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# $\alpha_2$-Adrenoceptors Do Not Regulate Catecholamine Secretion by Bovine Adrenal Medullary Cells: A Study with Clonidine

D. A. POWIS[1] and P. F. BAKER

*Department of Physiology, King's College London, Strand, London, WC2R 2LS, United Kingdom*

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**SUMMARY**

Experiments have been performed with perfused bovine adrenal glands, with freshly isolated chromaffin cells, and with chromaffin cells maintained in tissue culture to investigate the suggestion that there are $\alpha$-adrenoceptors present which regulate catecholamine secretion. Only one set of observations has lent support to this suggestion: the rather specific $\alpha_2$-adrenoceptor agonist clonidine inhibits catecholamine secretion evoked by the physiological secretogogue, acetylcholine, and by the related nicotinic agonists, carbachol and nicotine. All other observations detract from the suggestion. Other $\alpha$-adrenoceptor agonists (noradrenaline, adrenaline, tramazoline, phenylephrine, and $\alpha$-methyl-noradrenaline) are virtually ineffective at inhibiting secretion evoked by carbachol. In addition, the $\alpha$-adrenoceptor antagonists phentolamine, phenoxybenzamine, and yohimbine not only fail to enhance the secretion of catecholamines evoked by carbachol but also fail to offset the inhibitory action of clonidine. The data suggest that functional $\alpha_2$-adrenoceptors of the classical type are not present upon bovine chromaffin cells and that, in this tissue, clonidine must act in some other way. In the bovine adrenal medullary chromaffin cell clonidine probably acts at the nicotinic receptor because it does not reduce catecholamine secretion evoked by depolarizing concentrations of potassium or veratridine but does reduce the carbachol-evoked influx of $^{22}Na$ that can be measured in the presence of tetrodotoxin and ouabain and which probably reflects entry through the nicotinic channel. Furthermore, clonidine can abolish, in reversible fashion, the acetylcholine-activated inward current determined with patch-clamp.

It is now 15 years since the suggestion was made that there are $\alpha$-adrenoceptors upon sympathetic, postganglionic nerve terminals (see, for example, Refs. 1–3). Stimulation of these receptors by newly released or circulating noradrenaline has been shown to effect a reduction in subsequent neuronal release evoked by depolarizing stimuli. The present experiments were undertaken to determine whether such adrenoceptors are present also on the chromaffin cells of the adrenal medulla.

There are reports already in the literature dealing with this question, but investigating groups differ in their conclusions. There are those who claim the presence of $\alpha$-adrenoceptors on adrenal medullary cells (4–10), others whose data do not exclude the possibility (11), and yet others whose data deny their presence (12, 13).

The present study provides conclusive evidence to deny the presence of $\alpha$-adrenoceptors, as currently defined pharmacologically, upon bovine adrenal medullary chromaffin cells.

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Some of the present data have been presented in preliminary form (14).

## Materials and Methods

### Experimental Preparations

Bovine adrenal glands were obtained from the abbatoir. They were removed as soon as possible after death, dissected free of perinephric fat and connective tissue, and flushed via the central vein to remove blood. The flushing solution used was ice-cold Locke's solution (composition below) containing 0.2% (w/v) bovine serum albumin. Glands intended for tissue culture were flushed instead with a sterile solution as above containing, additionally, penicillin, streptomycin, and amphotericin B (100 IU/ml, 100 $\mu$g/ml, and 0.25 $\mu$g/ml, respectively; Sigma A9909). The glands were transported to the laboratory on ice.

**Intact adrenal glands.** Adrenal glands were perfused retrogradely via the central vein with bicarbonate-buffered solutions according to the method of Banks (15) as modified by Baker and Rink (16). The perfusion rate was maintained at 10 ± 2 ml/min. Catecholamine secretion was measured in samples of the gland effluent by fluorimetry (see below).

**Isolated adrenal medullary cells.** Chromaffin cells were isolated from slices (0.5 mm thick) of adrenal medullae by a method essentially the same as that described by Knight and Baker (17), except that digestion was achieved with 0.05% protease (type XIV; Sigma P5147),

**ABBREVIATIONS:** Hepes, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; ACh, acetylcholine; CCh, carbamylcholine; DHE, dihydroergocryptine.

