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# Cholinoceptor regulation of cyclic AMP levels in bovine adrenal medullary cells

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**1** The regulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels by cholinoceptors has been studied in cultured bovine adrenal medullary cells.

**2** Acetylcholine (100 μM), nicotine (10 μM) and dimethylphenylpiperazinium (20 μM) each increased cellular cyclic AMP levels 2 to 4 fold over 5 min in the absence of phosphodiesterase inhibitors. The muscarinic agonist acetyl-β-methylcholine (100 μM) had no effect either on its own or on the response to nicotine. The responses to acetylcholine and nicotine were unaffected by atropine (1 μM) but were abolished by mecamylamine (5 μM).

**3** Cellular cyclic AMP increased transiently during continuous exposure to nicotine (1– 20 μM), with the largest response seen after 5 min, a smaller response after 20 min, and no change in cyclic AMP levels seen after 90 or 180 min. The maximal response after 5 min stimulation was seen with 5– 10 μM nicotine and the $EC_{50}$ was about 2 μM. In contrast, extracellular cyclic AMP levels did not change after 5 or 20 min stimulation with nicotine, but increased slightly after 90 min and further after 180 min.

**4** The cellular cyclic AMP response to nicotine (10 μM) was unchanged or weakly enhanced in the presence of the unselective phosphodiesterase inhibitor, isobutylmethylxanthine, and was unchanged in the presence of rolipram. Nicotine did not interact synergistically with low concentrations of forskolin. The response was however completely abolished in the absence of extracellular $Ca^{2+}$.

**5** The nicotinic cyclic AMP response was almost abolished by sphingosine (30 μM), which did not inhibit the cyclic AMP response to phorbol-12,13-dibutyrate (PDB). The nicotinic response was reduced by 55% by the calmodulin antagonist W7 at 3 μM, and by >90% at 10 μM W7. At these concentrations, W7 had no effect on the cyclic AMP response to 100 nM PDB. The nicotinic response was also selectively abolished by another calmodulin antagonist, trifluoperazine (10 μM).

**6** The results indicate that nicotinic cholinoceptors can increase cyclic AMP production in chromaffin cells by a mechanism that does not directly involve $G_S$, that is dependent on extracellular $Ca^{2+}$ and that is sensitive to the calmodulin antagonists W7 and trifluoperazine. We propose that a $Ca^{2+}$/calmodulin-sensitive adenylate cyclase may mediate the nicotinic cyclic AMP response in bovine chromaffin cells.

**Keywords:** Adrenal medulla; adenosine-3':5'-cyclic monophosphate; cholinoceptors; chromaffin cells; calcium; second messengers

## Introduction

During stimulation of secretion from the adrenal medulla, substantial quantities of catecholamines are released; however, little depletion of adrenaline or noradrenaline occurs from the adrenal gland. These findings suggested that, during periods of increased secretion, the adrenal chromaffin cells increase their synthesis of catecholamines to replenish and maintain their secretory reserves (Bygdeman & von Euler, 1958). The coupling between secretion and catecholamine biosynthesis ('stimulation-secretion-synthesis coupling') is likely to be the result of second messengers generated in the chromaffin cells. These act via kinases to regulate the phosphorylation state, and hence the activity, of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis (George *et al.*, 1989 and references therein).

A number of recent studies have indicated that tyrosine hydroxylase from bovine chromaffin cells can be phosphorylated by several different classes of endogenous kinases present in these cells, including adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent kinase, $Ca^{2+}$/calmodulin-dependent kinase and protein kinase C (Niggli *et al.*, 1984; Pocotte & Holz, 1986; George *et al.*, 1989). The enzyme can be phosphorylated on a number of distinct sites and several of these sites appear to play a role in regulating the activity of the enzyme (Haycock *et al.*, 1982; Meligeni *et al.*, 1982; Niggli *et al.*, 1984; Pocotte & Holz, 1986; Pocotte *et al.*, 1986; Waymire *et al.*, 1988; George *et al.*, 1989). Furthermore, the temporal pattern of phosphorylation of these different sites is not the same (Waymire *et al.*, 1988), suggesting that different kinases may regulate the activity of the enzyme at different times during the secretory response.

The splanchnic nerve transmitter acetylcholine not only evokes adrenal catecholamine secretion but also increases chromaffin cell tyrosine hydroxylase activity by affecting multiple-site phosphorylation of the enzyme (Masserano & Weiner, 1979; Amy & Kirshner, 1981; Haycock *et al.*, 1982; Meligeni, 1982; Pocotte *et al.*, 1986; Waymire, *et al.*, 1988). Cholinergic stimulation is known to increase cytosolic $Ca^{2+}$ levels, diacylglycerol formation and cyclic AMP levels in chromaffin cells (Shima *et al.*, 1976; Eiden *et al.*, 1984; Pocotte *et al.*, 1986; TerBush & Holz, 1986; Morita *et al.*, 1987; TerBush *et al.*, 1988; Wilson, 1988). Several kinases activated by these second messengers may therefore play a role in mediating cholinergic activation of tyrosine hydroxylase.

