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Inhibition of nicotinic responses of bovine adrenal chromaffin cells by the protein kinase C inhibitor, Ro 31-8220

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1 The effects of the protein kinase C inhibitor, Ro 31-8220, on the responses of cultured bovine adrenal chromaffin cells to nicotine, phorbol 12,13-dibutyrate (PDBu) and K^+ have been investigated.

2 Tyrosine hydroxylase activity was measured *in situ* in intact cells by measuring $^{14}CO_2$ evolved following the hydroxylation and rapid decarboxylation of [^{14}C]-tyrosine offered to the cells. Secretion of endogenous adrenaline and noradrenaline was measured by use of h.p.l.c. with electrochemical detection. Cyclic AMP levels were measured in cell extracts by RIA.

3 Ro 31-8220 produced a concentration-dependent inhibition of 300 nM PDBu-stimulated tyrosine hydroxylase activity with an IC_{50} of <2 μM and complete inhibition at 10 μM. It had no effect on the responses to forskolin.

4 Ro 31-8220 produced a concentration-dependent inhibition of 5 μM nicotine-stimulated tyrosine hydroxylase activity, adrenaline and noradrenaline secretion and cellular cyclic AMP levels, with an IC_{50} of about 3 μM and complete inhibition by 10 μM. At concentrations up to 10 μM, Ro 31-8220 had little or no effect on the corresponding responses to 50 mM K^+.

5 A structural analogue of Ro 31-8220, bisindolylmaleimide V, that lacks activity as a protein kinase C inhibitor, had no effect up to 10 μM on PDBu-stimulated tyrosine hydroxylase activity or on nicotine-stimulated cyclic AMP levels or noradrenaline secretion and only marginal inhibitory effects on nicotine-stimulated tyrosine hydroxylase activity and adrenaline secretion.

6 A structurally related protein kinase C inhibitor, bisindolylmaleimide I, inhibited PDBu-stimulated tyrosine hydroxylase activity with an IC_{50} of <1 μM and complete inhibition by 3 μM, but had essentially no effect on nicotine stimulated tyrosine hydroxylase activity or catecholamine secretion.

7 The results suggest that Ro 31-8220 is not only a protein kinase C inhibitor but is also a potent inhibitor of nicotinic receptor responses in adrenal chromaffin cells by a mechanism unrelated to protein kinase C inhibition. The results are consistent with Ro 31-8220 being a nicotinic receptor antagonist.

Keywords: Ro 31-8220; kinase inhibitors; chromaffin cells; nicotinic cholinoceptors; tyrosine hydroxylase; protein kinase C; nicotinic antagonists; bisindolylmaleimide

Introduction

Nicotinic stimulation of adrenal chromaffin cells causes acute activation of tyrosine hydroxylase (TOH), the rate-limiting enzyme for catecholamine biosynthesis (Haycock *et al.*, 1982; Zigmond *et al.*, 1989). This activation is due to increased phosphorylation of three serine residues in the N-terminal regulatory domain of TOH (Haycock & Wakade, 1992; Haycock, 1993). The increased phosphorylation of all three serine residues and the activation of TOH produced by nicotinic agonists are totally dependent on extracellular Ca^{2+} (Haycock *et al.*, 1982; Pocotte *et al.*, 1986; Waymire *et al.*, 1988). At present, it is not clear which kinases mediate the increased phosphorylations on the three serine residues and which of these phosphorylations are responsible for activating TOH.

Stimulation of chromaffin cells and PC12 cells with phorbol esters that activate protein kinase C (PK-C) also activates TOH (Pocotte & Holz, 1986; Tachikawa *et al.*, 1987; Haycock, 1990). This activation is accompanied by an increase in phosphorylation of two of the same serine residues in TOH that are phosphorylated by nicotinic stimulation (Haycock, 1993). Furthermore, nicotinic stimulation of chromaffin cells causes a Ca^{2+}-dependent activation of PK-C (TerBush & Holz, 1986). These findings suggest that PK-C may be one of the kinases involved in mediating the phosphorylation and activation of TOH by nicotinic stimulation.

We have used potent, selective, membrane permeant inhibitors of PK-C to investigate the role of PK-C in the nicotinic activation of TOH in intact bovine adrenal chromaffin cells. One of these PK-C inhibitors, Ro 31-8220, is a bisindolylmaleimide compound structurally related to the indolocarbazole staurosporine (Davis *et al.*, 1989; 1992b; Elliott *et al.*, 1990). Ro 31-8220 acts competitively with respect to ATP to inhibit purified rat brain PK-C *in vitro* with an IC_{50} of 10 nM (in the presence of 10 μM ATP: Elliott *et al.*, 1990; Davis *et al.*, 1992b), and shows little selectivity for different PK-C isozymes (Wilkinson *et al.*, 1993). It is more than 100 fold selective for PK-C compared with protein kinase A (PK-A) or Ca^{2+}/calmodulin dependent kinases such as phosphorylase kinase or Ca^{2+}/calmodulin-dependent protein kinase II (Davis *et al.*, 1989; Elliott *et al.*, 1990). We have investigated the effects of Ro 31-8220 on nicotinic responses of bovine chromaffin cells and have found it has actions that are not related to its ability to inhibit PK-C. Preliminary accounts of this work have been published (Marley *et al.*, 1994; 1995a).

Methods

Measurement of TOH activity in bovine chromaffin cells

Bovine adrenal chromaffin cells were isolated as described by Livett *et al.* (1987b) and cultured for two to four days at a density of 55×10^6 cells per 35 ml in 650 ml culture flasks. Cells were harvested, washed and used for determination of TOH activity *in situ* in the intact cells as described previously (Marley *et al.*, 1995b). The assay measures the production of $^{14}CO_2$ following the hydroxylation and rapid decarboxylation

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of L-[carboxyl-^{14}C]-tyrosine offered to the cells (see Meligeni 1982; Marley *et al.*, 1995b). TOH activity was measured at 37°C over a 10 min period in the presence of agonists. Kinase inhibitors or vehicle were present for a 15 min preincubation period and during the 10 min stimulation period. Stimulation with elevated K^+ concentrations was performed by replacing 50 mM of the NaCl in the buffer with 50 mM KCl.