0.05% collagenase (type II; Sigma C6885), 0.05% hyaluronidase (type 1S; Sigma H3506), and 0.003% DNase I (type III; sigma D4638). Each gland yielded typically in excess of $10^7$ cells estimated by standard hemocytometry of which more than 95% excluded trypan blue and were thus judged viable. Cells from the adrenal cortex were the major contaminant of the cells suspension amounting in some preparations to as much as 35–40% of the total cell count as assessed by neutral red staining of medullary cells (18). Preparations of cortical cells [98% pure as judged by neutral red staining (18)] did not secrete catecholamines and were considered unlikely to interfere with the experiments to be described.

**Isolated adrenal medullary cells maintained in primary tissue culture.** Chromaffin cells were isolated as above but under sterile conditions using aseptic techniques. All solutions contained 1% antibiotic/antimycotic mixture (Sigma A9909). After disaggregation, the cells were washed four times in Earle's balanced salts solution and finally resuspended in Dulbecco's modified Eagle's medium containing, in addition, Hepes (5 mM), penicillin G (100,000 IU/ml), gentamicin (4.9 mg/100 ml), 5-fluorodeoxyuridine (5 $\mu$M), cytosine arabinofuranoside (5 $\mu$M), and fetal calf serum (10%, v/v). The cells were counted in a hemocytometer and the suspension was diluted to yield 4–8 $\times$ $10^5$ cells/ml. The cells were cultured in 24-well cluster plates (4–8 $\times$ $10^5$ cells/well); cell attachment occurred by day 3, and half the medium was replaced every 3 days. Plated cells were used for experimentation between day 3 and day 22. In experiments where patch-clamp pipettes were to be used, cells were cultured on glass coverslips, one slip to each well. It was found to be unnecessary to pretreat the coverslips other than to clean and sterilize them; the cells attached well to untreated glass.

## Solutions, Materials, and Drugs

The primary solution used was Hepes-buffered Locke's solution containing (mM): NaCl, 144; KCl, 5.4; $MgCl_2$, 1.8; $CaCl_2$, 1.8; Hepes, 10; and glucose, 5 adjusted to pH 7.2 ± 0.1 using NaOH. This solution was equilibrated with 100% $O_2$. In experiments with perfused, intact adrenal glands, a bicarbonate-buffered solution was used. This contained (mM): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 1.8; $KH_2PO_4$, 1.2; $NaHCO_3$, 25.0; and glucose, 11 and was equilibrated with 5% $CO_2$/95%$O_2$. In solutions of increased potassium content, NaCl was replaced by KCl on an equimolar basis.

All inorganic chemicals were of analytical grade and were obtained either from B.D.H. or from Sigma; organic chemicals not specifically listed below, and enzymes, were obtained from Sigma. Radiochemicals ($^3$H-noradrenaline, $^3$H-ouabain, $^3$H-clonidine, $^3$H-DHE, and $^{22}$Na) were obtained from Amersham International.

Materials for tissue culture were obtained either from Flow Laboratories or from Sigma.

Drugs used were obtained as follows: ACh chloride, adrenaline bitartrate, CCh chloride, clonidine hydrochloride, noradrenaline bitartrate, ouabain octahydrate, phenylephrine hydrochloride, tetrodotoxin, and yohimbine hydrochloride, all from Sigma; nicotine, B.D.H.; phentolamine mesylate, Ciba; $\alpha$-methyl-noradrenaline, a gift from Hoechst; tramazoline hydrochloride (Karl Thomai GmbH), a gift from Dr. K. Starke; and phenoxybenzamine hydrochloride (Dibenyline Injection), a gift from S.K.F. All drugs were dissolved in distilled water except for yohimbine for which dimethyl sulfoxide (Sigma, final concentration dimethyl sulfoxide 0.1%) was used. Final dilutions were made in Locke's solution.

## Catecholamine Assay

**Perfused glands.** Sequential samples of the adrenal gland effluent were collected at 1-min intervals and assayed for catecholamines by the fluorimetric tri-hydroxyindole method of von Euler and Floding (19) against adrenaline standards. Excitation/emission wavelengths of 400/510 nm were selected on a Perkin-Elmer 204 fluorescence spectrometer.