Recently, it was observed that vasoactive intestinal polypeptide (VIP) also increases the activity of tyrosine hydroxylase in bovine chromaffin cells (Waymire *et al.*, 1991). This effect was quite distinct from cholinergic stimulation, in that the phosphorylation of only a single site in the enzyme was

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affected. The site was the same as the single site affected by cyclic AMP-dependent protein kinase (George *et al.*, 1989). Since VIP induces a cyclic AMP response in these cells (Wilson, 1988), VIP may act exclusively via cyclic AMP to regulate tyrosine hydroxylase activity.

In view of this, and to gain further insight into the possible contribution of cyclic AMP to the cholinergic regulation of adrenal tyrosine hydroxylase activity, we have investigated the mechanism of cholinoceptor regulation of cyclic AMP levels in bovine chromaffin cells.

## Methods

### *Incubation of bovine cultured adrenal chromaffin cells*

Adrenal medullary cells were isolated from bovine adrenal glands by collagenase digestion, purified by Percoll density gradient centrifugation and cultured at a density of $1.25 \times 10^6$/well in 6-well plastic culture plates, as described in detail previously (Livett *et al.*, 1987a). Cells prepared in this manner are typically 85% chromaffin cells (Marley *et al.*, 1989).

Cells were cultured for three days before use. They were then given three consecutive incubations at 37°C in 1 ml of buffer comprising (mM); NaCl 154, KCl 2.6, $K_2HPO_4$ 2.15, $KH_2PO_4$ 0.85, $MgSO_4$ 1.18, $CaCl_2$ 2.2 and D-glucose 10, supplemented with 0.5% bovine serum albumin and adjusted to pH 7.4. The first incubation was a 5 min wash and the second a 5, 10 or 15 min equilibration with buffer or appropriate antagonists, as indicated. The third incubation was for the times indicated in the presence of agonists together with antagonists, forskolin or phosphodiesterase inhibitors, where used.

In experiments where $Ca^{2+}$-free buffer was used, the 2.2 mM $CaCl_2$ in the incubation buffer was replaced with 2.2 mM $MgCl_2$, and all three incubations were performed in $Ca^{2+}$-free buffer.

### *Determination of cyclic AMP and catecholamines in cell and medium extracts*

Following the third incubation, the incubation medium was removed and the cellular cyclic AMP extracted in acid ethanol. When the incubation medium was also saved for cyclic AMP assay, the medium was also extracted. Extracts were dried down in a vacuum centrifuge, reconstituted in assay buffer and their cyclic AMP content determined by radioimmunoassay. Full details of the extraction and assay of cyclic AMP are given elsewhere (Keogh & Marley, 1991; Marley *et al.*, 1991).

The cyclic AMP radioimmunoassay had an $IC_{50}$ for cyclic AMP of 160 fmol/100 µl sample, and a detection limit of approximately 5 fmol. The assay had less than 0.05% cross-reactivity with cyclic GMP and essentially no (<0.0005%) crossreactivity with ATP, ADP, AMP, adenosine, GTP, GDP, GMP or guanosine. In addition, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine caused no interference in the assay when used in the experiments at a concentration of 1 mM.

The release of endogenous adrenaline and noradrenaline was studied as described previously (Livett *et al.*, 1987b).

### *Data presentation and statistics*

All results are presented as means ± s.e.mean for the stated number of observations. Due to the variability of basal cyclic AMP levels between cell preparations, results have been normalised to the basal levels for each cell preparation. Statistical significance had been assessed by Student's *t* test for two-sample comparisons, and the modified *t* statistic provided by Fisher's least significant difference (LSD) test, protected by one-way analysis of variance, for multiple comparisons. Interactions between combinations of agonists were assessed by determining whether the observed effect of the combination of drugs lay outside the 95% confidence limits for the expected effect if the two agents were simply additive (see Moeller *et al.*, 1989).

## Materials

Acetyl-β-methylcholine chloride, acetylcholine chloride, atropine sulphate, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), 3-isobutyl-1-methylxanthine (IBMX), mecamylamine, nicotine, phorbol-12,13-dibutyrate, phorbol-13-monoacetate and W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide HCl) were from Sigma Chemical Co. Forskolin and D-erythrosphingosine (bovine brain, approximately 70% erythro- and 30% threo-) were from Calbiochem. Trifluoperazine dihydrochloride was from Boehringer Mannheim. Rolipram was a generous gift from Dr G. Riisfeldt (Schering Pty. Ltd).