Catecholamine secretion and cellular cyclic AMP responses in bovine chromaffin cells

Bovine chromaffin cells were prepared as described by Livett *et al.* (1987b) and cultured in 24 well or 6 well tissue culture plates for measurement of catecholamine secretion or cellular cyclic AMP responses, respectively. Cells were used two to four days after plating. Cell washes and incubations were performed in the same HEPES-buffered saline (HBS) solution used for the TOH assays (Marley *et al.*, 1995b). Catecholamine secretion and cellular cyclic AMP responses were measured at 37°C over a 10 min period in the presence of agonists. Kinase inhibitors or vehicle were present for a 15 min preincubation period as well as during the 10 min stimulation period. Endogenous catecholamines secreted into the incubation buffer and in cell extracts were measured by h.p.l.c. with electrochemical detection, essentially as described previously (Livett *et al.*, 1987a). Cellular cyclic AMP levels were determined in cell extracts by radioimmunoassay as described in detail previously (Marley *et al.*, 1991).

Data presentation and statistics

TOH activity is presented as a % of the basal $^{14}CO_2$ produced over a 10 min period, which was typically 500–2000 c.p.m. 10 min^{-1} per 0.6×10^6 cells. Catecholamine release is expressed as % of cell content released per 10 min. Cellular cyclic AMP levels are expressed as fmol cyclic AMP per 10^6 cells. All results are presented as mean ± s.e.mean for the stated number of determinations from a single preparation of cells. The number of cell preparations on which similar observations were made is given in the figure legends. Statistical significance for multiple comparisons has been assessed by the modified *t*-statistic provided by Fisher's least significant difference (LSD) test, protected by a one-way analysis of variance (ANOVA). In all cases, a P value of <0.05 was taken to indicate statistical significance.

Drugs

Some of the Ro 31-8220 (also known as bisindolylmaleimide IX) used in this study was a generous gift from Dr G. Lawton, Roche Products Ltd, UK. Bisindolylmaleimide I (also known as GF 109203X and Gö 6850), bisindolylmaleimide V (also known as Ro 31-6045), forskolin and the remaining Ro 31-8220 were from Calbiochem-Novobiochem Pty Ltd, Australia. Nicotine and phorbol 12,13-dibutyrate were from Sigma Chemical Co, U.S.A. L-[carboxyl-^{14}C]-tyrosine, specific activity 2.18 GBq $mmol^{-1}$, was from Amersham International Plc, U.K. Ro 31-8220, bisindolylmaleimides I and V, phorbol 12,13-dibutyrate and forskolin were each dissolved in dimethylsulphoxide before dilution in buffer. Appropriate solvent (vehicle) controls were performed in every experiment.

Results

Effects of Ro 31-8220 on responses to phorbol ester

Ro 31-8220 produced a concentration-dependent inhibition of TOH activation by the PK-C activator phorbol 12,13-dibutyrate (PDBu, Figure 1a). Ro 31-8220 had an IC_{50} of <2 μM and inhibited the PDBu response by 73.5 ± 4.7% and by 87 ± 3.7% at 3 and 10 μM, respectively (mean from 4 cell preparations). It had no significant effect on basal TOH activity at concentrations up to 3 μM and at 10 μM weakly inhibited basal activity in only 3 out of 15 experiments (compare Figures 1a, 2a and 2b).

The specificity of the action of Ro 31-8220 in inhibiting the phorbol ester response was assessed in two ways. Firstly, it was tested for its effect on protein kinase A, by using forskolin to activate TOH. Forskolin is known to stimulate cyclic AMP formation by adenylate cyclase, and phosphorylates and activates TOH exclusively through stimulation of PK-A (see George *et al.*, 1989; Haycock, 1993; Marley *et al.*, 1995b). However, at concentrations up to 10 μM, Ro 31-8220 had no

