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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

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Abstract—The effects of a number of α- and β-adrenoceptor agonists and antagonists on the modulation of secretion from bovine adrenal chromaffin cells were investigated. Secretion was induced by nicotine, 56 mM K^+, histamine or Ba^{2+} and was detected by the ATP luciferin-luciferase bioluminescence technique or by the measurement of endogenous catecholamines (CA) by HPLC coupled with electrochemical detection. ATP release from freshly isolated cells by 5 μM nicotine was only weakly inhibited by adrenaline and noradrenaline and even then required high concentrations (>500 μM), while dopamine (1 μM–1 mM) and isoproterenol (100 μM) had no effect. Clonidine (100 μM), oxymetazoline (100 μM), yohimbine (100 μM), and propranolol (5 μM) all produced inhibition of nicotine-induced ATP release with the order of potency: propranolol > oxymetazoline > clonidine = yohimbine. The inhibitory effect by propranolol could not be reversed by high concentrations of adrenaline or isoproterenol. In chromaffin cell monolayer cultures, all α_2-adrenoceptor agents tested (clonidine, oxymetazoline and yohimbine), produced a dose-dependent, Na^+-sensitive, non-competitive inhibition of nicotine-induced catecholamine release with little effect on the catecholamine release induced by K^+ (56 mM), histamine (10 μM) or Ba^{2+} (2.2 mM). (±)Propranolol caused a similar pattern of inhibition, however, this inhibition was also observed by (+)propranolol, an isomer with little β-adrenoceptor antagonist activity. The effects of clonidine and propranolol on desensitization of nicotine-induced CA secretion were also investigated. The degree of desensitization of the nicotinic response was dependent on the concentration of nicotine to which the cells were pre-exposed. Desensitization was detected as the decrease in response to a near EC_{50} concentration of nicotine (5 μM) following pre-incubation of cells to nicotine in the range of 0.3–300 μM. The desensitization had a threshold of 1 μM nicotine and was maximal at 3 μM nicotine in the pre-incubation. Both clonidine (50 μM) and (±)propranolol (5 μM) inhibited CA secretion induced by nicotine (0.3 μM–300 μM) during the pre-incubation period. However, regardless of this inhibition of secretion, neither clonidine nor propranolol had an effect on either the onset, or the rate of nicotine-evoked desensitization subsequently observed. These data suggest that inhibition of the nicotinic response and desensitization of the nicotinic response are regulated independently. These findings do not support the existence of α- or β-adrenoceptor modulation of secretion from isolated bovine chromaffin cells.

The chromaffin cells of the adrenal medulla and peripheral noradrenergic neurons have a common embryological origin in the neural crest [1]. Catecholamines are synthesized and stored in vesicles in these cells and are released by a process of exocytosis in which extracellular calcium is required [2]. In view of these common features, it has been proposed that catecholamine release from the adrenal medulla may be subject to regulatory control similar to that seen in sympathetic nerves. There is very substantial evidence for the existence of presynaptic adrenoceptors on noradrenergic nerve terminals regulating noradrenaline release in peripheral sympathetic nerves [3–6]. The existence of adrenoceptors on adrenal medullary cells regulating the release of adrenal catecholamines in a similar fashion is, however, controversial. On the one hand there are reports claiming that α- and β-adrenoceptors play a part in the fine control of catecholamine secretion from adrenal medullary cells [7–12] and on the other hand those whose data do not support such a role [13–19]. The purpose of the present study was to re-examine the effects of a number of α- and β-adrenoceptor agonists and antagonists in modulating secretion from adrenal chromaffin cells by exploiting the experimental system afforded by isolated bovine adrenal chromaffin cells and by measuring two indices of secretion, ATP and endogenous catecholamines in response to different secretagogues. The results of this study do not support the hypothesis that α- or β-adrenoceptor feedback operates in the regulation of adrenal medullary secretion. A preliminary report of part of this work has been published [20].

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MATERIALS AND METHODS

Materials

Drugs used were obtained as follows: nicotine,

adrenaline bitartrate, noradrenaline bitartrate, (±)isoproterenol hydrochloride, (±)propranolol hydrochloride, clonidine hydrochloride, oxymetazoline hydrochloride, phenylephrine hydrochloride, histamine hydrochloride and dopamine hydrochloride were purchased from Sigma Chemical Corporation. Yohimbine hydrochloride and tolazoline hydrochloride were obtained from Aldrich and (+)propranolol hydrochloride was from Imperial Chemical Industries. All drugs were dissolved in distilled water to give 50 or 100 mM stock solution except for yohimbine hydrochloride which was dissolved in 50% (v/v) dimethyl sulphoxide. The drug solutions were prepared freshly on the day of experiment. Final dilutions were made in assay buffer.

Methods

Preparation of isolated bovine adrenal chromaffin cells. Isolated bovine adrenal chromaffin cells were prepared by collagenase digestion of adrenal glands as previously described [21]. For measurement of ATP release, the freshly isolated cells were dispersed and resuspended at a density of 2×10^6 cells/ml in HEPES–Locke's buffer of the following composition (mM): NaCl 154, KCl 2.55, K_2HPO_4 2.13, KH_2PO_4 0.88, D-glucose 10, $CaCl_2$ 2.2, HEPES 15.1, BSA 0.5% (w/v) pH 7.4. Cells were allowed to stabilize at room temperature for 2–3 hr and used on the day of preparation or on the following day. There was no significant difference in the sensitivity of cells to nicotine within the two days [22]. For primary monolayer cultures, the cells were further purified by Percoll(TM) gradient centrifugation (47,000 *g* for 20 min) and then plated on rat-tail collagen precoated 24 well plastic culture dishes at a density of 2.5×10^5 cells/well. The cells were maintained in a humid 10% CO_2 atmosphere at 37° in 0.4 ml medium comprising Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (1:1 v/v) supplemented with 10% fetal calf serum, 2.5 μg/ml flurodeoxyuridine, 2.5 μg/ml cytosine arabinoside, 2.5 μg/ml uridine, 5 μg/ml nystatin, 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were used for catecholamine release experiments on the third day after plating.

Measurement of ATP release. The ATP released from freshly isolated cell suspensions by nicotinic stimulation was measured by the luciferin–luciferase bioluminescence method [22, 23]. Cells (about 1×10^6 cells in 500 μl) were placed in a 6 × 50 mm glass cuvette followed by 20 μl of D-luciferin–luciferase solution which was prepared freshly by mixing 1 ml of firefly lantern extract (Sigma FLE-50, 25 mg/ml distilled water) with 0.5 ml of D-luciferin (Sigma, 5 mg/ml distilled water) and clarified by centrifugation. The cuvette was placed in the light-tight reaction chamber of an Aminco fluorocolorimeter modified to bring the cuvette closer to the photomultiplier. The output from the photomultiplier was amplified and recorded on a chart recorder. Addition of drugs to the cell suspension was achieved by injecting the drug solutions into the cuvette through a light-tight septum. At 3.5 min after mixing the cells with luciferin–luciferase, 10 μl of tested drug, or 10 μl of buffer alone as control, was injected, followed by 10 μl of nicotine 1 min later to evoke secretion. ATP release was estimated from light emission resulting from its reaction with luciferin–luciferase. Ten microlitres of ATP standard (5 μM) was then injected 1.5 min after the nicotine injection. The maximum light output, recorded as peak height on the recorder following nicotine injection, was compared with that due to the ATP standard. To minimize the systematic variations of the assay, the effect of drugs on nicotine-evoked ATP release was assessed by a series of paired trials, one of which was performed in the presence of drugs and one in its absence. The significance of the difference between the test and the control pairs was assessed by the Wilcoxon matched-paired signed rank test at 95% confidence [22].