Forskolin, sphingosine and W7 were dissolved in ethanol and IBMX, rolipram and the phorbol esters were dissolved in dimethylsulphoxide before dilution in buffer to their working concentrations. Appropriate solvent controls were performed in every experiment.

## Results

### *Cholinoceptor pharmacology of cyclic AMP response*

As shown by others, acetylcholine (100 µM) increased cellular cyclic AMP levels in bovine chromaffin cells (Table 1). This effect was mimicked by the selective nicotinic receptor agonists dimethylphenylpiperazinium (DMPP, 20 µM) and nicotine (10 µM) but not by the muscarinic agonist acetyl-β-methylcholine (100 µM) which had no significant effect. The lack of involvement of muscarinic receptors was confirmed by the lack of effect of atropine on the response to acetylcholine and the complete antagonism of the response by mecamylamine (Figure 1a,b). In addition, the response to nicotine was unaffected by addition of acetyl-β-methylcholine (Figure 1c), indicating muscarinic receptors do not modulate the cyclic AMP response to nicotinic receptor stimulation.

The cellular cyclic AMP response to nicotine showed a steep concentration-dependence, with a maximal response at 5–10 µM and an $EC_{50}$ of around 2 µM (Figure 2a). The response was also transient, with the cellular cyclic AMP levels declining between 5 and 20 min, and returning to basal levels by 90 min (Figure 2a).

**Table 1** Effect of cholinoceptor agonists on cellular cyclic AMP levels in bovine adrenal medullary cells

| *Agonist* | *Cellular cyclic AMP levels* (% of basal) | |
|---|---|---|
| Acetylcholine (100 µM) | *557 ± 13 | (4) |
| Nicotine (10 µM) | *320 ± 10 | (6) |
| DMPP (20 µM) | *281 ± 6 | (3) |
| Acetyl-β-methylcholine (100 µM) | 136 ± 10 | (6) |

Cells were incubated with the indicated drugs for 5 min. Cyclic AMP levels are expressed as a percentage of the basal levels, which were in the range 65–950 fmol/$10^6$ cells (mean ± s.e.mean 308 ± 52 fmol/$10^6$ cells for 22 cell preparations). Data presented are means ± s.e.mean for the indicated number of observations, each from single but different cell preparations and representative of similar results from at least four cell preparations for each drug. Fisher's protected LSD: *$P<0.01$ compared to basal.
DMPP: dimethylphenylpiperazinium.

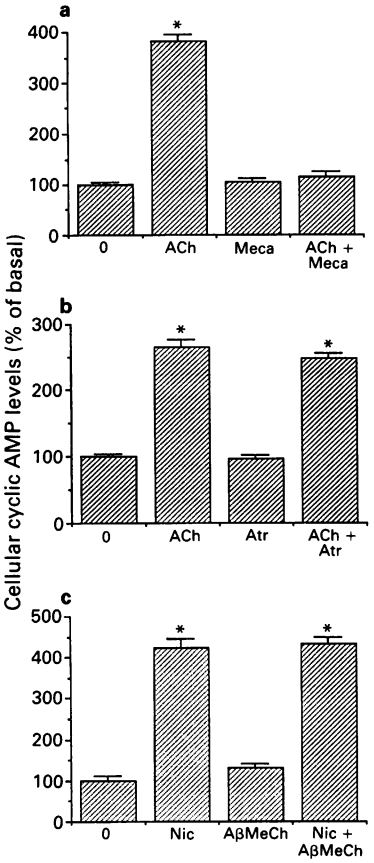


**Figure 1** Pharmacology of cholinoceptor-mediated cellular cyclic AMP responses in bovine adrenal medullary cells. (a, b) Cells were pretreated for 15 min with control buffer or with mecamylamine (Meca, 5 μM) or atropine (Atr, 1 μM) before a 5 min incubation with buffer (0), acetylcholine (ACh, 100 μM) or acetylcholine together with the antagonists. (c) Cells were incubated with control buffer (0), nicotine (Nic, 10 μM), acetyl-β-methylcholine (AβMeCh, 100 μM) or both drugs for 5 min. Results are means (s.e.mean shown by vertical bars) for $n = 4-6$; data from a single cell preparation and representative of similar results on four cell preparations. Fisher's protected LSD: (a) $*P<0.01$ compared to all other treatments. (b) $*P<0.01$ compared to basal and atropine alone. (c) $*P<0.01$ compared to basal and AβMeCh alone.