**Freshly isolated cells and isolated, cultured cells.** Catecholamine secretion was determined by measuring the release either of endogenous catecholamines (19) or of $^3$H release from cells previously labeled with $^3$H-noradrenaline. Freshly isolated cells were labeled by incubation for 1.5 hr with $^3$H-noradrenaline (1 $\mu$Ci/ml) in Locke's solution containing also ascorbic acid (1 mM), which not only inhibits oxidation of the amine but also promotes its uptake into the cell (20). Cells in culture were similarly labeled (1 $\mu$Ci/well) with the $^3$H-noradrenaline in Dulbecco's modified Eagle's medium. Labeled cells were washed three times with Locke's solution at room temperature to remove excess $^3$H.

## Measurement of Secretory Activity

**Cell suspensions.** After resuspension of the washed cells in the appropriate medium they were submitted to the desired experimental maneuver in plastic microcentrifuge tubes (1.5 ml). Catecholamine secretion was estimated by centrifuging samples (1500 $\times$ $g$ for 2 min) and assaying catecholamine present in the supernatant. Samples of uncentrifuged cell suspensions, lysed with 0.05% Triton X-100, were assayed to provide a measure of total cellular catecholamine. Secretion was expressed as a percentage of total cellular catecholamine [see also Ref. 17).

**Cultured cells.** After any required preincubation of cultured cells with drugs, the well contents were drained by aspiration and the cells were subjected to the desired experimental maneuver by inverting the multiwell dish over another containing in its wells the secretogogue in the appropriate experimental medium. The stimulus was terminated by reinversion to drain the medium from the cells. Catecholamines were assayed in samples of the supernatant. The catecholamine remaining in the attached cells was extracted either with 0.05% Triton X-100 in 1 M sodium acetate buffer, pH 6.5 (for endogenous catecholamine) or with 10% trichloracetic acid (for $^3$H estimation).

## Uptake of $^{22}$Na

$^{22}$Na uptake was measured in freshly isolated cells by one of the methods described by Pocock (21). Two-ml aliquots of cell suspension were first preincubated for 10 min with a mixture of tetrodotoxin (10 $\mu$M) and ouabain (100 $\mu$M) and then exposed to $^{22}$Na plus the compound under study. After 1.5 min, triplicate 200-$\mu$l samples were withdrawn and layered on to microcentrifuge tubes containing 0.9 ml of Locke's solution + 0.3 ml of a mixture comprising 10 parts dibutylphthalate to 1 part light liquid paraffin (SG 0.83–0.89). Whereas the cells will pass through this mixture during centrifugation, the supernatant containing excess $^{22}$Na remains above the oil and can be aspirated. The cells were removed by freezing the whole tube and cutting off the tip containing the pellet. The $^{22}$Na uptake into the cells was calculated according to the method described by Pocock (21).

## Ligand Binding Studies

Ligand binding studies were performed with (a) membranes prepared from freshly isolated adrenal medullary chromaffin cells, (b) intact, cultured chromaffin cells, and (c) lysed, cultured cells produced by exposing intact cells to distilled water for 30 min. The following radioisotopically labeled ligands were used: $^3$H-clonidine (a–c) and $^3$H-DHE (b). Established procedures were used throughout, being modified appropriately for the tissue from methods discussed by Baker and Willis (22); Wada *et al.* (9), Williams and Lefkowitz (23), and U'Prichard *et al.* (24, 25). For methods b and c, cells or cell membranes attached to the wells of culture dishes were exposed to the appropriate $^3$H-ligand for 30 min at room temperature. After rapid washing (three times) with Locke's solution (4°), the tissue was harvested by addition of 10% trichloracetic acid and agitation with a rubber policeman. The amount of $^3$H-ligand bound was determined by scintillation spectrometry.

## Patch-Clamp Studies

A few experiments using the technique of whole-cell voltage clamp (26, 27) were carried out with intact, cultured chromaffin cells to determine the effects of clonidine upon the ACh-activated inward current.

## Results

### Effects of Nicotinic Secretogogues upon Adrenal Medullary Catecholamine Secretion

**Isolated perfused glands.** The nicotinic agonist, CCh, injected over approximately 1 sec as a bolus (0.1 ml) into the perfusing fluid immediately proximal to the adrenal gland, caused a concentration-dependent secretion of catecholamine (see also Ref. 15 and references therein). The characteristics of this secretion were examined in two glands. Increasing concentrations of CCh caused both an increase in the rate of catecholamine secretion and an increase in the total amount released. Half-maximal secretion was achieved by injection of approximately 0.2 μmol of CCh (i.e., 0.1 ml of 2 mM CCh). At this concentration the time constant for the decay of secretion was about 215 sec. A maximal secretory response was invariably obtained by injection of 10 μmol of CCh.