Measurement of endogenous catecholamine release. The secretion of endogenous adrenaline (A) and noradrenaline (NA) from monolayer cultures of chromaffin cells was measured by use of HPLC with electrochemical detection (HPLC/ED). On the third day after plating, the culture dishes were removed from the incubator and allowed to equilibrate at room temperature for 5 min. Unless otherwise stated, each well then received a 5 min wash in 0.4 ml Locke's buffer of the following composition (mM): NaCl 154, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85, $MgSO_4$ 1.18, D-glucose 10, $CaCl_2$ 2.2, BSA 0.5% w/v, pH 7.4. This was followed by a 5 min pre-incubation in the presence of the test drug and then by a 10 min stimulation period with various secretagogues in the presence of test drug. The buffer from the stimulation period was collected and mixed with perchloric acid (PCA) to 0.4 M final concentration. Cellular catecholamines were extracted with 0.01 M PCA and then acidified to 0.4 M PCA. The protein precipitate was removed by centrifugation and the endogenous NA and A from the stimulation buffer and cell extracts were assayed by separation with HPLC (Bio-Rad VMA C18 reverse phase column; mobile phase 70 mM KH_2PO_4, 0.1 mM EDTA, 0.2% sodium heptane sulfonate, 5–10% methanol in deionised water; flow rate 1 ml/min) and quantitation by electrochemical oxidation (BAS LC-3A detector, 650 mV). Dihydroxybenzylamine (DHBA) was used as an internal standard to correct for injection volume variations. Results were expressed as the percent release of total cellular NA and A content. The significance of the difference between the test and the control was assessed by the nonparametric Kruskal–Wallis test.

Desensitization experiment protocol. The protocol for testing the effect of clonidine and propranolol on nicotine-induced desensitization of catecholamine release was modified from that described by Boksa and Livett [24]. Experiments were performed at room temperature (20–25°). The 3-day-old monolayer chromaffin cell cultures first received a 5 min wash in 0.4 ml Locke's buffer, followed by either a 5 min wash with Locke's buffer (control) or a 5 min pre-incubation of adrenoceptor agent. The cells in each group were then pre-stimulated with a range of nicotine concentrations (0.3–300 μM) in the presence (test) or absence (control) of drug for 10 min (incubation I, Inc I), rapidly (<30 sec) washed twice with Locke's buffer, and both control and test cells re-

stimulated with 5 μM nicotine *without* adrenoceptor drug for 10 min (Incubation II, Inc II). When studying the effects of different concentrations of clonidine on desensitization, the cells were stimulated with 3 μM nicotine in the presence of various concentrations of clonidine (0.01 μM–1 mM) in Inc I for 10 min, washed and subsequently stimulated with 5 μM nicotine alone. CA release during Inc I and Inc II was measured in samples of supernatant and samples of the cell lysates in 1 ml of 0.4 M perchloric acid by use of HPLC with electrochemical detection (HPLC/ED). Results were expressed in terms of the percentage of cellular noradrenaline (NA) and adrenaline (A) content released in 10 min.

RESULTS

Effects of adrenoceptor agents on nicotine-induced ATP release

Figure 1 shows the effects of exogenous catecholamines on ATP release evoked by 5 μM nicotine on freshly isolated chromaffin cells. Adrenaline over the range of 1 μM–0.1 mM had no effect on nicotine-induced ATP release. However, at higher concentrations (0.5 mM–1 mM), it produced about 10–20% inhibition ($P < 0.01$). A similar result was obtained for noradrenaline. Dopamine, on the other hand, failed to modify nicotinic stimulation over the 1 μM–1 mM range tested.

The ability of a number of adrenoceptor agents to modify nicotine-induced ATP release was also investigated. As shown in Fig. 2, (±)isoproterenol (100 μM), a β-adrenoceptor agonist, had no effect on ATP release induced by nicotine, whereas (±)propranolol, a β-adrenoceptor antagonist, markedly inhibited the release by about 50% at 5 μM. Both clonidine (100 μM) and oxymetazoline (100 μM), the α_2-adrenoceptor agonists, produced significant inhibition and the latter appeared to be more potent. Yohimbine (100 μM), an α_2-adrenoceptor antagonist, however, showed similar inhibitory effects to clonidine (100 μM). None of these α_2-adrenoceptor agents had any significant inhibitory effect at concentrations less than 1 μM (results not shown).

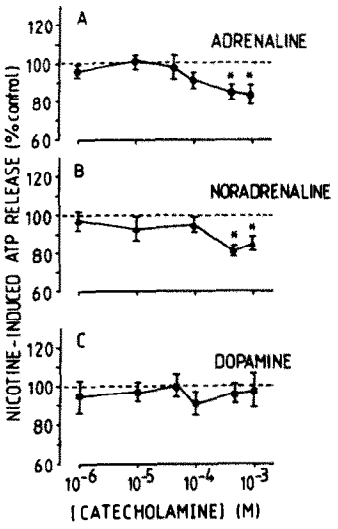

Fig. 1. Dose effects of exogenous adrenaline (●), noradrenaline (▲) and dopamine (■) on ATP release induced by 5 μM nicotine from freshly isolated bovine adrenal chromaffin cells. ATP release was expressed as percentage of control release determined in a series of paired trials (see Methods). Values are means ± SEM from 8–16 determinations. * denotes a significant difference from the control ($P < 0.01$ by Wilcoxon rank-sum test).

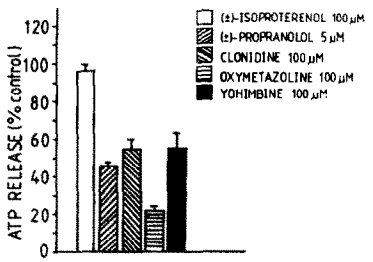

Fig. 2. Effect of adrenoceptor agents on ATP release induced by 5 μM nicotine from freshly isolated bovine adrenal chromaffin cells. Details as for Fig. 1. The histograms represent the means ± SEM from 8–11 determinations.

As shown in Fig. 2, (±)propranolol is a potent β-adrenoceptor antagonist able to inhibit ATP release induced by nicotine. In order to examine whether the inhibitory effect of (±)propranolol was acting via blockade of the putative β-adrenoceptor receptor on the chromaffin cells, the ability of other β-adrenoceptor agonists to reverse the inhibition by (±)propranolol was examined. As shown in Fig. 3, high concentrations of adrenaline (100 μM) and (±)isoproterenol (100 μM) failed to reverse the inhibition produced by (±)propranolol.