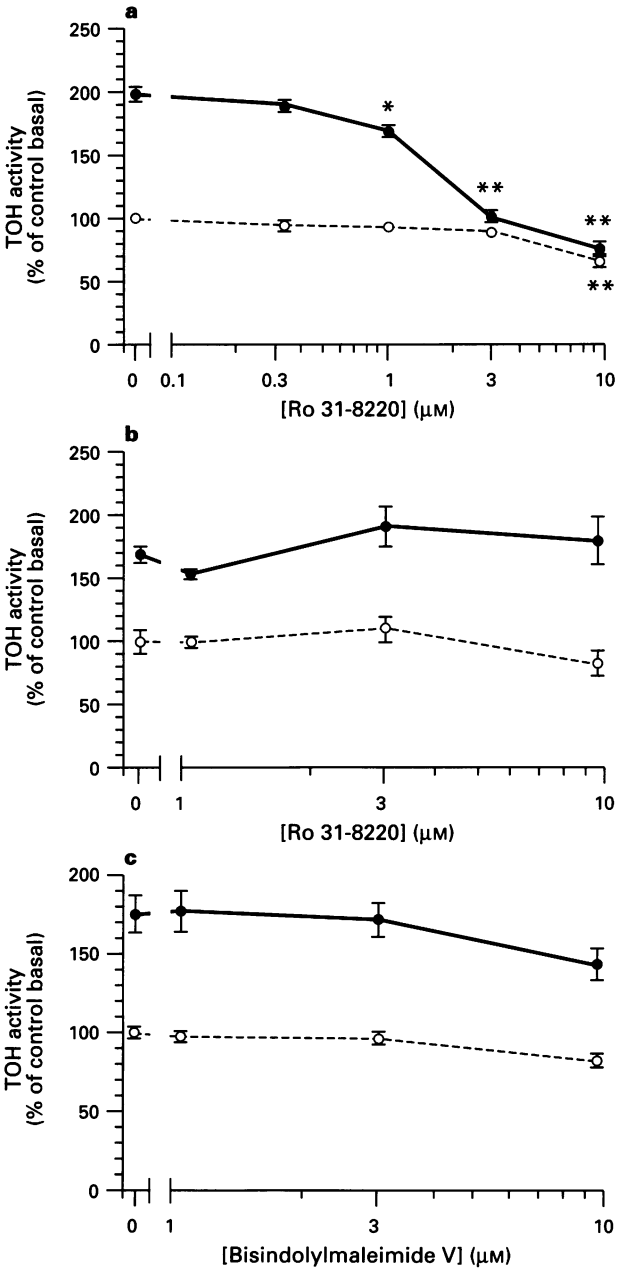

Figure 1 Effects of Ro 31-8220 (a,b) or bisindolylmaleimide V (c) on tyrosine hydroxylase activity in the absence and presence of 300 nM phorbol 12,13-dibutyrate (PDBu: a,c) or 3 μM forskolin (b): (●—●): in the presence of PDBu or forskolin; (○- - -○) in their absence (controls). Results are mean ± s.e.mean for $n = 5-7$ from a single preparation of cells and are representative of similar data from 2–4 cell preparations. $^*P<0.05$, $^{**}P<0.01$ compared with the corresponding TOH activity without Ro 31-8220 (Fisher LSD test). In the presence of 10 μM Ro 31-8220, PDBu did not significantly stimulate TOH activity.

effect on the response to forskolin (Figure 1b). Secondly, another bisindolylmaleimide compound, bisindolylmaleimide V (also known as Ro 31-6045), that is structurally related to Ro 31-8220 but lacks activity as a PK-C inhibitor (Elliott *et al.*, 1990; Twomey *et al.*, 1990), was tested for its effects on the PDBu stimulation of TOH activity. At concentrations up to 10 μM, bisindolylmaleimide V had no significant effects on PDBu-stimulated TOH activity (Figure 1c).

Effects of Ro 31-8220 on responses to nicotine and K^+

Ro 31-8220 produced a concentration-dependent inhibition of 5 μM nicotine-stimulated TOH activity (Figure 2a). It had an IC_{50} of about 3 μM and inhibited the nicotinic response by $56.3 \pm 8.2\%$ and $85 \pm 6.0\%$ at 3 and 10 μM, respectively ($n=6$ cell preparations). These values were not significantly different from the corresponding values with PDBu as the stimulant ($P>0.05$, Student's t test).

Depolarization of chromaffin cells with elevated K^+ mimics the effects of nicotinic stimulation in causing a Ca^{2+}-dependent increase in phosphorylation of the same three serine residues in the N-terminal of TOH and a Ca^{2+}-dependent increase in TOH activity (Haycock *et al.*, 1982; Haycock, 1993). However, at concentrations up to 10 μM, Ro 31-8220 did not inhibit TOH activation by 50 mM K^+ (Figure 2b).

The differential effects of Ro 31-8220 on the actions of nicotine and K^+ depolarization were unexpected, since these two agents are usually considered to give similar effects on chromaffin cells except that nicotine depolarizes the cells through activating nicotinic cholinoceptors while elevated [K^+] depolarizes the cells passively by changing the K^+ equilibrium potential. To determine whether the differential effects of Ro 31-8220 were restricted to the effects of nicotine on TOH activation, we tested Ro 31-8220 on two other responses of chromaffin cells, catecholamine secretion and cellular cyclic AMP levels. Over the range 1 – 10 μM, Ro 31-8220 produced a concentration-dependent inhibition of nicotine-induced noradrenaline and adrenaline secretion, without causing any significant inhibition of adrenaline or noradrenaline secretion produced by K^+ depolarization (Figure 3). Ro 31-8220 also caused a concentration-dependent inhibition of nicotine-induced cellular cyclic AMP levels without affecting the response to K^+ depolarization (Figure 4). Ro 31-8220 had no significant effect on basal catecholamine secretion (not shown) or on basal cellular cyclic AMP levels at concentrations up to 10 μM (Figure 4).

Effects of bisindolylmaleimides I and V on nicotinic responses

Bisindolylmaleimide V, the structural analogue of Ro 31-8220 that lacks activity as a PK-C inhibitor, had little or no effect on nicotine-induced activation of TOH, catecholamine secretion or cellular cyclic AMP levels (Figure 5). This suggests that the effects of Ro 31-8220 on the nicotinic responses are structurally specific. To determine whether Ro 31-8220 needed to inhibit

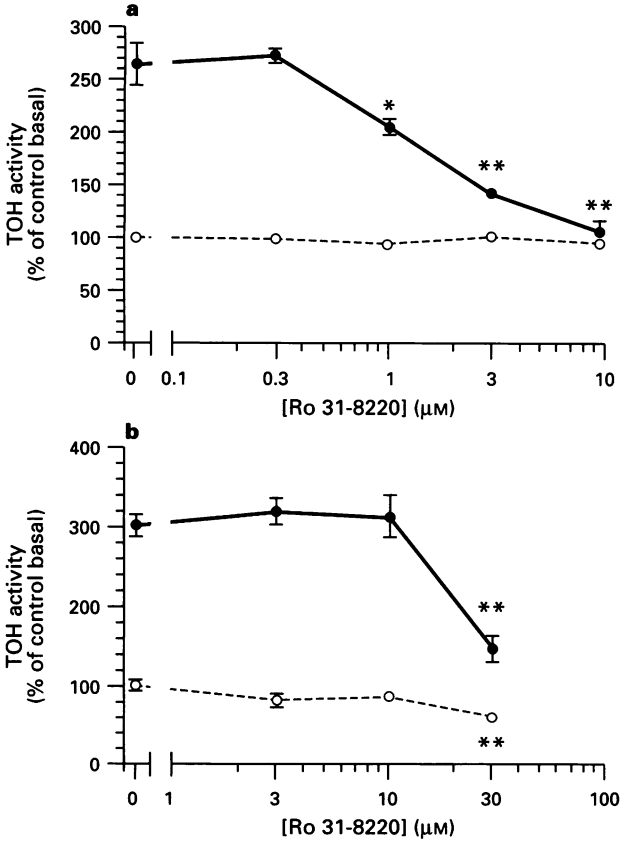

Figure 2 Effects of Ro 31-8220 on tyrosine hydroxylase activity in the absence and presence of 5 μM nicotine (a) or 50 mM additional K^+ (b). (●—●): in the presence of agonists; (○- - -○): in their absence (controls). Results are mean ± s.e.mean for $n=5-6$, from a single preparation of cells and are representative of similar results from at least 3 cell preparations. $^*P<0.05$ $^{**}P<0.01$ compared with the corresponding TOH activity in the absence of Ro 31-8220 (Fisher LSD test). In the presence of 10 μM Ro 31-8220, nicotine did not significantly stimulate TOH activity.