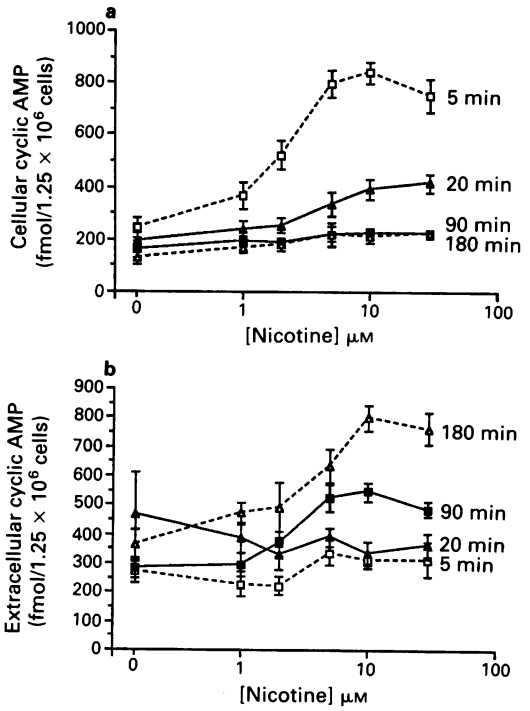


**Figure 2** Concentration-dependence and time course of nicotine-induced cellular (a) and extracellular (b) cyclic AMP responses in bovine adrenal medullary cells. Cells were incubated for various times with different concentrations of nicotine. The incubation medium and cells were then extracted separately for cyclic AMP determination. Results are means (s.e.mean shown by vertical bars) for $n = 4$ from a single cell preparation. Similar results were obtained on a second cell preparation.

### *The mechanism of nicotinic cyclic AMP responses*

Nicotine might increase cellular cyclic AMP by increasing cyclic AMP formation, reducing cyclic AMP degradation or by inhibiting cyclic AMP release from the cells.

At the early time points (5–20 min), nicotine had no effect on the export ('egress') of cyclic AMP from the cells into the incubation medium, however after 90 or 180 min significant increases in extracellular cyclic AMP were seen (Figure 2b). These observations indicate that the nicotine-induced increase in cellular cyclic AMP was not due to inhibition of cyclic AMP export from the cells.

Nicotine was still able to increase cellular cyclic AMP levels in bovine adrenal medullary cells after inhibition of all phosphodiesterase isozymes with the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) or after inhibition of the cyclic AMP-selective phosphodiesterase isozyme with rolipram (Figure 3a). These findings suggest that nicotine increases cellular cyclic AMP levels by increasing the rate of cyclic AMP formation.

Nicotine gave additive response with IBMX in 5 of 8 cell preparations, and was enhanced by 2–3 fold in the other three. Nicotine gave additive responses with rolipram in 3 of 4 preparations, and was enhanced by only 31% in the fourth. IBMX (1 mM) and rolipram (100 μM) increased basal cyclic AMP levels in all four cell preparations on which they were both tested, by 189 ± 42% and 64 ± 20%, respectively.

To investigate the mechanism by which nicotine increased adenylate cyclase activity, cyclic AMP levels were studied in the presence of both nicotine and the diterpine, forskolin. The latter acts directly on adenylate cyclase to stimulate the enzyme, and has been reported to bind preferentially and activate adenylate cyclase when the adenylate cyclase is bound to the stimulatory G protein $G_S$ (i.e.: during activation of adenylate cyclase by agents acting via $G_S$, Battaglia *et al.*, 1986; Robinson *et al.*, 1989). Consequently forskolin interacts synergistically with agents that activate adenylate cyclase via $G_S$ (Seamon & Daly, 1983). However, nicotine gave additive responses with a low concentration of forskolin in 4 of 5 preparations and was enhanced by only 25% in the fifth (Figure 3b). In contrast, forskolin enhanced the responses of these cells to the $H_2$-histamine agonist, dimaprit, by more than 10 fold (Marley *et al.*, 1991) and to 1 μM prostaglandin $E_1$ by almost 6 fold (Marley, unpublished observations), two agents that act via $G_S$-coupled receptors in other tissues. This suggests that nicotine increases adenylate cyclase activity in chromaffin cells independently of $G_S$.