Effects of adrenoceptor agents on CA secretion induced by nicotine

In order to characterize further adrenoceptor modulation of secretion on the chromaffin cells, a series of experiments were performed to measure endogenous CA secretion from 3-day-old monolayer cultures. Figure 4 shows the effect of α_2-adrenoceptor agents on NA and A secretion induced by 5 μM nicotine. This concentration of nicotine produced approximately half maximal secretion of NA and A. Basal NA and A secretion were usually in

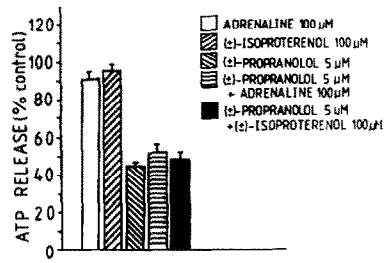

Fig. 3. Lack of antagonism by adrenaline (100 μM) or isoproterenol (100 μM) on the inhibition by (±)propranolol of nicotine-induced ATP release. ATP release was expressed as a percentage of control. Adrenaline or isoproterenol were injected to the cell suspensions 1 min prior to the injection of propranolol. Histograms represent the means ± SEM from 8–10 determinations.

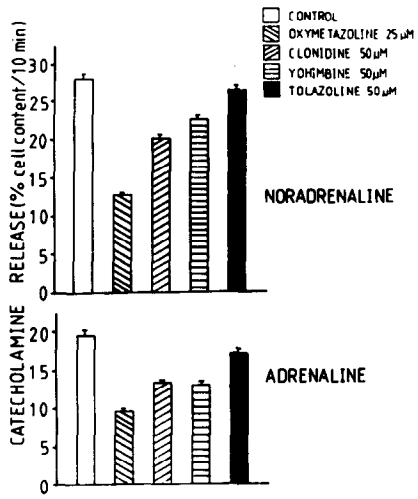

Fig. 4. Effect of α-adrenoceptor agents on catecholamine release induced by 5 μM nicotine from 3-day-old cultured, bovine adrenal chromaffin cells. Results were plotted as the % release of cellular content. Top panel: noradrenaline release. Bottom panel: adrenaline release. Data are means ± SEM from 6 determinations.

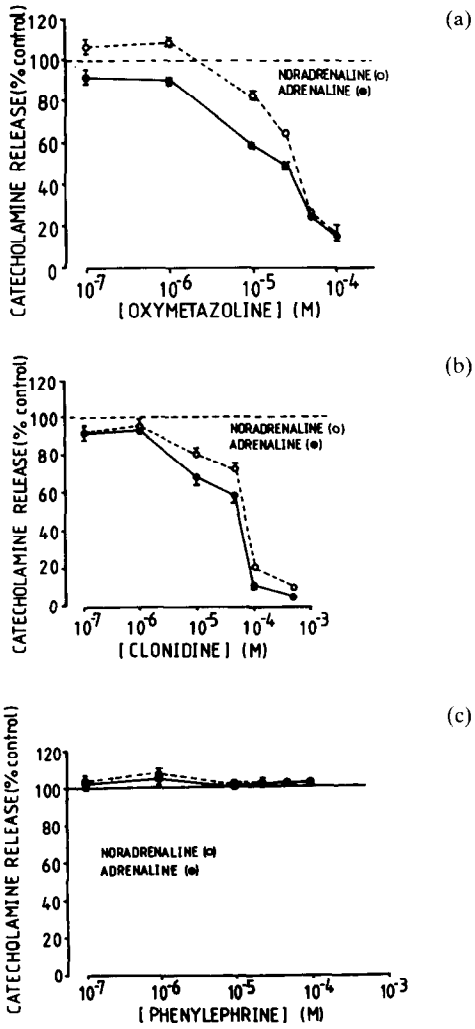

Fig. 5. Dose effects of oxymetazoline, clonidine and phenylephrine on 5 μM nicotine-induced catecholine release from cultured bovine adrenal chromaffin cells. Results are plotted as the % of control release in the absence of adrenoceptor agent. (a) Oxymetazoline (10^{-7}–10^{-4} M); (b) clonidine (10^{-7}–10^{-3} M); (c) phenylephrine (10^{-7}–10^{-4} M). Data points are means ± SEM from 4 determinations.

the range of 0.3–0.8% of cellular content and NA and A release evoked by 5 μM nicotine for 10 min were typically 25–30% and 15–20% of cell content respectively. Consistent with the results of inhibition of nicotine-induced release of ATP, oxymetazoline (25 μM), clonidine (50 μM) and yohimbine (50 μM) all inhibited nicotine-induced NA and A release. Tolazoline, another α-antagonist, however, showed little inhibitory activity. The order of inhibitory potency was: oxymetazoline > clonidine > yohimbine > tolazoline.

Inhibition of nicotine-induced CA secretion by oxymetazoline and clonidine were concentration-dependent with threshold effective concentrations at 1 μM (Fig. 5). By contrast, phenylephrine, an α_1-adrenoceptor agonist, failed to affect nicotine-induced CA release over the range of 0.1 μM–100 μM (Fig. 5c).

As for the effect of β-adrenoceptor agents, (±)propranolol (5 μM) markedly inhibited CA release produced by nicotine while salbutamol, a β_1-adrenoceptor agonist, was without effect at 50 μM concentration (Fig. 6). None of these agents in the concentrations tested had any effects on basal CA secretion. The inhibitory effects of (±)propranolol was concentration-dependent with an IC_{50} about 2 μM (Fig. 7a). Nevertheless, (+)propranolol, an isomer with little β-adrenoceptor antagonist activity [25, 26], produced a quantitatively similar inhibition (Fig. 7b).

Effects of adrenoceptor agents on CA secretion induced by K^+ *depolarization, histamine and* Ba^{2+}

CA secretion from the chromaffin cells can also be evoked by K^+ depolarization [27], histamine [28] and Ba^{2+} [29, 30]. Experiments were performed to investigate if adrenoceptor agents can also affect CA secretion induced by these secretagogues. The results are shown in Table 1. NA and A release induced by 56 mM K^+ for 10 min were typically about 15% and 8% of cellular content respectively. Adrenoceptor agents at the concentrations that effectively inhibited nicotine-induced CA secretion failed to affect secretion caused by high K^+.

Histamine has recently been shown to induce a concentration-dependent, Ca^{2+}-dependent CA secretion from cultured bovine adrenal chromaffin cells with an EC_{50} of 150 nM and maximal secretion at 10 μM. Histamine-induced CA secretion is mediated by H1-histamine receptors as the release was completely blocked by mepyramine and was unaffected by cimetidine [28]. NA and A secretion induced by 10 μM histamine for 20 min. were about 4% and 3% of cellular content respectively. Both the α_2-adrenoceptor agonists, oxymetazoline (50 μM) and clonidine (50 μM) failed to modify either NA or A secretion. Yohimbine (50 μM)

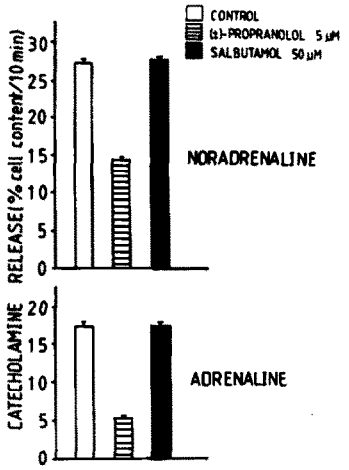

Fig. 6. Effects of (±)propranolol and salbutamol on 5 μM nicotine-induced catecholamine release from cultured bovine adrenal chromaffin cells. Top panel: noradrenaline release; bottom panel: adrenaline release. Results are plotted as the % of cellular content released/10 min. Data points are means ± SEM from six determinations.

slightly enhanced NA secretion with little effect on A secretion. The β-adrenoceptor agents, (±)propranolol (5 μM), salbutamol (50 μM) and (±)isoproterenol (50 μM) were all without effect.