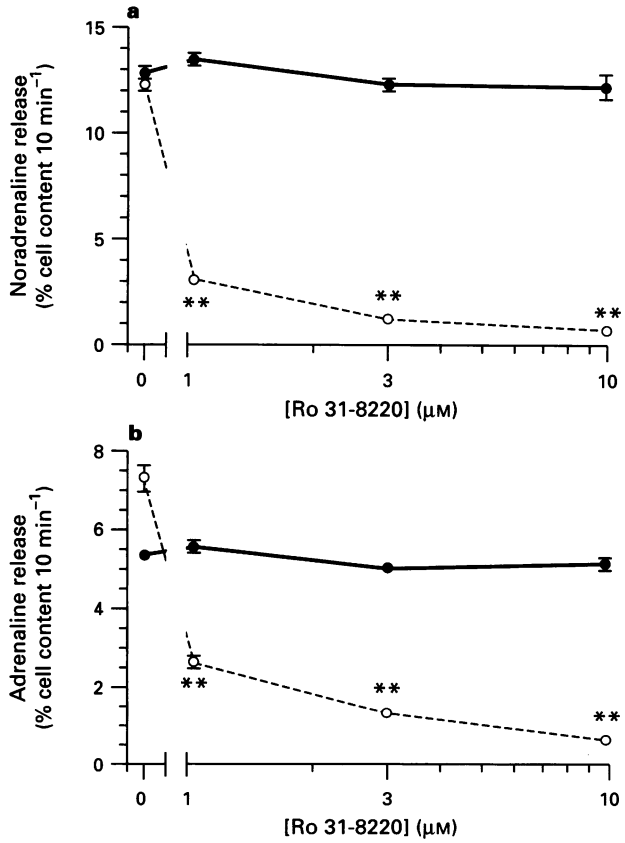

Figure 3 Effect of Ro 31-8220 on noradrenaline (a) and adrenaline secretion (b) in the presence of 5 μM nicotine (○) or 50 mM additional K^+ (●). Results are mean ± s.e.mean for $n=6$ from a single cell preparation and are representative of similar data from 2 cell preparations. Ro 31-8220 had no effect on basal noradrenaline or adrenaline secretion over the concentrations tested (data not shown). $^{**}P<0.01$ compared with response in the absence of Ro 31-8220 (Fisher's LSD test).

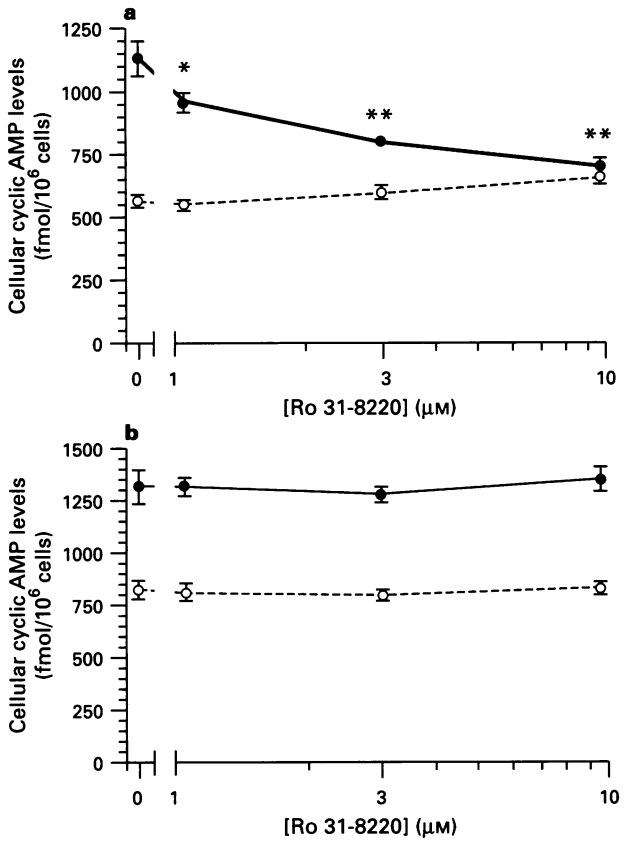

Figure 4 Effects of Ro 31-8220 on cellular cyclic AMP levels under control conditions (○- - -○) or in the presence of 5 μM nicotine (a: (●—●) or 50 mM additional K^+ (b: ●—●). Results are mean ± s.e.mean for $n=5$ from a single cell preparation and are representative of similar data from 2 (b) or 3 (a) cell preparations. $^*P<0.05$, $^{**}P<0.01$ compared with corresponding levels in the absence of Ro 31-8220 (Fisher's LSD test). In the presence of 10 μM Ro 31-8220, nicotine did not increase cellular cyclic AMP levels significantly.