In agreement with a previous study using the non-selective cholinoceptor agonist, carbachol (Pocotte *et al.*, 1986), the cyclic AMP response to nicotine was completely abolished by omitting extracellular $Ca^{2+}$ (Figure 3c), which as expected abolished the nicotine-induced secretion of catecholamines (data not shown). Also in agreement with the previous study, we found that incubation medium collected from cells that had been stimulated with 10 μM nicotine in the presence of $Ca^{2+}$ failed to produce a cyclic AMP response in a second batch of cells in the presence of 5 μM mecamylamine (data not shown). Furthermore, alteration of the incubation volume from 1 ml to 0.5 ml or 2 ml, to change the dilution of any released substances, did not affect the cyclic AMP res-

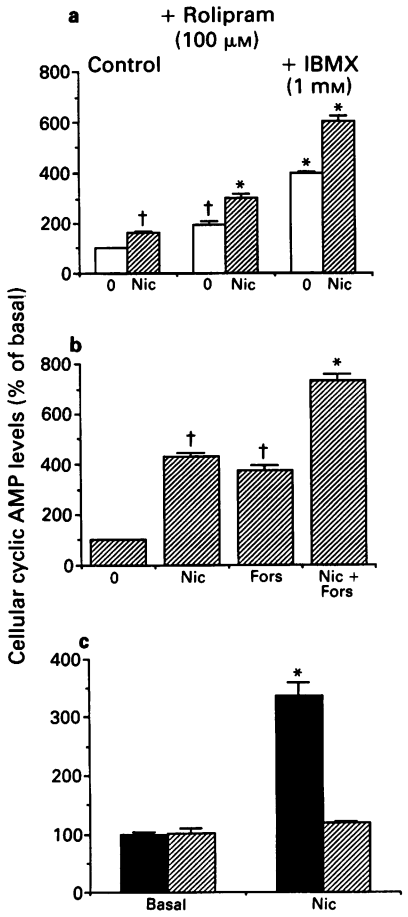


**Figure 3** Effect of nicotine (Nic, 10 μM) on cellular cyclic AMP response over 5 min in bovine adrenal medullary cells in the absence and presence of (a) 3-isobutyl-1-methylxanthine (IBMX, 1 mM) or rolipram (100 μM), (b) forskolin (Fors, 0.3 μM) or (c) extracellular calcium (2.2 mM, solid columns). (a, b) Results are means (s.e.mean shown by vertical bars) for $n = 4-6$; data from a single cell preparation and representative of results on four to eight cell preparations (see text). In the results shown, nicotine gave additive responses with rolipram and forskolin and was enhanced 3.2 fold by IBMX, the maximum enhancement observed (see Moeller *et al.*, 1989). (c) In experiments in the absence of extracellular $Ca^{2+}$ (hatched columns), cells received two 5 min washes and a 5 min stimulation in buffer in which the 2.2 mM $CaCl_2$ was replaced with $MgCl_2$. Results are means (s.e.mean shown by vertical bars) for $n = 5-6$, data from a single cell preparation and representative of results on three cell preparations. Fisher's protected LSD: $*P<0.01$ compared to all other treatments; † $P<0.01$ compared with all other conditions except each other.

ponse to nicotine (data not shown). These observations indicate that chromaffin cell products secreted during nicotinic stimulation do not contribute significantly to the cyclic AMP response.

Calcium could regulate adenylate cyclase activity by a number of mechanisms. These include direct allosteric activation via calmodulin and indirect effects mediated via protein kinase C, which is known to be activated by $Ca^{2+}$ in chromaffin cells. The possibility that nicotinic receptors regulate chromaffin cell adenylate cyclase by $Ca^{2+}$-dependent activation of protein kinase C was studied by examining the effect of the tumour-promoting phorbol ester, phorbol-12,13-dibutyrate (PDB). PDB (100 nM) increased cyclic AMP in these cells to 140 ± 7% of basal ($n = 17$, data pooled from 3 cell preparations, Fisher's protected LSD: $*P<0.01$ compared with basal or phorbol-13-monoacetate), but the inactive phorbol ester phorbol-13-monoacetate (PM) had no effect (101 ± 5% of basal, $n = 19$, data pooled from 3 cell preparations).

When protein kinase C was down-regulated by chronic treatment with a high concentration of PDB (500 nM for 24 h, see Wilson, 1990), the acute cyclic AMP response to PDB was abolished whilst that to nicotine was not (data not shown). The chronic phorbol ester treatment itself, however, caused cellular cyclic AMP levels to double, which made it difficult to conclude whether protein kinase C had been effectively down-regulated by this procedure.

To investigate further a possible role for protein kinase C in the cyclic AMP response to nicotine, putative protein kinase C inhibitors were used. H7 (20 μM) did not inhibit the response to 10 μM nicotine in each of 3 cell preparations, and it also failed to inhibit the response to 100 nM PDB in four preparations (data not shown). In view of the poor selectivity of H7 for protein kinase C over other kinases *in vitro* (Hidaka *et al.*, 1984) and its ability to inhibit equally responses to forskolin and phorbol esters in chromaffin cells (Waymire *et al.*, 1991), higher concentrations were not tested here.