Ba^{2+} (2.2 mM) caused a differential release of NA (30% cell content) over A (20% cell content) during a 10 min incubation period in the absence of extracellular Ca^{2+}. None of the adrenoceptor agents

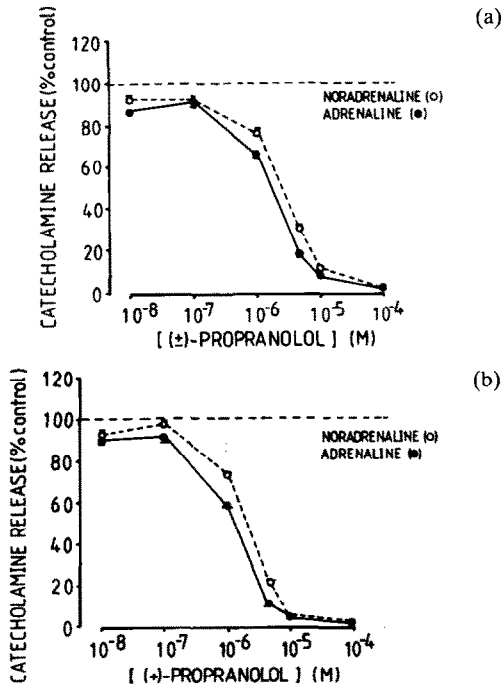

Fig. 7. Effects of (±)propranolol and (+)propranolol on 5 μM nicotine-induced catecholamine release from cultured bovine adrenal chromaffin cells. Results are plotted as the % of control release in the absence of adrenoceptor agents. (a) (±)Propranolol (10^{-8}–10^{-4} M); (b) (+)propranolol (10^{-8}–10^{-4} M). Data points are means ± SEM from four determinations.

Table 1. Effect of α- and β-adrenoceptor agents on 56 mM K^+, 10 μM histamine and 2.2 mM Ba^{2+}-induced CA secretion

	56 mM K^+		Histamine		Ba^{2+}	
	CA release (% content/10 min)		CA release (% content/20 min)		CA release (% content/10 min)	
	NA	A	NA	A	NA	A
α-Adrenoceptor agents						
Control	15.6 ± 0.3	8.1 ± 0.3	3.7 ± 0.1	3.6 ± 0.1	30.9 ± 0.7	21.8 ± 0.5
Oxymetazoline (50 μM)	15.2 ± 0.7	8.4 ± 0.5	3.4 ± 0.2	3.3 ± 0.2	32.3 ± 0.5	23.3 ± 0.5
Clonidine (50 μM)	15.1 ± 0.3	7.9 ± 0.2	3.4 ± 0.2	3.3 ± 0.1	32.2 ± 0.5	22.2 ± 0.5
Yohimbine (50 μM)	13.6 ± 0.2	6.5 ± 0.2	4.5 ± 0.2	3.3 ± 0.1	31.2 ± 1.1	21.3 ± 0.5
Talozoline (50 μM)	16.0 ± 0.4	7.5 ± 0.3	n.d.	n.d.	n.d.	n.d.
β-Adrenoceptor agents						
Control	16.8 ± 0.7	7.8 ± 0.6	4.0 ± 0.1	3.2 ± 0.1	30.6 ± 0.8	21.3 ± 0.7
(±)Propranolol (5 μM)	15.8 ± 0.5	7.2 ± 0.4	3.9 ± 0.2	3.1 ± 0.2	30.7 ± 0.9	20.8 ± 0.6
(±)Isoproterenol (50 μM)	n.d.	n.d.	3.9 ± 0.1	3.2 ± 0.1	32.8 ± 0.8	22.8 ± 0.5
Salbutamol (50 μM)	16.1 ± 0.5	7.7 ± 0.5	4.0 ± 0.1	3.4 ± 0.1	n.d.	n.d.

Data are means ± SEM from N = 6. In 56 mM K^+-induced secretion, oxymetazoline concentration was 25 μM instead.
n.d. = not determined.

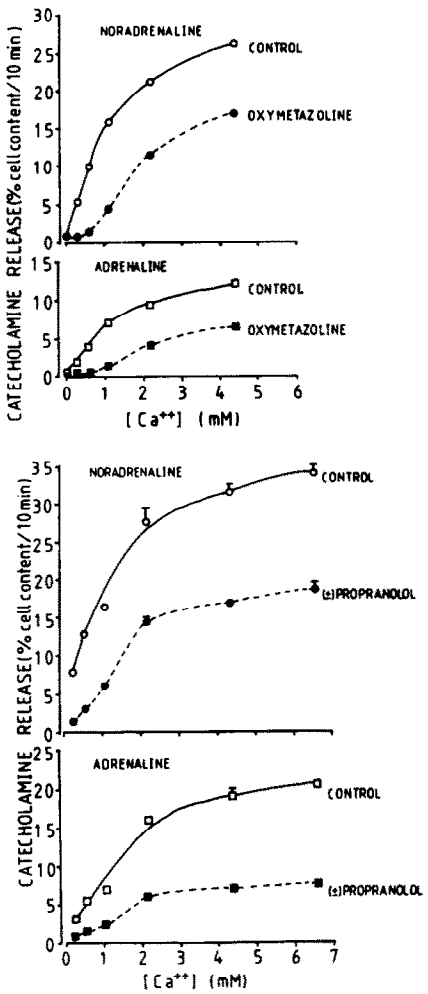

Fig. 8. Ca^{2+}-dependence of the inhibition by oxymetazoline and (±)propranolol of nicotine-induced catecholamine release from cultured bovine adrenal chromaffin cells. Results are expressed as the % of cellular content released/10 min. (a) Effects of oxymetazoline; (b) effects of (±)propranolol. Data points are means ± SEM from four determinations.

tested at concentrations which affect nicotinic responses had any effect on Ba^{2+}-induced secretion.

Ca^{2+} *dependence of the inhibitory response*

Figure 8 shows the inhibitory effects of oxymetazoline and propranolol on nicotine-induced CA secretion at different concentrations of extracellular Ca^{2+}. Nicotine caused a Ca^{2+}-dependent CA secretion. The inhibitory effects of oxymetazoline and propranolol were also Ca^{2+}-dependent. A higher percentage of inhibition was achieved by these agents at lower Ca^{2+} concentration. The inhibitory effects, however, could not totally be reversed by higher concentrations of Ca^{2+}.