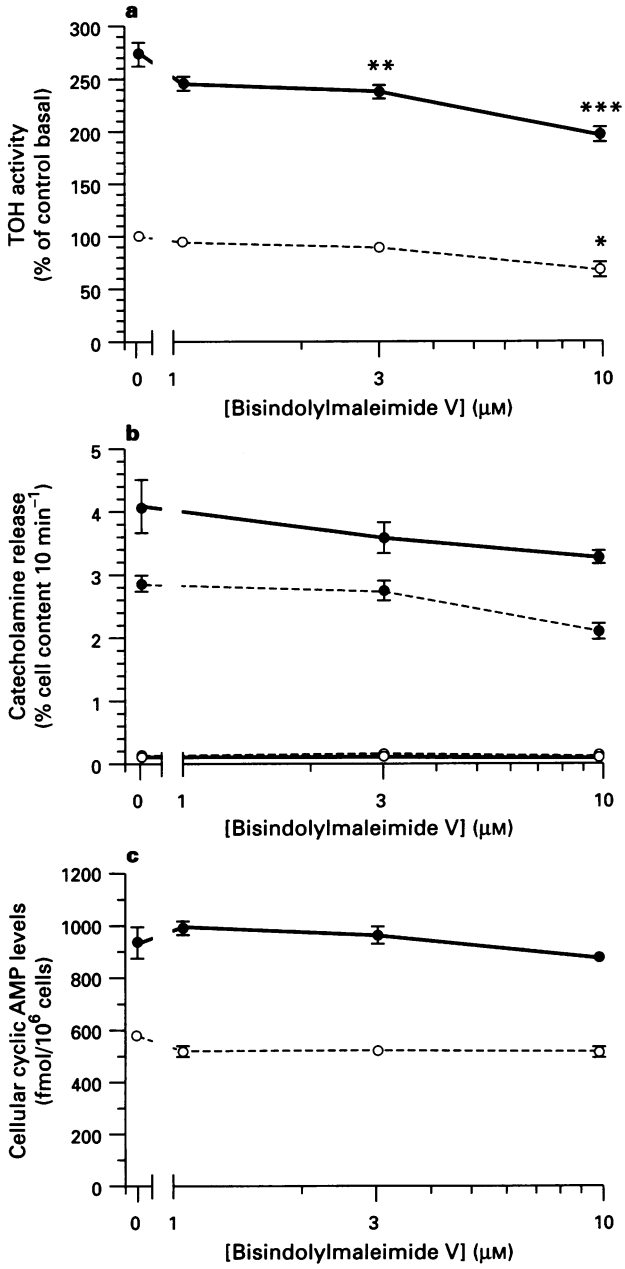

Figure 5 Effect of bisindolylmaleimide V, an analogue of Ro 31-8220 that lacks activity as a PK-C inhibitor, on tyrosine hydroxylase activity (a), catecholamine secretion (b) and cellular cyclic AMP levels (c) under basal conditions (○) or in the presence of 5 μM nicotine (●). Solid lines in (b) are noradrenaline release, dotted lines are adrenaline release. Results are mean ± s.e.mean for $n=4-6$ from a single preparation of cells and are representative of similar data from 2–5 cell preparations. $^*P<0.05$, $^{**}P<0.01$ compared with the corresponding result in the absence of bisindolylmaleimide V (Fisher's LSD test).

PK-C to produce its effects on the nicotinic responses, another bisindolylmaleimide inhibitor of PK-C was used, bisindolylmaleimide I (also known as GF 109203X and Gö 6850: Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993). This compound produced a concentration-dependent inhibition of TOH activation by PDBu with an IC_{50} of <1 μM (Figure 6a), but had essentially no effect on either TOH activation (Figure 6b) or catecholamine secretion (Figure 7) induced by nicotine.

Discussion

The present results suggest that Ro 31-8220 is not only an inhibitor of PK-C but is also an antagonist of nicotinic cholinoceptors on adrenal chromaffin cells. Its inhibitory action on the nicotinic receptors is not due to inhibition of PK-C since inhibition of PK-C with bisindolylmaleimide I did not mimic the effects of Ro 31-8220 (Figures 6 and 7).

Ro 31-8220 as a PK-C inhibitor on chromaffin cells

The inhibitory effect of Ro 31-8220 on TOH activation by PDBu is consistent with its known actions as a PK-C inhibitor. Our previous studies have shown that the cyclic AMP-dependent protein kinase (PK-A) appears to be an absolute requirement for activation of TOH by many agents including nicotine, histamine, PACAP-27, PDBu and forskolin (Marley *et al.*, 1994; 1995a,b; 1996). However, the effect of Ro 31-8220 on the PDBu response cannot be explained by it inhibiting PK-A because it failed to inhibit TOH activation by forskolin, an agent known to activate TOH exclusively through PK-A activation (George *et al.*, 1989); Haycock, 1993). In addition, the lack of effect of Ro 31-8220 on basal TOH activity and TOH activation by forskolin and K^+ indicates it does not non-specifically inhibit the tyrosine transporter in the plasma membrane or the TOH enzyme itself, or voltage-sensitive Ca^{2+} channels. Furthermore, its actions are mimicked by another known PK-C inhibitor, bisindolylmaleimide I, but not by the structurally related compound, bisindolylmaleimide V which lacks activity as a PK-C inhibitor. Taken together, these

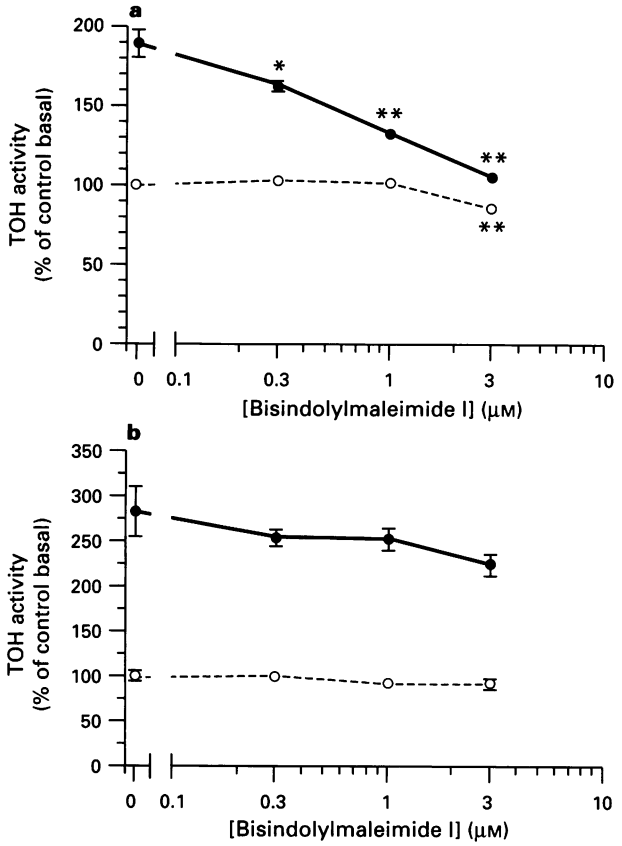

Figure 6 Effects of bisindolylmaleimide I on tyrosine hydroxylase activity in the absence (○- - -○) and presence (●—●) of 300 nM phorbol 12,13-dibutyrate (a: PDBu) or 5 μM nicotine (b). Results are mean ± s.e.mean for $n=6$ from a single preparation of cells and are representative of similar data from 3 cell preparations. $^{*}P<0.05$, $^{**}P<0.01$ compared with the corresponding TOH activity in the absence of bisindolylmaleimide I (Fisher's LSD). In the presence of 3 μM bisindolylmaleimide I, PDBu did not significantly stimulate TOH activity.