To investigate the possible involvement of calmodulin in the nicotinic cyclic AMP response, the selective calmodulin antagonist, W7, was used. At 100 μM, W7 had no effect on resting cellular cyclic AMP levels and completely abolished the cyclic AMP response to 10 μM nicotine (Figure 4a). Although preferentially an inhibitor of calmodulin, due to its lipophilic structure W7 can also inhibit protein kinase C (Hidaka & Hagiwara, 1987). Consequently, the concentration-dependence of the effect of W7 on nicotine- and PDB-induced cyclic AMP response was investigated (Figure 4a). The nicotinic response was inhibited by >90% at 10 μM W7 and by 55% at 3 μM W7. At these concentrations, W7 had no effect on the cyclic AMP response to PDB, which was only inhibited at much higher W7 concentrations.

Another calmodulin antagonist, trifluoperazine (10 μM), also completely blocked the cyclic AMP response to nicotine without affecting either basal cyclic AMP levels or the response to PDB (Figure 4b). Sphingosine, a kinase inhibitor with some selectivity for protein kinase C, failed at 30 μM to affect the cyclic AMP response to PDB but reduced the response to nicotine by 83% (mean of three cell preparations, Figure 4c).

## Discussion

### *Cholinoceptor-mediated cyclic AMP responses in adrenal medullary cells*

Previous studies have found conflicting evidence on the ability of cholinoceptors to increase cyclic AMP levels in chromaffin cells. A number of authors found that carbachol or acetylcholine had no effect on adenylate cyclase activity in membranes prepared from bovine adrenal medulla (Serck-Hanssen *et al.*, 1972; Hurko *et al.*, 1974; Boonyaviroj & Gutman, 1977) while others found no effect of carbachol, acetylcholine, nicotine or DMPP on cyclic AMP levels in slices or isolated cells from this tissue (Shima *et al.*, 1976; Niggli *et al.*, 1984; Chern *et al.*, 1988; Marriott *et al.*, 1988). In contrast, several reports have recorded robust cyclic AMP responses to cholinoceptor agonists in slices of bovine adrenal medulla or in cultured bovine chromaffin cells (Shima *et al.*, 1976; Eiden *et al.*, 1984; Pocotte *et al.*, 1986; Morita *et al.*, 1987; Wilson, 1988). Furthermore, cholinoceptor cyclic AMP responses have also been reported in rat, cat and dog adrenal medulla (Guidotti & Costa, 1973; Jaanus & Rubin, 1974; Shima *et al.*, 1978; Tsujimoto *et al.*, 1980). The reason for the varied results on bovine adrenal medullary preparations is not clear.

In the present study using bovine cultured chromaffin cells, we found a reproducible cellular cyclic AMP response to nicotine, DMPP and acetylcholine, although as noted by others (Tsujimoto *et al.*, 1980; Pocotte *et al.*, 1986; Morita *et al.*, 1987), this reponse was transient: at times longer than