Na^+-*dependence of the inhibitory response*

In the experiments above, it was found that the adrenoceptor agents affect *only* nicotine-induced secretion. It seems likely that these adrenoceptor agents, instead of acting directly on the nicotine binding sites, bind to a second site on the nicotinic receptor which caused the impairment of subsequent ion movement through the nicotinic receptor–ionophore complex. To address this possibility, the effect of some adrenoceptor agents on nicotinic responses in normal and Na^+-free medium was compared. Na^+ has been shown to compete with Ca^{2+} for the nicotinic receptor ionophore complex and depletion of Na^+ would thus enhance the influx of Ca^{2+} through the nicotinic receptor–ionophore complex, thereby increasing CA secretion. Two different Na^+-free buffers were tested. Initially, the Na^+-free buffer was prepared according to Wada *et al.* [31] with the following composition: 10 mM Tris–HCl, pH 7.4, 270 mM sucrose, KCl 2.6, $MgSO_4$ 1.18, D-glucose 10, $CaCl_2$ 2.2, BSA 0.5% w/v. As shown in Table 2, the nicotine-induced secretion was significantly reduced in Tris–sucrose buffer system. In the presence of adrenoceptor agents, a higher percentage of inhibition was seen in Tris–sucrose buffer. One interpretation of this data is that Na^+-depletion caused a reduction in nicotine-induced secretion. However, when the experiments were performed using Locke's sucrose buffer (in which NaCl was

Table 2. Effect of adrenoceptor agents on nicotine-induced CA secretion in Locke's and Na^+-free Tris–sucrose buffer

	Catecholamine release (% cell content/10 min)			
	Locke's buffer		Na^+-free Tris–sucrose	
Treatment	NA	A	NA	A
Control	28 ± 1.7	19.7 ± 1.6	13.0 ± 0.5†	7.7 ± 0.4†
Oxymetazoline	12.6 ± 0.5*	7.9 ± 0.3*	1.7 ± 0.03*	0.9 ± 0.01*
Clonidine	20.3 ± 0.9*	13.4 ± 0.8*	3.9 ± 0.02*	1.4 ± 0.2*
Yohimbine	22.8 ± 1.3*	13.1 ± 1.3*	7.7 ± 0.1*	3.2 ± 0.01*
(±)Propranolol	14.3 ± 0.6*	5.2 ± 0.4*	1.9 ± 0.01*	0.8 ± 0.1*
Salbutamol	27.8 ± 0.1	17.5 ± 0.5	10.0 ± 0.3	3.7 ± 0.1*
Tolazoline	26.4 ± 0.7	15.4 ± 0.5	6.2 ± 0.8*	1.9 ± 0.4*

Data are mean ± SEM of four determinations.

* $P < 0.001$ from the control.

† $P < 0.001$ when compared to the Locke's buffer control.

Concentrations of the agents are: oxymetazoline (25 μM), clonidine (50 μM), yohimbine (50 μM), (±)propranolol (5 μM), salbutamol (50 μM), tolazoline (50 μM). Cells were stimulated with 5 μM nicotine for 10 min.

replaced by 270 mM sucrose in Locke's buffer to maintain isotonicity) in this buffer nicotine-induced secretion was not reduced, in spite of the absence of Na^+. Once more, however, the inhibitory effects of the adrenoceptor agents were greater in the Na^+-free Locke's-sucrose buffer than in control Locke's buffer (Fig. 9, compared to Fig. 4 and Fig. 6).

Effects of clonidine and propranolol on desensitization of the nicotinic response

The release of CA from chromaffin cell cultures during Inc I and Inc II in response to various concentrations of nicotine in the absence and presence of clonidine (50 μM) is shown in Fig. 10. During the first incubation (Inc I), there was a concentration-dependent release of NA and A with a threshold nicotine concentration of 0.3 μM and with a maximum at 10–30 μM. The EC_{50} for nicotine-evoked CA release was about 3 μM. Exposure to higher concentrations of nicotine (> 30 μM) caused a decreased release of both A and NA indicative of

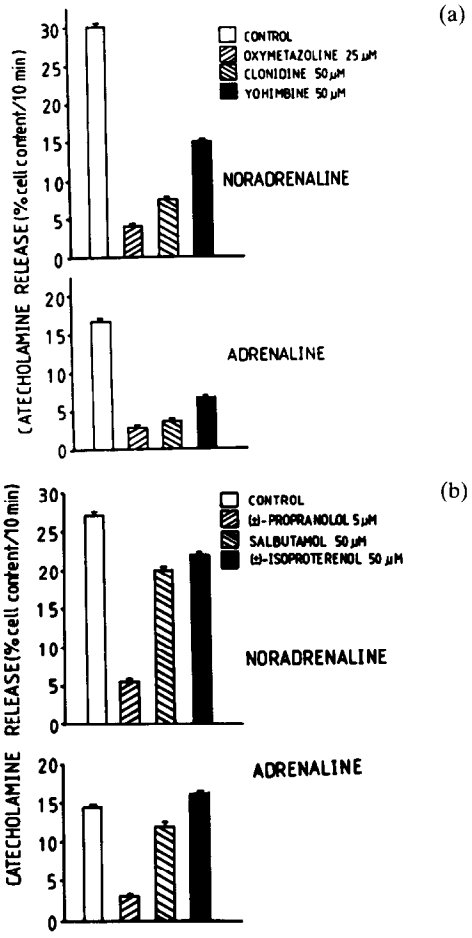

Fig. 9. Effects of α- and β-adrenoceptor agents on 5 μM nicotine-induced catecholamine release from cultured bovine adrenal chromaffin cells in Na-free Locke's sucrose buffer. Results are expressed as the % of cellular content released/10 min. Top panels: noradrenaline release. Bottom panels: adrenaline release. (a) Effects of α-adrenoceptor agents; (b) effects of β-adrenoceptor agents. Data points are means ± SEM from six determinations.

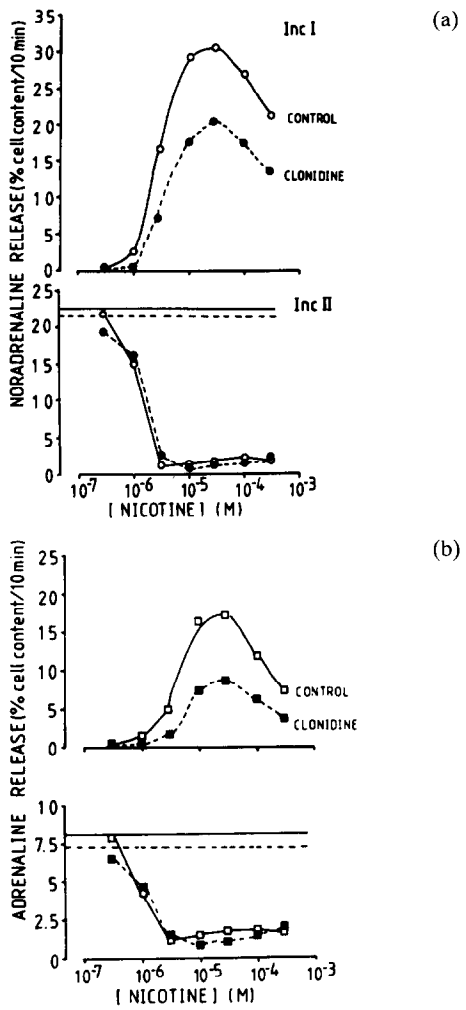

Fig. 10. Effect of clonidine on nicotine-evoked release and nicotine-induced desensitization of (a) noradrenaline and (b) adrenaline release. Chromaffin cell cultures were incubated for 10 min with nicotine (0.3–300 μM) with (●, ■) or without (○, □) clonidine (50 μM) (Inc I), rapidly washed and re-stimulated for 10 min with 5 μM nicotine (Inc II). CA release during Inc I and Inc II are expressed as a percentage of the cellular CA content present at the beginning of each incubation period. Each point represents the mean ± SEM of six determinations. In all cases the error bars lie within the extent of the symbols. The solid horizontal line in Inc II represents control release (no nicotine, no clonidine in Inc I), and the dashed horizontal line represents the release from cells pre-incubated with 50 μM clonidine (no nicotine) in Inc I.