Fig. 1 shows the catecholamine released from one gland following bolus injections of CCh (1 μmol) at 10-min intervals. Successive injections resulted in a secretion that declined only gradually in magnitude. However, after the gland had been perfused for 5 min with Locke's solution containing clonidine (10 μM), a markedly reduced secretory response to CCh was obtained. After clonidine was removed from the perfusing fluid, recovery of the secretory response to CCh was slow and incomplete. These observations were repeated in six adrenal glands. Clonidine (1–10 μM) consistently depressed the secretory response to CCh given both as bolus injections (0.1–10 μmol) or as a continued infusion (10 μM for 5 min).

It was presumed that clonidine exerted this inhibitory effect upon CCh-evoked secretion of catecholamine by acting at $\alpha_2$-adrenoceptors on the chromaffin cells. It was expected, therefore, that an enhancement of CCh-evoked catecholamine secretion would be obtained following an infusion of the α-adrenoceptor antagonist phentolamine. Eight glands were perfused with phentolamine (10 μM). In only one gland was a clear enhancement of the secretory response to CCh (0.1 μmol) observed: three successive challenges with CCh at 11-min intervals after perfusion of the gland with phentolamine gave catecholamine secretions that were 53%, 74%, and 115% larger than that observed prior to phentolamine. In two other glands only a small enhancement of the secretory response to a CCh challenge was seen (<25%), and even then the enhancement was not consistently observed with successive CCh challenges. In the remaining five glands phentolamine had no significant effect upon the secretion evoked by CCh (0.1–10 μmol). In one gland the $\alpha_2$-adrenoceptor antagonist, yohimbine (10 μM) had no effect upon the catecholamine secretion caused by CCh (0.3 μmol).

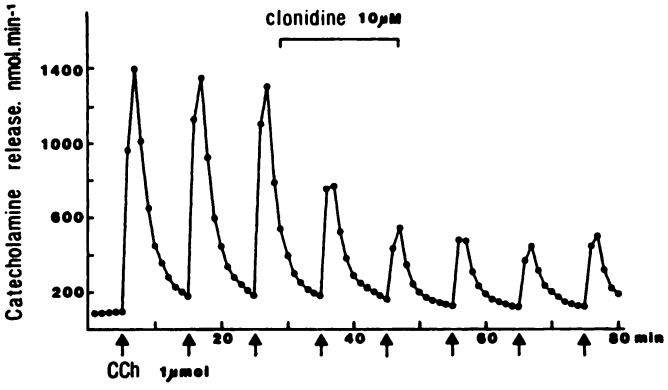


**Fig. 1.** Effects of clonidine upon CCh-evoked catecholamine release from a perfused adrenal gland. Bolus injections (0.1 ml) of CCh (10 mM, 1 μmol) were made at 10-min intervals (*arrows*) into the perfusing fluid immediately proximal to the gland. Samples of effluent were collected at 1-min intervals and assayed fluorimetrically for catecholamines against an adrenaline standard. Catecholamine release was calculated from the adrenaline concentration of the effluent and adrenal gland perfusion rate. Clonidine (10 μM) was added to the perfusion fluid for an 18-min period as shown.

**Isolated chromaffin cells.** CCh is an effective secretogogue both in freshly isolated chromaffin cells and in those cells maintained in primary culture for periods of up to 22 days (see also Ref. 17). Fig. 2A shows the concentration-response relationship for CCh in 11 experiments using freshly isolated cells. The $EC_{50}$ for CCh on catecholamine release derived from this curve is 30 μM. In these preparations basal secretion of catecholamine over 15 min was 3.7 ± 0.43% of total cellular catecholamine ($n$ = 11); maximum secretion evoked over the same period by CCh was 9.9 ± 0.60%.

As with the perfused adrenal gland, clonidine depressed the secretory response to CCh. Fig. 2A shows that the inhibition is apparently noncompetitive and, furthermore, that clonidine (20 μM) completely abolished the CCh-evoked secretion. Fig. 2B indicates that the $IC_{50}$ for the clonidine inhibition is 0.7 μM.

