroxylase activity, in an attempt to identify its intracellular site of action. High extracellular K^+ stimulates tyrosine hydroxylase phosphorylation on the same three serine residues as nicotine (see Introduction) and causes a Ca^{2+}-dependent activation of tyrosine hydroxylase (Haycock et al., 1982; Haycock, 1993). Elevated K^+ stimulated tyrosine hydroxylase activity with an EC_{50} of 15–20 mM (data not shown). Using either 15 or 20 mM additional K^+, CGP 41521 caused a concentration-dependent inhibition of tyrosine hydroxylase activation but only at concentrations > 1 μM (Fig. 2A). The inhibition was less than 50% even at 10 μM CGP 41251.

Forskolin stimulates tyrosine hydroxylase phosphorylation and activity exclusively through protein kinase A, which phosphorylates Ser^{40} (George et al., 1989; Haycock, 1993; Marley et al., 1995b). In the present experiments, tyrosine hydroxylase activation by 100 μM forskolin was taken as maximal. Forskolin at 4 μM stimulated tyrosine hydroxylase to 45.9 ± 7.8% of this level (data from 5 cell preparations: data not shown). At concentrations > 1 μM, CGP 41251 produced a concentration-dependent inhibition of tyrosine hydroxylase activation by 4 μM forskolin (Fig. 2B). The inhibition reached 50% at 10 μM CGP 41251 (mean from 3 cell preparations).

Since CGP 41251 is an ATP binding site inhibitor (see Introduction), the inhibition of the response to forskolin may be due to inhibition of adenylate cyclase which also has an ATP binding site. To bypass adenylate cyclase, the membrane-permeant cyclic AMP analogue 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cyclic AMP) was used. A concentration of 2 mM 8-Br-cyclic AMP produced 57.3 ± 10.2% of the response to 100 μM forskolin (data from 3 cell preparations; data not shown). The effect of CGP 41251 on tyrosine hydroxylase stimulation by 2 mM 8-Br-cyclic AMP was very similar to its effects on the responses to nicotine, K^+ and forskolin with no inhibition at concentrations < 1 μM and only 65% inhibition at 10 μM (Fig. 2C).

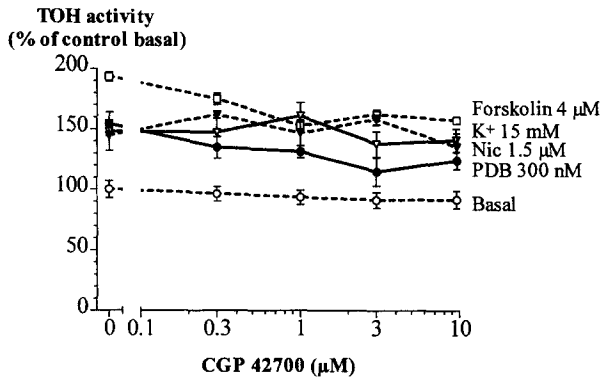

Fig. 3. Effect of CGP 42700 on basal TOH activity and TOH activity stimulated with 300 nM PDB, 1.5 μM nicotine (Nic), 15 mM extra K^+ or 4 μM forskolin. Open circles and dotted line: basal. Solid circles and line: PDB. Solid triangles and dotted line: Nic. Open triangles and solid line: K^+. Open squares and dotted line: forskolin. Data are means ± S.E.M. for $n = 4–5$ from a single preparation of cells each and are respresentative of similar data from 2 or 3 cell preparations. Control basal TOH activity was 6372 (±451) cpm $^{14}CO_2$ produced/10 min/10^6 cells. CGP 42700 had no significant effect on basal or any of the agonist responses ($P > 0.05$ for one-way ANOVA on basal or on each agonist-stimulated response).

Table 1
Effect of CGP 41251, CGP 42700 and α-methyl-*p*-tyrosine on the in vitro activity of rat recombinant full-length tyrosine hydroxylase

Condition	Tyrosine hydroxylase activity (pmol tyrosine hydroxylated /9.25 μg TOH/6 min) mean (±S.E.M.)	*n*
Control	1326 (±35)	10
CGP 41251 (3 μM)	1352 (±48)	7
CGP 41251 (10 μM)	1422 (±36)	8
CGP 42700 (3 μM)	1352 (±36)	8
CGP 42700 (10 μM)	1444 (±55)	8
α-Methyl-*p*-tyrosine (10 μM)	419 (±62) *	4
α-Methyl-*p*-tyrosine (100 μM)	134 (±30) *	4

* $P < 0.01$ compared with control (Dunnett's multiple comparison test).