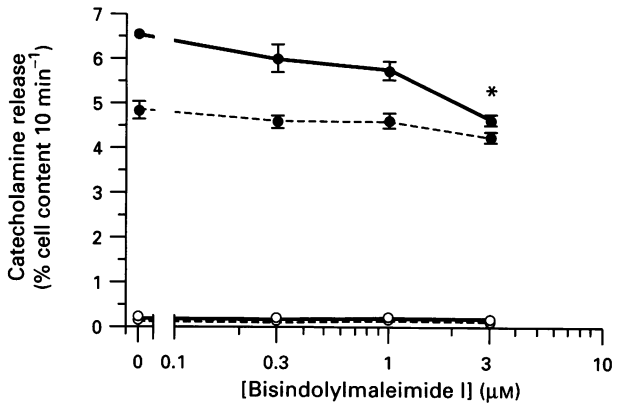

Figure 7 Effect of bisindolylmaleimide I on noradrenaline (●—●) and adrenaline (●- - -●) release in the absence (controls: ○- - -○) and presence of 5 μM nicotine (●). Results are mean ± s.e.mean for $n=6$ from a single preparation of cells and are representative of similar data from 2 cell preparations. $^{*}P<0.05$ compared with the corresponding result in the absence of bisindolylmaleimide I (Fisher LSD test).

findings are consistent with Ro 31-8220 inhibiting PDBu activation of TOH by inhibition of PK-C. Its potency in this action was some 50 fold lower than the reported IC_{50} for Ro 31-8220 inhibiting purified PK-C isozymes *in vitro* (5–27 nM: Wilkinson *et al.*, 1993), consistent with the 2–4 mM ATP concentrations found in intact cells with which it competes to inhibit PK-C.

Ro 31-8220 as a nicotinic receptor antagonist

Both nicotine and K^+ cause TOH activation, catecholamine secretion and elevated cyclic AMP levels in chromaffin cells by activating Ca^{2+} channels in the membrane which allow extracellular Ca^{2+} influx (see Haycock *et al.*, 1982; Burgoyne, 1991; Keogh & Marley, 1991; Anderson *et al.*, 1992). The selective inhibition of these three chromaffin cell responses to nicotine over the corresponding responses to K^+ indicates that Ro 31-8220 acts at a site prior to the activation of the voltage-sensitive Ca^{2+} channels. This suggests Ro 31-8220 inhibits the function of the nicotinic receptor/ionophore complex. The failure of Ro 31-8220 to inhibit TOH activation by histamine and PACAP-27 (Marley *et al.*, 1995a; 1996), which act through other receptor types, is consistent with this conclusion.

There are two possible explanations for the selectivity of Ro 31-8220 in inhibiting the nicotinic responses over those of K^+ depolarization. Firstly, Ro 31-8220 may specifically inhibit PK-C and PK-C may need to phosphorylate the nicotinic receptors before they are responsive to nicotinic agonists. Alternatively, Ro 31-8220 may be a direct inhibitor of the chromaffin cell nicotinic receptor by an action unrelated to PK-C inhibition.

Several of the subunits that comprise nicotinic cholinoceptors are known to be phosphoproteins that are substrates for a variety of kinases including PK-A, Ca^{2+}/calmodulin-dependent protein kinase II, a tyrosine kinase related to $PP60^{c\text{-}src}$ and PK-C (Huganir & Greengard, 1987). PK-C is known to phosphorylate the α and δ subunits of the neuromuscular junction type of nicotinic receptor, and nicotinic receptor phosphorylation has been shown to increase desensitization of the receptor, hence reducing its responsiveness to nicotinic agonists (see Huganir & Greengard, 1987; 1990). It is not yet known which types of nicotinic receptor subunits are expressed in chromaffin cells, their stoichiometry in forming functional chromaffin cell nicotinic receptors or whether they are substrates for PK-C. Consequently, it is not clear whether PK-C can regulate nicotinic receptor function in chromaffin cells. In addition, phorbol esters enhance Ca^{2+}-dependent catecholamine secretion from chromaffin cells (Bittner & Holz, 1990), suggesting PK-C may have actions other than enhancing nicotinic receptor desensitization in these cells.

If Ro 31-8220 were inhibiting the nicotinic responses of chromaffin cells by inhibiting nicotinic receptor phosphorylation by PK-C, its actions should be mimicked by other PK-C inhibitors. This was not the case: bisindolylmaleimide I almost completely inhibited TOH activation by PDBu at concentrations which had no effect on the nicotinic responses (Figures 6 and 7). Furthermore, we have also studied the indolocarbazole PK-C inhibitor, CGP 41251 (Meyer *et al.*, 1989), and this compound also inhibited the response to PDBu at concentrations that did not affect the responses to nicotine (Loneragan & Marley, in press). This strongly suggests that the inhibition of the nicotinic responses of chromaffin cells by Ro 31-8220 is unrelated to its ability to inhibit PK-C.

Taken together, the results indicate that Ro 31-8220 is an antagonist of nicotinic cholinoceptors on chromaffin cells. Although this effect is unrelated to its ability to inhibit PK-C, it is clearly a structurally specific effect since it is not shared by the related compounds bisindolylmaleimides I or V (Figures 5–7) or by CGP 41251 (Loneragan & Marley, in press). It is also relatively potent, having an IC_{50} of about 3 μM which is similar to hexamethonium (Mizobe *et al.*, 1979; Bourke *et al.*, 1988). Ro 31-8220 may represent a new class of compounds that function as nicotinic receptor antagonists and joins a large list of compounds characterized as antagonists of nicotinic receptors on bovine chromaffin cells, including substance P, somatostatin, opioid peptides, fragments of β-amyloid protein,

clonidine, phentolamine, yohimbine, propranolol, oxymetazoline and neosurugatoxin (Mizobe *et al.*, 1979; Marley *et al.*, 1986; Powis & Baker, 1986; Orts *et al.*, 1987; Bourke *et al.*, 1988; Wan *et al.*, 1988; Cheung *et al.*, 1993). Further studies using either ligand binding techniques or isolated-patch recording of nicotinic receptor channel currents are required to confirm that Ro 31-8220 acts directly on the nicotinic receptor protein to inhibit its response to agonist. A wide range of analogues of Ro 31-8220 have been synthesized for studies on PK-C (e.g., see Toullec *et al.*, 1991; Davis *et al.*, 1992a,b; Bit *et al.*, 1993) and many of these are available for further characterization of its structure-activity profile against nicotinic receptors. It will also be of interest to determine the selectivity of Ro 31-8220 for the chromaffin cell form of nicotinic receptor compared with the neuromuscular junction, autonomic ganglia and CNS types of nicotinic receptor.