Precisely comparable data were obtained with cultured chromaffin cells. In eight experiments the $EC_{50}$ for the CCh-evoked catecholamine secretion was 38 μM; the $ICc_{50}$ for clonidine inhibition of the CCh-evoked secretion was 1.4 μM. In cultured cells basal secretion was 9.7 + 1.54% and maximum secretion evoked by CCh was 23.8 ± 1.89% ($n$ = 8). Note that, although both basal and evoked secretion of catecholamines is higher in cultured than in freshly isolated cells, the characteristics of the secretion evoked by CCh and its inhibition by clonidine in the two preparations are comparable.

The secretory response evoked by other nicotinic agonists, including the physiological secretogogue, ACh, is inhibited by clonidine in the same way as the response to CCh. In a single experiment with freshly isolated cells it was found that nicotine, ACh, and CCh (60 μM) each evoked a secretion of very similar magnitude. In the same experiment it was found also that the secretion of catecholamine evoked by each agonist was inhibited by clonidine (1 μM) by 67%, 57%, and 75%, respectively, for nicotine, ACh, and CCh. At this concentration clonidine itself did not affect basal secretion of catecholamine.

### Effects of Other α-Adrenoceptor Agonists upon CCh-evoked Catecholamine Secretion

Clonidine is purported to be a relatively specific $\alpha_2$-adrenoceptor agonist. To see if other α-adrenoceptor agonists have a comparable effect on CCh-evoked secretion, experiments were conducted in which cells were challenged with CCh after exposure for 10 min to various α-adrenoceptor agaonists. Fig. 3 shows the results of three experiments with freshly isolated cells in which $^3$H-(noradrenaline) release evoked by CCh was examined after exposure of aliquots of the same population of cells to adrenaline (100 μM), noradrenaline (100 μM), or clonidine (1 μM). Neither of the physiological agonists had an effect comparable to that of clonidine despite the high concentration used. In other experiments adrenaline and noradrenaline at 10 μM were also without effect upon the CCh-evoked catecholamine secretion.

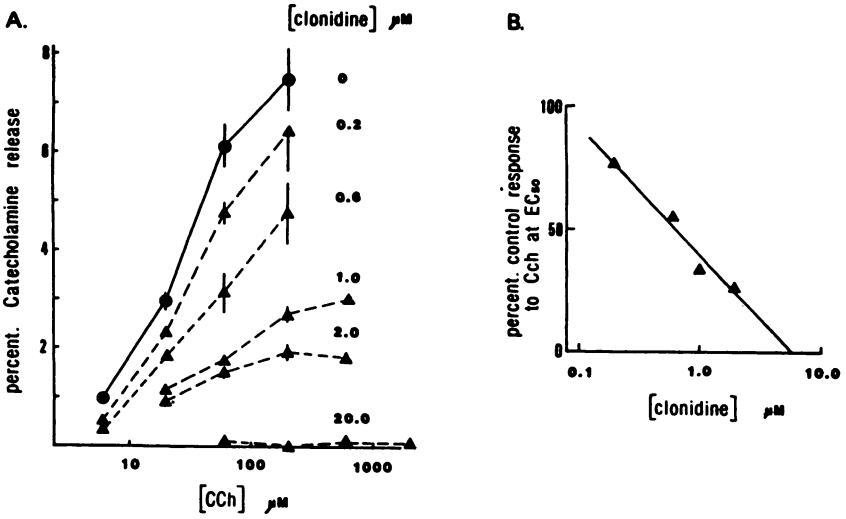


**Fig. 2.** Effects of clonidine upon CCh-evoked catecholamine release from isolated adrenal medullary chromaffin cells. A. Illustrated is the concentration-response relationship for CCh upon catecholamine release (measured over 15 min at room temperature; ●) and the effects upon CCh-evoked release of preincubation of chromaffin cells with clonidine for 10 min (▲). Catecholamine release is that present in the supernatant after centrifugation expressed as a percentage of that in a sample of uncentrifuged suspension of chromaffin cells. Basal release over the 15-min period was estimated in a sample of cells from the same population not exposed to CCh and was subtracted to give the net CCh-evoked release shown on the *ordinate*. Clonidine itself, even at the highest concentration, did not affect basal secretion. $EC_{50}$ for CCh-evoked release is 30 μM. Each *point* represents the mean ± SE; each *curve* is constructed from data obtained from between 3 and 11 experiments. B. Relationship between clonidine concentration and inhibition of catecholamine release evoked by CCh at $EC_{50}$. The $IC_{50}$ for clonidine is 0.7 μM and CCh-evoked secretion would be completely inhibited at a clonidine concentration of 5.7 μM. Data were taken from Fig. 2A.