At concentrations up to 10 μM, CGP 42700 (19,31-dibenzoyl-staurosporine), a structural analogue of CGP 41251 that lacks activity as a protein kinase C inhibitor (Meyer et al., 1989; Marte et al., 1994), had no significant effect on basal tyrosine hydroxylase activity or on tyrosine hydroxylase activation produced by PDB, nicotine, forskolin or K^+ (Fig. 3). Neither CGP 41251 nor CGP 42700 had any effect on in vitro hydroxylation of tyrosine by recombinant tyrosine hydroxylase at concentrations up to 10 μM (Table 1). In contrast the well-characterised tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine readily inhibited the recombinant enzyme in vitro (Table 1).

4. Discussion

The present study shows that nicotine-stimulated tyrosine hydroxylase activation and catecholamine secretion are unaffected by CGP 41251 at concentrations that fully block phorbol ester activation of tyrosine hydroxylase. This suggests that phorbol ester-sensitive isozymes of protein kinase C do not play a substantial role in either of these nicotinic responses in bovine chromaffin cells.

4.1. Protein kinase C and tyrosine hydroxylase activation

CGP 41251 inhibited PDB-stimulated tyrosine hydroxylase activity with an IC_{50} close to 0.3 μM (Fig. 1). This

effect of CGP 41251 is likely to be due to protein kinase C inhibition for four reasons. Firstly, its action was not mimicked by CGP 42700 (Fig. 3), a structurally similar indolocarbazole that is not a protein kinase C inhibitor (Meyer et al., 1989; Marte et al., 1994). Secondly, at concentrations that fully blocked the phorbol ester response, CGP 41251 had no effect on tyrosine hydroxylase activation by forskolin or 8-Br-cyclic AMP, two agents that activate tyrosine hydroxylase exclusively through protein kinase A activation (George et al., 1989; Haycock, 1993; Marley et al., 1995b). This indicates that at these concentrations CGP 41251 does not inhibit protein kinase A, nor does it inhibit tyrosine transport into chromaffin cells or the tyrosine hydroxylase enzyme itself. Thirdly, the effects of CGP 41251 on the phorbol ester response were mimicked by two other protein kinase C inhibitors, Ro 31-8220 and bisindolylmaleimide I (Marley and Thomson, 1996; see Marley et al., 1994, 1995a).

Finally, the potency of CGP 41251 in inhibiting the PDB response was similar to that reported previously for CGP 41251 effects on other intact cells. CGP 41251 inhibited phorbol ester responses of human monocytes with an IC_{50} of 0.15 μM (Meyer et al., 1989), reversed multidrug resistance in lymphoblastoid multidrug resistant cells with an IC_{50} of ~0.2 μM (Utz et al., 1994) and inhibited proliferation of human bladder carcinoma cells, human promyelocytic leukemia cells and bovine corneal endothelial cells with an IC_{50} of 0.2 μM, 0.3 μM and 0.2 μM, respectively (Meyer et al., 1989). In contrast, much higher CGP 41251 concentrations of 10 μM were required to inhibit phorbol ester-induced *c-fos* mRNA expression in mouse epidermal keratinocytes (Andrejauskas-Buchdunger and Regenass, 1992). The different potency of CGP 41251 against different responses may be due to the protein kinase C isozyme involved in these responses: CGP 41251 is more potent against the classical protein kinase C isozymes α, βI, βII, and γ and the novel isozyme protein kinase Cη than against other novel isozymes such as protein kinase Cδ or ε and is almost inactive against the atypical isozyme protein kinase Cξ (Marte et al., 1994). Alternatively, it could be because protein kinase C produces some cellular effects by acting in the cytosol and others by acting in a membrane environment: CGP 41251 is more potent against cytosolic protein kinase C than the membrane-associated kinase (Budworth and Gescher, 1995).

The failure of CGP 41251 to inhibit the nicotinic activation of tyrosine hydroxylase at concentrations that fully blocked the phorbol ester response suggests that phorbol ester-sensitive classical and novel isozymes of protein kinase C do not play a substantial role in this nicotinic response. Previous studies have shown that phorbol ester treatment stimulated tyrosine hydroxylase phosphorylation on two of the same serine residues phosphorylated during nicotinic stimulation or K^+ depolarisation (Haycock, 1993). Chronic phorbol ester treatment, which reduced protein kinase C activity by 90%, greatly reduced the phosphorylation of tyrosine hydroxylase by K^+ depolarisation (Cahill et al., 1989), suggesting protein kinase C may be involved in tyrosine hydroxylase activation by depolarising stimuli such as K^+ and nicotine. However, chronic phorbol ester treatment has numerous effects on chromaffin cells due to the long-term activation of protein kinase C that precedes its down-regulation. Indeed, in chromaffin cells treated for 24 h with 1 μM PDB, we found that forskolin was unable to activate tyrosine hydroxylase (Marley et al., 1996), suggesting the effects of chronic phorbol ester treatment are not restricted to impairing the function of protein kinase C.