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References

ANDERSON, K., ROBINSON, P.J. & MARLEY, P.D. (1992). Cholinoceptor regulation of cyclic AMP levels in bovine adrenal medullary cells. *Br. J. Pharmacol.*, **106**, 360–366.

BIT, R.A., DAVIS, P.D., ELLIOTT, L.H., HARRIS, W., HILL, C.H., KEECH, E., KUMAR, H., LAWTON, G., MAW, A., NIXON, J.S., VESEY, D.R., WADSWORTH, J. & WILKINSON, S.E. (1993). Inhibitors of protein kinase C. 3. Potent and highly selective bisindolylmaleimides by conformational restriction. *J. Med. Chem.*, **36**, 21–29.

BITTNER, M.A. & HOLZ, R.W. (1990). Phorbol esters enhance exocytosis from chromaffin cells by two mechanisms. *J. Neurochem.*, **54**, 205–210.

BOURKE, J.E., BUNN, S.J., MARLEY, P.D. & LIVETT, B.G. (1988). The effects of neosurugatoxin on evoked catecholamine secretion from bovine adrenal chromaffin cells. *Br. J. Pharmacol.*, **93**, 275–280.

BURGOYNE, R.D. (1991). Control of exocytosis in adrenal chromaffin cells. *Biochim. Biophys. Acta*, **1071**, 174–202.

CHEUNG, N.S., SMALL, D.H. & LIVETT, B.G. (1993). An amyloid peptide, βA_4 25-35, mimics the function of substance P on modulation of nicotine-evoked secretion and desensitization in cultured bovine adrenal chromaffin cells. *J. Neurochem.*, **60**, 1163–1166.

DAVIS, P.D., ELLIOTT, L.H., HARRIS, W., HILL, C.H., HURST, S.A., KEECH, E., KUMAR, M.K.H., LAWTON, G., NIXON, J.S. & WILKINSON, S.E. (1992b). Inhibitors of protein kinase C. 2. Substituted bisindolylmaleimides with improved potency and selectivity. *J. Med. Chem.*, **35**, 994–1001.

DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, A.D., WADSWORTH, J., WESTMACOTT, D.W. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Lett.*, **259**, 61–63.

DAVIS, P.D., HILL, C.H., LAWTON, G., NIXON, J.S., WILKINSON, S.E., HURST, S.A., KEECH, E. & TURNER, S.E. (1992a). Inhibitors of protein kinase C. 1. 2,3-Bisarylmaleimides. *J. Med. Chem.*, **35**, 177–184.

ELLIOT, L.H., WILKINSON, S.E., SEDGWICK, A.D., HILL, C.E., LAWTON, G., DAVIS, P.D. & NIXON, J.S. (1990). K252a is a potent and selective inhibitor of phosphorylase kinase. *Biochem. Biophys, Res. Commun.*, **171**, 148–154.

GEORGE, R.J., HAYCOCK, J.W., JOHNSTON, J.P., CRAVISO, G.L. & WAYMIRE, J.C. (1989). In vitro phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase by endogenous protein kinases. *J. Neurochem.*, **52**, 274–284.

HAYCOCK, J.W. (1990). Phosphorylation of tyrosine hydroxylase *in situ* at serines 8,19,31, and 40. *J. Biol. Chem.*, **265**, 11682–11691.

HAYCOCK, J.W. (1993). Multiple signalling pathways in bovine chromaffin cells regulate tyrosine hydroxylase phosphorylation on Ser^{19}, Ser^{31}, and Ser^{40}. *Neurochem. Res.*, **18**, 15–26.

HAYCOCK, J.W., MELIGENI, J.A., BENNETT, W.F. & WAYMIRE, J.C. (1982). Phosphorylation and activation of tyrosine hydroxylase mediate the acetylcholine-induced increase on catecholamine biosynthesis in adrenal chromaffin cells. *J. Biol. Chem.*, **257**, 12641–12648.

HAYCOCK, J.W. & WAKADE, A.R. (1992). Activation and multiple-site phosphorylation of tyrosine hydroxylase in perfused rat adrenal glands. *J. Neurochem.*, **58**, 57–64.

HUGANIR, R.L. & GREENGARD, P. (1987). Regulation of receptor function by protein phosphorylation. *Trends Pharmacol. Sci.*, **8**, 472–477.

HUGANIR, R.L. & GREENGARD, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron*, **5**, 555–567.

KEOGH, R. & MARLEY, P.D. (1991). Regulation of cyclic AMP levels by calcium in bovine adrenal medullary cells. *J. Neurochem.*, **57**, 1721–1728.

LIVETT, B.G., MARLEY, P.D., MITCHELHILL, K.I., WAN, D.C.C. & WHITE, T.D. (1987a). Assessment of adrenal chromaffin cell secretion: presentation of four techniques. In *The Secretory Process*, Vol 3, *In Vitro Methods for Studying Secretion*, ed. Poisner A.M. & Trifaro, J.-M., pp. 177–204. Amsterdam: Elsevier.

LIVETT, B.G., MITCHELHILL, K. & DEAN, D.M. (1987b). Adrenal chromaffin cells - their isolation and culture. In *The Secretory Process*, Vol 3, *In Vitro Methods for Studying Secretion*, ed. Poisner, A.M. & Trifaro, J.-M., pp. 171–175. Amsterdam: Elsevier.