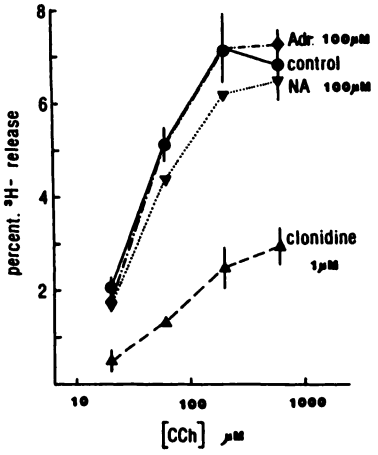


**Fig. 3.** Effects of physiological α-adrenoceptor agonists upon CCh-evoked release of $^3$H-(noradrenaline) from isolated adrenal medullary chromaffin cells. Illustrated is the concentration-response relationship for CCh upon $^3$H release from cells loaded with $^3$H-noradrenaline (●) and the effects on such release of preincubation of the cells with adrenaline (*Adr.*, 100 μM ◆), noradrenaline (*NA*, 100 μM, ▼), or clonidine (1 μM, ▲), each for 10 min. The mean results (±SE) of three experiments are shown. Standard errors for the points obtained in the presence of *Adr.* and *NA* have been omitted for clarity.

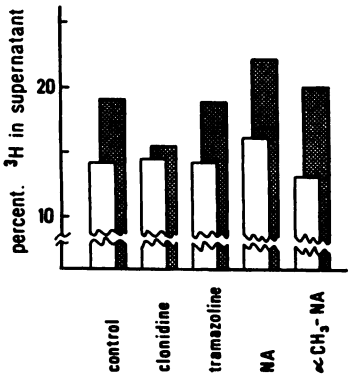


**Fig. 4.** Effects of α-adrenoceptor agonists upon CCh-evoked release of $^3$H-(noradrenaline) from adrenal medullary chromaffin cells in culture. Basal release of $^3$H (*open bars*) and that evoked by CCh at $EC_{50}$ (*shaded bars*; 15 min at room temperature) are shown for cells in culture that were loaded earlier with $^3$H-noradrenaline and then preincubated (for 10 min) with Locke's solution alone (control) or with that containing either clonidine, tramazoline, noradrenaline (*NA*), or α-methyl-noradrenaline (*αCH₃-NA*), each at 1 μM. The mean results of three experiments are shown. The standard error bars, which were large because of the wide variation between absolute values in the different experiments, have been omitted.

Precisely similar results were obtained with cultured cells. Fig. 4 shows the mean data from three experiments in which cultured cells were stimulated with CCh at $EC_{50}$. The figure shows clearly that only clonidine depresses the CCh-evoked secretion of $^3$H-noradrenaline; tramazoline, α-methyl-noradrenaline, and noradrenaline itself were all ineffective.

### Effects of α-Adrenoceptor Antagonists upon CCh-evoked Catecholamine Secretion

If α-adrenoceptors which regulate catecholamine secretion evoked by depolarizing stimuli are present upon the chromaffin cell, then one would expect, in the presence of α-adrenoceptor antagonists, an enhancement of CCh-evoked catecholamine secretion. This possibility was examined with freshly isolated cells preincubated for 10 min with either phentolamine, yohimbine, or phenoxybenzamine (0.1–10 μM). With none of these three antagonists was there an enhancement of CCh-evoked secretion (fig. 5); on the contrary, in most cases an inhibition of evoked secretion was observed, particularly at the higher concentrations of antagonist. Basal secretion was unaffected by the antagonists (data not shown).