desensitization. Clonidine (50 μM) inhibited NA release produced by nicotine during Inc I in a non-competitive manner. Desensitization caused by exposure to the higher concentrations of nicotine in Inc I was unaffected by clonidine.

On re-stimulation of the cells (Inc II) with a sub-maximal concentration of nicotine (5 μM), NA release was reduced in cultures that had been stimulated in Inc I with 0.3 μM to 3 μM nicotine (Fig. 10a). Desensitization of the nicotinic response in Inc II was seen when nicotine concentrations in Inc I were above 0.1 μM. At 1 μM nicotine in Inc I, the response to 5 μM nicotine in Inc II was reduced by about 30%

and at 3 μM nicotine and above in Inc I it was reduced by 95% in Inc II (> 95% desensitized). It should be noted that 1 μM nicotine in Inc I caused about 1/10 of maximal release, yet on re-stimulation with 5 μM nicotine in Inc II there was about 30% desensitization of the secretory response. Moreover, at 3 μM nicotine during Inc I, NA release was only half the maximal release (i.e. that seen with 10 μM nicotine), yet produced the same degree of desensitization. This clearly shows that nicotine-evoked desensitization is not merely due to depletion of the CA pool upon pre-exposure to nicotine. Those cultures that had been exposed to nicotine in the presence of clonidine during Inc I showed a similar degree of desensitization of NA release as cultures exposed to nicotine alone regardless of the substantial inhibition of NA release by clonidine at each nicotine concentrations. This indicates that clonidine neither enhances, nor protects against the nicotine-induced desensitization. A similar result was observed with A release (Fig. 10b); again clonidine had no effect on nicotine-induced desensitization. It should be noted that in the cultures exposed to clonidine during Inc I, there was a small residual inhibitory effect on evoked CA release during Inc II despite two rapid washes to remove clonidine. This is evident from the slight reduction in catecholamine release compared with control (about 5% for NA and 12% for adrenaline) in Inc II in cultures that had been exposed to clonidine alone in Inc I. To ensure a comparison of the effects of clonidine present in Inc I on the degree of desensitization in Inc II, the data were normalized with respect to control values (Fig. 11). This presentation more clearly shows the lack of effect of clonidine on the desensitization produced by nicotine.

As seen in Fig. 10, desensitization in Inc II occurred in response to preincubation with nicotine in Inc I over the range of 0.3–3 μM. In order to investigate whether different concentrations of clonidine would affect the extent of desensitization, cells were stimulated with 3 μM nicotine in the presence of different concentrations of clonidine in Inc I and re-stimulated with 5 μM nicotine in Inc II. Figure 12 shows that clonidine caused a concentration-dependent inhibition of nicotine-induced NA and A secretion. A total inhibition of CA secretion was achieved at 1 mM clonidine. However, clonidine even at 1 mM had no substantial effect on subsequent desensitization of the nicotinic response.

Figure 13 shows the effect of (±)propranolol on desensitization of the nicotinic secretory response. As with clonidine, (±)propranolol (5 μM) produced a non-competitive inhibition of both NA and A secretion caused by nicotine over the range of 0.3 μM–1 mM in Inc I. A slight shift in the maximum nicotinic response to higher nicotine concentrations was observed in the presence of propranolol which was not seen with clonidine. During Inc II, the nicotine-evoked secretion of both NA and A was reduced by about 30% for NA and 35% for A in cells that had been pre-incubated with propranolol alone as compared to the controls. This is probably due to the residual effect of propranolol by carry-over from Inc I. In spite of this, propranolol pretreated cells followed a parallel rate of desensitization to control cells when stimulated with nicotine in Inc II, indicating that propranolol did not affect the onset or rate of desensitization. Normalization of data showed that (±)propranolol enhanced desensitization of the nicotinic secretory

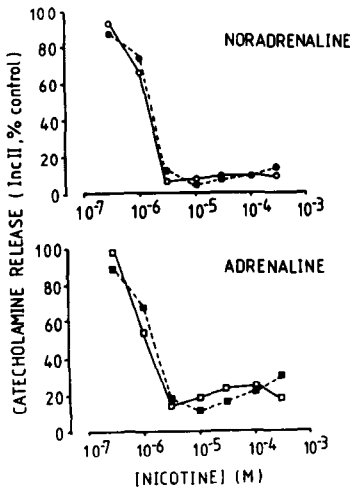

Fig. 11. Effect of clonidine on nicotine-induced desensitization of noradrenaline and adrenaline release in Inc II. Data from Fig. 10, lower panels has been normalized to 100% of control release. The percentage of total noradrenaline released during Inc II from cells not exposed to nicotine in Inc I was 22.9 ± 0.4 and 21.9 ± 0.3 in the absence (○) and presence (●) of clonidine respectively and the percentage of A release was 8.2 ± 0.2 and 7.2 ± 0.1 in the absence (□) and presence (■) of clonidine respectively.

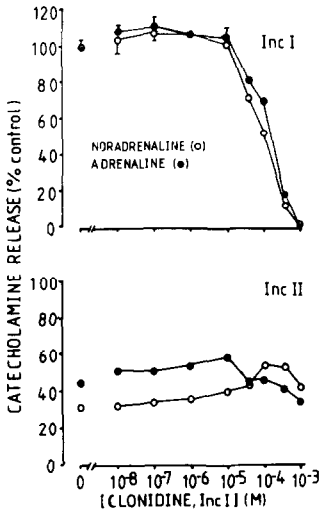

Fig. 12. Effect of clonidine on nicotine-evoked release and nicotine-induced desensitization. Chromaffin cell cultures were incubated for 10 min with 3 μM nicotine in the presence of 10 nM–1 mM clonidine (Inc I), rapidly washed and re-stimulated for 10 min with 5 μM nicotine (Inc II). CA release during Inc I and Inc II are expressed as a percentage of the cellular CA content present at the beginning of each incubation period. Each point represents the mean ± SEM of results from four determinations. Error bars were omitted where they lie within the extent of the symbols.