MARLEY, P.D., CHEUNG, C.Y., THOMSON, K.A. & MURPHY, R. (1996). Activation of tyrosine hydroxylase by pituitary adenylate cyclase-activating polypeptide (PACAP-27) in bovine adrenal chromaffin cells. *J. Autonom. Nerv. Syst.*, (in press).

MARLEY, P.D., MITCHELHILL, K.I. & LIVETT, B.G. (1986). Effects of opioid peptides containing the sequence of Met^5-enkephalin or Leu^5-enkephalin on nicotine-induced secretion from bovine adrenal chromaffin cells. *J. Neurochem.*, **46**, 1–11.

MARLEY, P.D., ROBOTIS, R., THOMSON, K.A. & CHEUNG, C. (1995a). Effects of protein kinase inhibitors on tyrosine hydroxylase activity in bovine chromaffin cells. *Proc. 8th Int. Symp. Chromaffin Cell Biol.*, Edinburgh 6th–10th August, abstract S-20.

MARLEY, P.D., THOMSON, K. & BRALOW, R. (1994). Pharmacological inhibition of protein kinases in adrenal chromaffin cells: effects on tyrosine hydroxylase activation. *Can. J. Physiol. Pharmacol.*, **72**, Suppl. 1, P18.10.11.

MARLEY, P.D., THOMSON, K.A. & BRALOW, R. (1995b). Protein kinase A and nicotinic activation of bovine adrenal tyrosine hydroxylase. *Br. J. Pharmacol.*, **114**, 1687–1693.

MARLEY, P.D., THOMSON, K.A., JACHNO, K. & JOHNSTON, M.J. (1991). Histamine-induced increases in cyclic AMP levels in bovine adrenal medullary cells. *Br. J. Pharmacol.*, **104**, 839–846.

MARTINY-BARON, G., KAZANIETZ, M.G., MISCHAK, H., BLUMBERG, P.M., KOCHS, G., HUG, H., MARMÉ, D. & SCHÄCHTELE, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.*, **268**, 9194–9197.

MELIGENI, J.A., HAYCOCK, J.W., BENNETT, W.F. & WAYMIRE, J.C. (1982). Phosphorylation and activation of tyrosine hydroxylase mediate the cAMP-induced increase in catecholamine biosynthesis in adrenal chromaffin cells. *J. Biol. Chem.*, **257**, 12632–12640.

MEYER, T., REGENASS, U., FABBRO, D., ALTERI, E., RÖSEL, J., MÜLLER, M., CARAVATTI, G. & MATTER, A. (1989). A derivative of staurosporine (CGP 41251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumour activity. *Int. J. Cancer*, **43**, 851–856.

MIZOBE, F., KOZOUSEK, V., DEAN, D.M. & LIVETT,B.G. (1979). Pharmacological characterization of adrenal paraneurons: substance P and somatostatin as inhibitory modulators of the nicotinic response. *Brain Res.*, **178**, 555–566.

ORTS, A., ORELLANA, C., CANTO, T., CENA, V., GONZALEZ-GARCIA, C. & GARCIA, A.G. (1987). Inhibition of adrenomedullary catecholamine release by propranolol isomers and clonidine involving mechanisms unrelated to adrenoceptors. *Br. J. Pharmacol.*, **92,** 795–801.

POCOTTE, S.L. & HOLZ, R.W. (1986). Effects of phorbol esters on tyrosine hydroxylase phosphorylation and activation in cultured bovine adrenal chromaffin cells. *J. Biol. Chem.*, **261,** 1873–1877.

POCOTTE, S.L., HOLZ, R.W. & UEDA, T. (1986). Cholinergic receptor-mediated phosphorylation and activation of tyrosine hydroxylase in cultured adrenal chromaffin cells. *J. Neurochem.*, **46,** 610–622.

POWIS, D.A. & BAKER, P.F. (1986). α_2-Adrenoceptors do not regulate catecholamine secretion by bovine adrenal medullary cells: a study with clonidine. *Mol. Pharmacol.*, **29,** 134–141.

TACHIKAWA, E., TANK, A.W., WEINER, D.H., MOSIMANN, W.F., YANAGIHARA, N. & WEINER, N. (1987). Tyrosine hydroxylase is activated and phosphorylated on different sites in rat pheochromocytoma PC12 cells treated with phorbol ester and forskolin. *J. Neurochem.*, **48,** 1366–1376.

TERBUSH, D.R. & HOLZ, R.W. (1986). Effects of phorbol esters, diglyceride, and cholinergic agonists on the subcellular distribution of protein kinase C in intact or digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.*, **261,** 17099–17106.

TOULLEC, D., PIANETTI, P., COSTE, H., BELLEVERGUE, P., GRAND-PERRET, T., AJAKANE, M., BAUDET, V., BOISSIN, P., BOURSIER, E., LORIOLLE, F., DUHAMEL, L., CHARON, D. & KIRILOVSKY, J. (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.*, **266,** 15771–15781.

TWOMEY, B., MUID, R.E., NIXON, J.S., SEDGWICK, A.D., WILKINSON, S.E. & DALE, M.M. (1990). The effect of new potent selective inhibitors of protein kinase C on the neurotrophil respiratory burst. *Biochem. Biophys. Res. Comm.*, **171,** 1087–1092.

WAN, D.C.C., POWIS, D.A., MARLEY, P.D. & LIVETT, B.G. (1988). Effects of α- and β-adrenoceptor agonists and antagonists on ATP- and catecholamine release and desensitization of the nicotinic response in bovine adrenal chromaffin cells. *Biochem. Pharmacol.*, **37,** 725–736.

WAYMIRE, J.C., JOHNSTON, J.P., HUMMER-LICKTEIG, K., LLOYD, A., VIGNY, A. & CRAVISO, G.L. (1988). Phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase. Temporal correlation of acetylcholine's effect on site phosphorylation, enzyme activation, and catecholamine synthesis. *J. Biol. Chem.*, **263,** 12439–12447.

WILKINSON, S.E., PARKER, P.J. & NIXON, J.S. (1993). Isozyme selectivity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.*, **294,** 335–337.

ZIGMOND, R.E., SCHWARZSCHILD, M.A. & RITTENHOUSE, A.R. (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. *Annu. Rev. Neurosci.*, **12,** 415–461.

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