### Effects of α-Adrenoceptor Agonists and Antagonists upon the Inhibition by Clonidine of CCh-evoked Catecholamine Secretion

If it is the α-adrenoceptor that mediates the clonidine inhibition of catecholamine secretion evoked by CCh, then one would expect that preincubation of chromaffin cells with α-adrenoceptor antagonists or with other agonists should modify the inhibition. Fig. 6 shows clearly that preincubation of chromaffin cells with phentolamine (1 μM) does not block the clonidine (0.6–2 μM) effect; indeed the intrinsic inhibitory effect of phentolamine upon CCh-evoked secretion (see Fig. 5) is

approximately additive to that of clonidine and shifts the concentration-secretion curve of CCh further to the right. A similar observation was made with phenoxybenzamine (1 μM): after preincubation with this antagonist the clonidine inhibition of the CCh-evoked catecholamine secretion was not prevented (data not shown).

Preincubation of chromaffin cells with the α-adrenoceptor agonist noradrenaline (100 μM) did not offset at all the inhibition by clonidine (1 μM) of the CCh-evoked catecholamine secretion (Table 1).

### Effect of Clonidine upon Secretion of Catecholamines Evoked by Raised K

Whereas clonidine effectively inhibits catecholamine secretion evoked by CCh and by other nicotinic agonists ($IC_{50}$ 0.7 μM), it does not inhibit secretion evoked by depolarizing concentrations of K. Fig. 7 shows the catecholamine release caused by raised K in the absence and presence of clonidine (2 μM). Clonidine does not inhibit at all the K-evoked secretion.

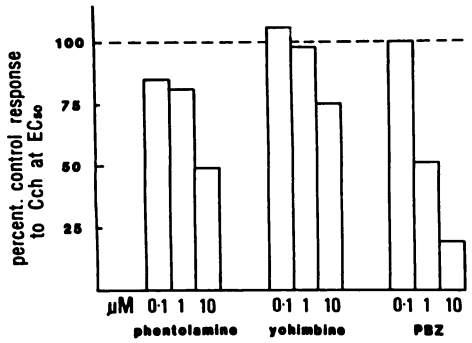


**Fig. 5.** Effects of α-adrenoceptor antagonists upon CCh-evoked release of catecholamines from isolated adrenal medullary chromaffin cells. The effects of preincubation (10 min) with phentolamine, yohimbine, or phenoxybenzamine (*PBZ*; 0.1–10 μM) on the catecholamine release evoked over 15 min at room temperature by CCh at $EC_{50}$ are shown. Catecholamine release evoked in the absence of the drugs is assigned a value of 100%. Each *bar* summarizes data from one to four experiments.

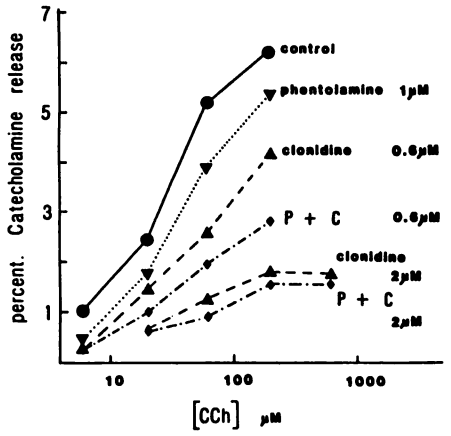


**Fig. 6.** Effects of phentolamine upon clonidine inhibition of CCh-evoked catecholamine release from isolated adrenal medullary chromaffin cells. The figure shows the concentration-response relationship for CCh on catecholamine release over 15 min at room temperature (●) and the effects on this relationship of phentolamine alone (▼, 1 μM), clonidine alone (▲, 0.6 or 2 μM), and phentolamine and clonidine together (◆). All data were gathered from samples of a single population of cells in one experiment.

TABLE 1

**The effects of noradrenaline upon the inhibition by clonidine of CCh-evoked catecholamine secretion from chromaffin cells**

In a single experiment aliquots of a suspension of freshly isolated cells were preincubated (10 min) either with noradrenaline (100 μM) or with Locke's solution before addition of clonidine (1 μM) for 10 min. Incubation with CCh at the concentrations shown (for 15 min at room temperature) resulted in catecholamine secretion which is expressed as a percentage of cellular catecholamine content with basal secretion subtracted.

| CCh concentration | % Secretion of catecholamine after exposure to: | |
|---|---|---|
| | Clonidine alone | Noradrenaline followed by clonidine |
| μM | | |
| 20 | +1.0 | +1.0 |
| 60 | +1.5 | +1.7 |
| 200 | +3.2 | +3.3 |
| 600 | +3.7 | +3.7 |