Higher concentrations of CGP 41251, from 1 to 10 μM, did substantially inhibit tyrosine hydroxylase activation by nicotine, but at these concentrations CGP 41251 also inhibited the responses to K^+, forskolin and 8-Br-cyclic AMP, and reduced basal tyrosine hydroxylase activity. In each case the IC_{50} was 3–10 μM. The inhibition of nicotinic tyrosine hydroxylase activation at these higher concentrations is not due to non-specific actions of CGP 41251 inhibiting the tyrosine hydroxylase enzyme directly (Table 1) or to inhibition of nicotinic receptors or blocking calcium channels (which are required for nicotinic activation of tyrosine hydroxylase – see Haycock et al., 1982; Pocotte and Holz, 1986), since CGP 41251 did not block catecholamine secretion by nicotinic receptors, and the responses to forskolin and 8-Br-cyclic AMP do not require extracellular Ca^{2+} (Marley et al., 1996). These effects are likely to be due to inhibition of protein kinase A by these higher CGP 41251 concentrations, since selective, potent, membrane-permeant protein kinase A inhibitors mimic all of these effects, including the suppression of basal tyrosine hydroxylase activity (Marley et al., 1995b), and because CGP 41251 is known to be a weak inhibitor of protein kinase A (Meyer et al., 1989). CGP 42700, which not only lacks activity as a protein kinase C inhibitor but is also inactive as a protein kinase A inhibitor (Meyer et al., 1989), did not affect tyrosine hydroxylase activation by nicotine, K^+, forskolin or 8-Br-cyclic AMP, or basal tyrosine hydroxylase activity.

If protein kinase C does not mediate nicotinic stimulation of tyrosine hydroxylase activation, alternative signaling pathways must be involved. We have previously shown that the nicotinic response has a complete dependence on protein kinase A activity (Marley et al., 1995b). Since protein kinase A phosphorylates Ser^{40} and this phosphorylation alone is adequate to activate tyrosine hydroxylase, the activation of tyrosine hydroxylase by nicotinic agonists is possibly mediated by protein kinase A. However, nicotinic stimulation also increases phosphorylation on Ser^{19} and Ser^{31} (see Introduction). It is possible that these phosphorylations are mediated by a Ca^{2+}-dependent activation of the ras/Raf/MEK/MAP kinase pathway (Rosen et al., 1994; Farnsworth et al., 1995), that could result in phosphorylation of these serines (Haycock et al., 1992;

Sutherland et al., 1993). In addition, Ca^{2+}/calmodulin-dependent protein kinase II could mediate the phosphorylation on Ser^{19} and contribute to tyrosine hydroxylase activation since two Ca^{2+}/calmodulin-dependent protein kinase II inhibitors, KN62 and KN93, have been reported to partially inhibit tyrosine hydroxylase activation by K^+ depolarisation and acetylcholine in rat PC12h cells (Ishii et al., 1991; Sumi et al., 1991). However, these drugs have non-specific actions that complicate the interpretation of these results (Marley and Thomson, 1996; Maurer et al., 1996).

4.2. CGP 41251 and catecholamine secretion

Previous studies have shown that phorbol esters can enhance Ca^{2+}-dependent exocytosis from bovine adrenal chromaffin cells (see Burgoyne, 1991). In isolated, perfused rat adrenal glands, catecholamine secretion in response to nicotine was more than doubled by 30 nM PDB (Wakade et al., 1986), while in permeabilised bovine chromaffin cells phorbol esters enhanced secretion evoked by Ca^{2+} (Knight and Baker, 1983; Pocotte et al., 1985; Bittner and Holz, 1990). Inhibition of protein kinase C by the non-specific kinase inhibitor staurosporine or by down-regulating protein kinase C by chronic phorbol ester treatment was previously shown to reduce Ca^{2+}-dependent secretion from permeabilised chromaffin cells, but the more selective pseudosubstrate protein kinase C inhibitor had little effect (see Burgoyne, 1991). The present results agree with these latter findings since high concentrations of CGP 41251 had no effect on this response. We have also found that another potent and selective protein kinase C inhibitor, bisindolylmaleimide I, had no effect on this nicotinic response (Marley and Thomson, unpublished observation). These data suggest that phorbol ester-sensitive isozymes of protein kinase C do not play a significant role in nicotine-evoked catecholamine secretion.

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