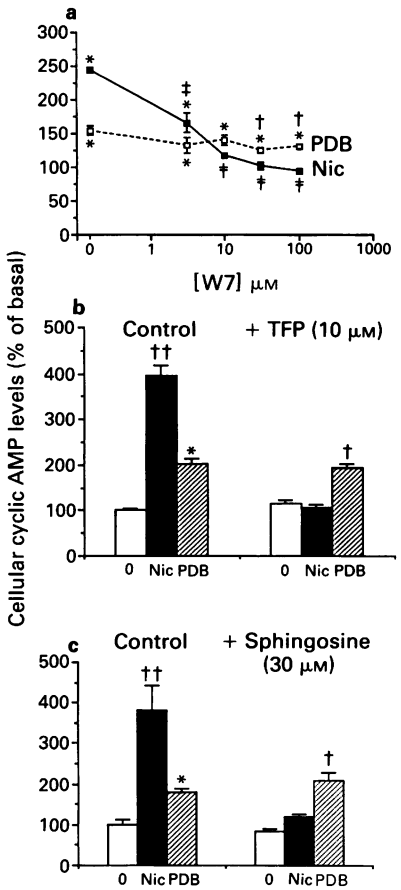


**Figure 4** Effect of (a) W7 (3–100 μM), (b) trifluoperazine (TFP, 10 μM) and (c) sphingosine (30 μM) on the cellular cyclic AMP response of bovine adrenal medullary cells to nicotine (Nic, 10 μM) or phorbol-12,13-dibutyrate (PDB, 100 nM in (a), 300 nM in (b) and (c)). Cells were pretreated for 10 or 15 min with the indicated concentrations of W7, TFP or sphingosine and then incubated for 5 min with nicotine (solid symbols, solid columns) or PDB (open symbols, hatched columns) together with W7, TFP or sphingosine. Results are means (s.e.mean shown by vertical bars) for $n = 4-6$, data from a single cell preparation and representative of similar results from (a) two or (b, c) three cell preparations. Fisher's protected LSD: (a) $*P<0.05$ compared to W7 alone, † $P<0.05$ compared with PDB alone, ‡ $P<0.01$ compared to nicotine alone. W7 alone at concentrations up to 100 μM had no effect on cellular cyclic AMP levels. (b) $*P<0.01$ compared to all treatments except PDB with TFP, † $P<0.01$ compared to all treatments except PDB alone, †† $P<0.01$ compared to all other treatments. (c) $*P<0.05$ compared to basal, $P<0.01$ compared with all other conditions except PDB with sphingosine; † $P<0.01$ with all other conditions except PDB alone; †† $P<0.01$ compared to all other conditions.

20 min cholinoceptor stimulation no longer increased cellular cyclic AMP levels. Consequently, the failure to observe a cyclic AMP response in some studies may have been due to the incubation period chosen. Shima *et al.* (1976) found they needed periods longer than 20 min before they saw any cyclic AMP response. However, they used slices of bovine adrenal medulla, which may only allow slow penetration of agonists into the tissue. The transient nature of the chromaffin cell cholinoceptor cyclic AMP response is in direct contrast to that produced by VIP (Wilson, 1988) and forskolin (Marley, Thomson & Jachno, unpublished observations) which are maintained for many hours.

Our results showing no synergism between nicotine and forskolin (see below), and the complete $Ca^{2+}$-dependence of the nicotinic cyclic AMP response suggest strongly that the nicotinic cholinoceptors do not act on adenylate cyclase via a $G_S$ protein, but by a mechanism involving $Ca^{2+}$ entry. Consequently, the failure to detect any effect of cholinoceptor stimulation on adenylate cyclase activity in adrenal medullary membrane preparations is not surprising (Serck-Hanssen *et al.*, 1972; Hurko *et al.*, 1974). Acetylcholine did however increase adenylate cyclase activity in intact rat adrenal glands (Shima *et al.*, 1978). Marriott *et al.* (1988) reported that nicotine had no effect on bovine chromaffin cell cyclic AMP levels on its own, but augmented the response to low concentrations of forskolin. The reason for the difference between results of that study and the present observations is not known.

The pharmacology of cholinoceptor cyclic AMP responses in bovine chromaffin cells has not been investigated in detail previously. Although carbachol, acetylcholine and DMPP have all been reported to induce a cyclic AMP response in these cells, a possible involvement of muscarinic receptors was not excluded (Eiden *et al.*, 1984; Pocotte *et al.*, 1986; Morita *et al.*, 1987). Wilson (1988) observed that although muscarine had no effect on its own, it enhanced the cyclic AMP response to VIP. The effect of muscarinic receptors on the nicotinic cyclic AMP response was not investigated, although muscarinic receptors can modulate other responses to nicotinic receptors in these cells (see Forsberg *et al.*, 1986). In the present study, muscarinic receptors were found not to contribute to the cyclic AMP response to acetylcholine (Figure 1a,b) or to modulate the cyclic AMP response to nicotine (Figure 1c), suggesting that the cholinoceptor cyclic AMP response is solely nicotinic.

### *The mechanism of nicotinic receptor-mediated cyclic AMP responses in bovine adrenal medullary cells*

Following the rise in cellular cyclic AMP, nicotinic stimulation was found to produce a delayed increase in cyclic AMP release suggesting that the cellular cyclic AMP response is not due to inhibition of cyclic AMP release. The phenomenon of evoked cyclic AMP release was noted over 25 years ago by Davoren & Sutherland (1963) and has been reported from rat, cat and dog adrenal glands and from bovine chromaffin cells (Jaanus & Rubin, 1974; Shima *et al.*, 1978; Tsujimoto *et al.*, 1980; 1986; Morita *et al.*, 1985; Chern *et al.*, 1988). Whether agonist-induced increases in cyclic AMP export from cells is simply a passive event secondary to the increase in cellular levels or due to a more active regulatory mechanism is not known.

The failure of phosphodiesterase inhibition with high concentrations of IBMX or rolipram to prevent the cyclic AMP response to nicotine, together with the finding that nicotine does not inhibit cyclic AMP release from the these cells, suggests that nicotine increases cellular cyclic AMP levels by increasing the rate of cyclic AMP formation (i.e. by increasing adenylate cyclase activity). It was noteworthy that the cyclic AMP response to nicotine was often not enhanced significantly by phosphodiesterase inhibition with IBMX or rolipram (Figure 3a). This may have been because the levels of cyclic AMP generated by nicotinic stimulation in some cell preparations were substantially below the $K_m$ of the phosphodiesterases, although this appears unlikely, since IBMX and rolipram both increased basal cyclic AMP levels in all cell preparations tested. Alternatively, the cyclic AMP generated by nicotine may be in an intracellular location protected from rapid degradation by phosphodiesterases.