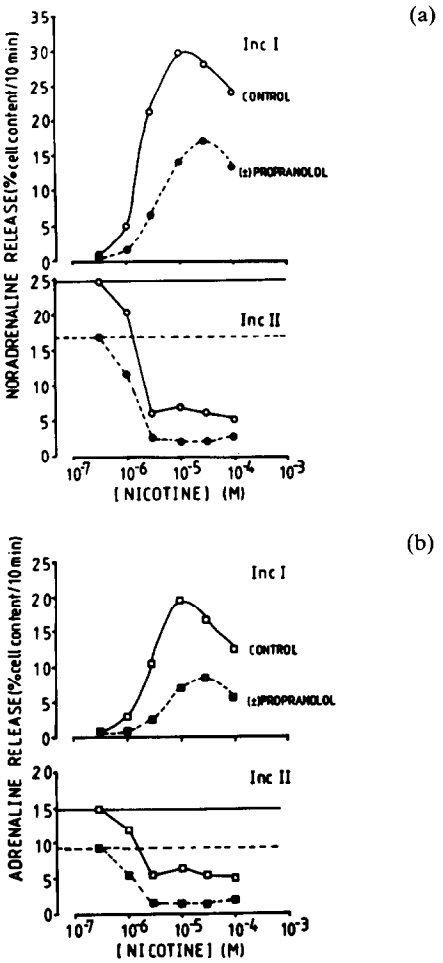

Fig. 13. Effect of (±)propranolol on nicotine-evoked release and nicotine-induced desensitization of (a) noradrenaline and (b) adrenaline release. Chromaffin cell cultures were incubated for 10 min with nicotine (0.3–300 μM) with (●, ■) or without (○, □) (±)propranolol (5 μM) (Inc I), rapidly washed and re-stimulated for 10 min with 5 μM nicotine (Inc II). CA release during Inc I and Inc II are expressed as a percentage of the cellular CA content present at the beginning of each incubation period. Each point represents the mean ± SEM of results from six determinations. In all cases the error bars lie within the extent of the symbols. The solid horizontal line in Inc II represents control release (no (±)propranolol, no nicotine in Inc I), and the dashed horizontal line represents the release from cells pre-incubated with 5 μM (±)propranolol (no nicotine) in Inc I.

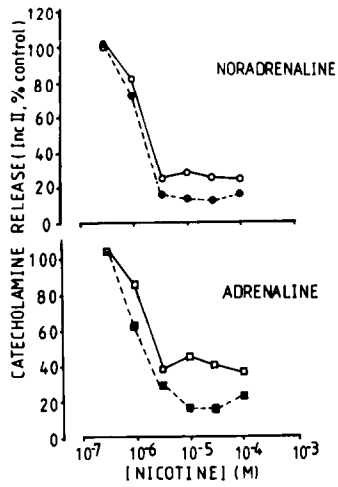

Fig. 14. Effect of (±)propranolol on nicotine-induced desensitization of noradrenaline and adrenaline release in Inc II. Data from Fig. 13, lower panel has been normalized to 100% of control release. The percentage of total noradrenaline released during Inc II from cells not exposed to nicotine in Inc I was 25 ± 0.2 and 16.1 ± 0.4 in the absence (○) and presence (●) of (±)propranolol respectively and the percentage of A release was 14.5 ± 0.3 and 8.6 ± 0.3 in the absence (□) and presence (■) of (±)propranolol respectively.

response at concentrations of nicotine > 10 μM (Fig. 14).

DISCUSSION

Previous studies on release of catecholamines from isolated bovine adrenal chromaffin cell suspensions have suggested the presence of both α- and β-adrenoceptor regulatory feedback mechanisms [10, 12]. This conclusion was based on the findings that some α_2-adrenoceptor agonists such as clonidine and oxymetazoline inhibited acetylcholine-induced catecholamine release. β-agonists, such as isoproterenol potentiated, while the β-antagonist, propranolol, inhibited acetylcholine-induced catecholamine release. However, no evidence of similar adrenoceptor regulation was found by other investigators using perfused rabbit or rat adrenal glands [13–16, 19]. In the present study, we re-investigated this controversy by using freshly prepared bovine adrenal chromaffin cells as well as monolayer cultures and have monitored secretion by two methods: (1) ATP bioluminescence and (2) measurement of endogenous catecholamines by HPLC/ED. The advantages of using monolayer cultures are twofold. First, monolayer cultures give a lower basal secretion than cell suspensions thereby increasing the sensitivity of detecting any drug effects on catecholamine release; second, the use of isolated cells allowed us to investigate only the post-synaptic actions without the complication of the possible presynaptic actions of adrenoceptor agents on splanchnic nerve terminals that may occur in perfused adrenal gland preparations.

Actions of adrenoceptor agents on ATP release from chromaffin cell suspensions

The validity of the luciferin–luciferase bioluminescence method for monitoring exocytotic release from chromaffin cells has been demonstrated and evaluated previously in our laboratory where it had been used to examine the ability of opioid peptides and tachykinins to modify nicotine-induced ATP release from chromaffin cels [22, 32]. In the present experiments, the ability of adrenoceptor agents to modify ATP release induced by 5 μM nicotine was examined. Both adrenaline, and noradrenaline were poor inhibitors of nicotine-induced ATP release, and dopamine had little effect at con-

centrations up to 1 mM. Although it has been reported that dopamine D2 receptors are present in the bovine adrenal medulla [33, 34], these receptors had very low affinity for dopamine. Our finding suggests that dopamine itself does not play a significant role in modifying CA secretion. The findings that noradrenaline and adrenaline were weak in modifying nicotine-induced release argues against an autoregulatory role *in vivo* (see also Ref. 18).

It might be argued that although noradrenaline and adrenaline were weak in modifying nicotine-induced secretion, there might still be a highly selective adrenoceptor present in these cells. We tested this by screening some typical α- and β-adrenoceptor agonists and antagonists for their ability to modify nicotine-induced ATP release. Contrary to the findings of Greenberg and Zinder [10], we found no evidence for facilitation of CA release by β-adrenoceptor activation. Isoproterenol (100 μM), a β-adrenoceptor agonist, did not modify nicotine-induced ATP release nor did it stimulate release on its own. On the other hand, we were able to confirm their findings on the marked inhibitory effect of propranolol (5 μM) on nicotine-induced ATP release. However, this inhibitory effect is unlikely to have been produced by β-adrenoceptor blockade since this inhibition could not be reversed even by high concentrations of β-agonists (e.g. adrenaline or isoproterenol).

In agreement with previous findings [10, 12, 18], we were also able to demonstrate an inhibition of nicotine-induced secretion by clonidine and oxymetazoline. Yohimbine, an α_2-adrenoceptor antagonist, had a similar inhibitory effect (see also Ref. 18). This lack of agonist–antagonist specificity indicated that these inhibitory effects are not mediated by a classical α_2-adrenoceptor interaction.

Actions of adrenoceptor agents on endogenous catecholamine secretion induced by different secretagogues

The experiments described above indicate that some adrenoceptor agents did have inhibitory effects in modifying nicotine-induced secretion of ATP from chromaffin cells. Further investigations were performed to determine their effects on evoked secretion of endogenous catecholamines. There were two main objectives of these experiments: (1) to examine if there were any differential effects on noradrenaline and adrenaline release induced by nicotine; and (2) to examine whether these inhibitory effects were restricted to nicotine-induced responses. The abilities of these adrenoceptor agents to modify endogenous catecholamine release induced by nicotine (5 μM), high K^+ (56 mM), Ba^{2+} (2.2 mM) and histamine (10 μM) were thus compared.

In agreement with the results from ATP release experiments, clonidine and oxymetazoline produced a concentration-dependent inhibition of NA and A release induced by 5 μM nicotine over the range 10–100 μM. These agents were more potent inhibitors of A than NA release. These inhibitory properties were shared by the α_2-adrenoceptor antagonist, yohimbine. The α_1-adrenoceptor agonist, phenylephrine, was, however, not effective in modifying nicotine-induced catecholamine release over the same concentration range. Tolazoline (50 μM), an α-antagonist was also without effect. Although it has been reported that [^{3}H]-clonidine binding to membrane fractions prepared from adrenal medullary cells is saturable, reversible and of high affinity consistent with α_2-adrenoceptor action [12], the high concentrations (> 10 μM) of adrenoceptor agents required for the inhibitory effects and the lack of receptor specificity argue against the hypothesis that the effects were produced by the activation of putative α_2-adrenoceptors by these adrenoceptor agents on the chromaffin cells.

Although (±)propranolol was found to be an effective inhibitor of catecholamine release induced by nicotine, the observation that (+)propranolol, an isomer with little β-adrenoceptor activity, was equally effective strongly argue against a classical β-adrenoceptor action.

All adrenoceptor agents tested at concentrations which caused significant inhibition of nicotine-induced release failed to affect release induced by high K^+, Ba^{2+} or histamine. The inhibitory effects on nicotine-induced secretion were dependent on extracellular Ca^{2+}, suggesting these drugs may exert their effects by blocking Ca^{2+} influx. However, the lack of effects of these agents on secretion induced by other secretagogues (both those with high secretory efficacy, e.g. Ba^{2+} or those with low efficacy, e.g. histamine) indicate that the Ca^{2+} channels associated with these agonists are not affected by these adrenoceptor agents. In contrast, Ca^{2+} entry through the ion channel associated with the nicotinic receptor–ionphore complex appears particularly sensitive to inhibition by these adrenoceptor agents. The non-competitive nature of the inhibitory effects on nicotine-induced release seem to rule out the possibility of direct competition with nicotinic binding sites on the receptor complex.

Actions of adrenoceptor agents on desensitization of the nicotinic response

Although desensitization of the nicotinic response has been much studied previously in perfused adrenal glands and isolated adrenal chromaffin cell preparations [24, 35–39], the mechanisms involved are still not known. Using isolated bovine adrenal chromaffin cells, a more thorough study was undertaken to characterize the mechanism of desensitization at the cellular level [24, 38]. It was shown that desensitization of CA release produced by ACh (or nicotine) had two components: one Ca^{2+}-dependent and the other Ca^{2+}-independent and depletion-independent. Substance P, a peptide endogenous to the splanchnic nerve innervating the adrenal medulla, inhibited nicotine-induced catecholamine release from chromaffin cells [40, 41] and in addition, protected against desensitization to subsequent cholinergic stimulation [24]. Whether a similar regulation occurs operated by endogenous CA was unknown. We have tested two adrenoceptor agents, clonidine and propranolol for their ability to modify desensitization of the secretory response induced by nicotine.

Evidence has been presented by previous investigators which suggests that depletion of releasable catecholamine stores alone is unlikely to account for

desensitization [36, 42]. The present data supports this view. Both clonidine (50 μM) and propranolol (5 μM) inhibited nicotine-evoked CA release from the cells in Inc I by more than 50%, thereby preserving a major proportion of the intracellular releasable CA pool, but upon re-stimulation (Inc II) the same degree of desensitization was seen in the adrenoceptor agent treated cells as in the controls. Furthermore, as shown in Fig. 12, a complete inhibition by clonidine (1 mM) of nicotine-induced secretion did not result in a protection of subsequent desensitization of the nicotine response despite no loss of CA stores from these cells during Inc I. It is clear that the extent of desensitization of the nicotinic response was more dependent on the concentration of nicotine in the pre-exposure period (Inc I) than on the extent of CA depletion. Stimulation in Inc I with nicotine (3–300 μM) released 15%–30% NA and 5%–17% A, yet all concentrations of nicotine in this range caused a similar degree of desensitization evident during re-stimulation.

Characterization by Boksa and Livett [38] of the cholinergic-agonist induced desensitization in isolated bovine chromaffin cells has shown that it has two components: one Ca^{2+}-dependent the other Ca^{2+}-independent. These authors suggested that cholinergic-agonist induced desensitization involves two separate events: (1) inactivation of a receptor-associated and voltage sensitive Ca^{2+} permeability, a process facilitated by Ca^{2+}, and (2) the transformation of the cholinergic receptor conformation from resting state to densensitized state, a process which is Ca^{2+}-independent but dependent on the concentration of cholinergic agonist present. Substance P is apparently able to protect both of the components involved in desensitization [24] but it is clear that adrenoceptor agents like clonidine and propranolol fail to protect either component. Similarly, a number of opioid compounds that have inhibitory actions on nicotine-induced CA secretion are also without effect or have only weak effects on desensitization of the nicotinic response [43]. Taken together, these results suggest that inhibition of CA secretion is not an obligatory requirement for protection against desensitization upon second stimulation. Whatever the mechanisms involved, these data indicated that nicotine-evoked secretion and nicotine-induced desensitization can be regulated differentially.

Are the inhibitory effects of adrenoceptor agents on CA release due to their (lipophilic) membrane stabilizing properties?

It has been proposed by others [15, 18, 44] that the inhibitory effects produced by propranolol and clonidine may be attributed to their lipophilic properties. Although it is still possible that some of the actions of these adrenergic agents on catecholamine release might be so attributed, the present findings indicate that drug lipophilicity cannot fully explain their inhibitory effects. This is based on: (1) The adrenoceptor agents affected only nicotinic receptor activated CA release with little effect on CA release induced by membrane depolarization or by other receptor-mediated secretagogues. If these drugs exerted their inhibitory effect by membrane stabilization, we would have expected them to affect secretory responses induced by these other secretagogues. (2) The inhibitory effect is not well correlated with the lipophilicity of the drug. Yohimbine, a drug which is highly lipophilic was a weaker inhibitor than the less lipophilic agents propranolol, clonidine or oxymetazoline. (3) The inhibitory effects of the adrenoceptor agents were dependent on the ionic composition of the release buffer. The findings that inhibition occurred in the absence of extracellular Na^+ further indicate that membrane depolarization is not a pre-requisite for the inhibitory effects. Instead, the mechanism may involve blockade of calcium influx through voltage-insensitive ion channels and this effect may be enhanced in the absence of Na^+. The observation that Na^+ competes with Ca^{2+} for influx through ion channel associated with the nicotinic receptor–ionophore complex [32] may help to explain why higher inhibitory effects by these adrenoceptor agents were observed in the absence of Na^+. Whatever the mechanism involved, our results do not support a role for α- and β-adrenoceptor regulation in the release of catecholamines from bovine chromaffin cells.

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