The lack of synergy with forskolin (Figure 3b), is in contrast with the results with histamine $H_2$-agonists (Marley *et al.*, 1991) and prostaglandin $E_1$ (agents that act through $G_S$ in other tissues), and suggests the nicotinic activation of adenylate cyclase is indirect and not via a $G_S$ protein (but see Marriott *et al.*, 1988). This is supported by the complete dependence of the nicotinic cyclic AMP response on extracellular $Ca^{2+}$ (see also Pocotte *et al.*, 1986). In parallel studies we have found that $Ca^{2+}$ entry into chromaffin cells is a sufficient and adequate stimulus to increase cyclic AMP for-

mation, whether it is induced by nicotine, histamine $H_1$-agonists, $K^+$ depolarization or veratridine (Keogh & Marley, 1991; Marley *et al.*, 1991).

Calcium is known to affect a number of second messenger transduction systems in chromaffin cells. It activates phopholipase C which generates inositol phosphates (Eberhard & Holz, 1987) together with diacylglycerol, an activator of protein kinase C. It also activates protein kinase C directly, by inducing translocation of the enzyme to the plasma membrane (TerBush & Holz, 1986; TerBush *et al.*, 1988). These effects are also produced by nicotinic stimulation in a $Ca^{2+}$-dependent manner (TerBush & Holz, 1986; Eberhard & Holz, 1987, TerBush *et al.*, 1988). Consequently, the $Ca^{2+}$-dependent nicotinic activation of adenylate cyclase could be mediated by protein kinase C. The ability of phorbol esters that activate protein kinase C to elevate chromaffin cell cyclic AMP levels supports this explanation. Protein kinase C has been reported to enhance cyclic AMP formation in a number of other cell types (see Yoshimasa *et al.*, 1987; Choi & Toscano, 1988).

Attempts to down-regulate protein kinase C by chronic treatment of the cells with high PDB concentrations (see Wilson, 1990), resulted itself in a two fold increase in cellular cyclic AMP levels. It was therefore not possible to determine whether the subsequent lack of effect of acute PDB stimulation was due to down-regulation of protein kinase C or occlusion of the PDB effect. Highly selective, cell-permeant inhibitors of protein kinase C are not yet commercially available and attempts to inhibit the cyclic AMP response to PDB with low concentrations of H7 were not successful (see Wright & Hoffman, 1980).

In contrast, the calmodulin antagonist, W7, almost completely inhibited the cyclic AMP response to nicotine at concentrations that did not reduce the response to PDB. W7 inhibits $Ca^{2+}$/calmodulin-dependent kinases with an $IC_{50}$ of around 10 μM while it inhibits protein kinase C with an $IC_{50}$ of 60 μM (Kuo *et al.*, 1984). The selective inhibition of the nicotinic cyclic AMP response over the phorbol ester response at W7 concentrations of 10 μM and below is compatible with the suggestion that nicotine is acting through a $Ca^{2+}$/calmodulin-dependent process. This conclusion is supported by the selective inhibition of the nicotinic cyclic AMP response by trifluoperazine, another calmodulin antagonist. Although sphingosine has been reported as an inhibitor of phorbol ester activation of protein kinase C (Hannun *et al.*, 1986), we found that at 30 μM it had no effect on the cyclic AMP response to PDB but almost completely inhibited that to nicotine (Figure 4c). Although the mechanism of inhibition of the nicotinic response by sphingosine is not clear, it is evident that it blocks the nicotinic response at concentrations at which protein kinase C is not inhibited.

Since a $Ca^{2+}$/calmodulin-dependent isozyme of adenylate cyclase has been identified in bovine adrenal medulla (LeDonne & Coffee, 1980), it is possible that nicotine activates this isozyme allosterically, following $Ca^{2+}$ entry and association with calmodulin, to produce its cyclic AMP response. The present data, however, do not rule out an involvement of protein kinase C in the cyclic AMP response to nicotinic agonists, in addition to the role of $Ca^{2+}$/calmodulin.

The present study suggests that nicotinic receptors mediate their cyclic AMP response secondarily to inducing $Ca^{2+}$ entry into chromaffin cells. The contribution of this secondary cyclic AMP response to the phosphorylation and activation of tyrosine hydroxylase induced by acetylcholine in chromaffin cells remains to be determined.

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