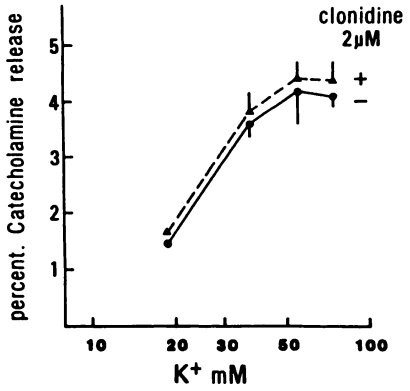


**Fig. 7.** Effects of clonidine upon catecholamine release from isolated adrenal medullary chromaffin cells evoked by potassium. The figure shows the concentration-response relationship for K-evoked catecholamine release (at 37° for 6 min) in the absence (●) and presence of clonidine (▲, 2 μM). The mean (±SE) data from three experiments are shown. The $EC_{50}$ for K is approximately 24 mM.

### Effects of α-Adrenoceptor Agonists and Antagonists on Secretion of Catecholamine Evoked by High-K Solutions

In two experiments preincubation of chromaffin cells with phentolamine (1 μM) prior to depolarization with K did not substantially alter the evoked secretion of catecholamines (data not shown).

In an experiment in which $^3H$ release was measured in cultured cells preloaded with $^3H$-noradrenaline, neither phenylephrine nor noradrenaline reduced the secretion evoked by K (18.6–56 mM) which might be expected if α-adrenoceptors were present. At $EC_{50}$ the K-evoked secretion in the presence of phenylephrine (10 μM) and noradrenaline (10 and 100 μM) was 124%, 98%, and 110% of control, respectively.

### Effect of Clonidine upon Veratridine-evoked Secretion of Catecholamine

Fig. 8 shows that, in four determinations with freshly isolated chromaffin cells, clonidine (1 μM) failed to inhibit the catecholamine secretion evoked by veratridine [75 μM for 15 min at 37°, a concentration shown by Knight and Baker (17) to be close to $EC_{50}$].

### Effect of Clonidine upon CCh-stimulated Uptake of $^{22}Na$ into Chromaffin Cells

In the presence of tetrodotoxin (10 μM), to block the uptake of Na through sodium channels, and ouabain sufficient to

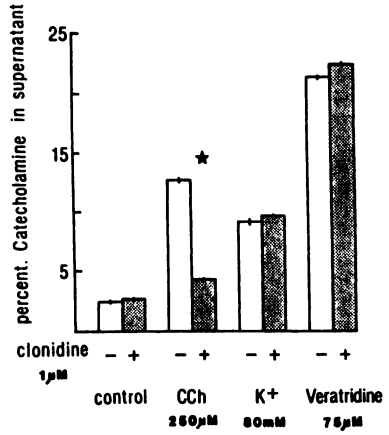


**Fig. 8.** Effects of clonidine upon catecholamine release from adrenal medullary cells evoked by various depolarizing stimuli. Illustrated are the effects of clonidine (1 μM) upon the catecholamine release (15 min at 37°) evoked by CCh, high K and veratridine. The means (±SE) of quadruplicate determinations in a single experiment are shown. ★, statistically significant reduction ($p < 0.0005$) by clonidine of the evoked release.

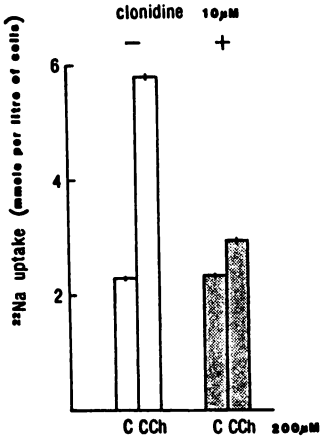


**Fig. 9.** Effects of clonidine upon CCh-stimulated uptake of $^{22}$Na uptake by adrenal medullary chromaffin cells. Illustrated are the effects of clonidine (10 μM for 10 min at room temperature; *hatched bars*) on both control uptake of $^{22}$Na (*C*; over a 2-min period at 37°) and that stimulated by CCh (200 μM) in cells pretreated with tetrodotoxin (2 μM) and ouabain (100 μM). The mean uptake (expressed in mmol of $^{22}$Na taken up per liter of chromaffin cells ±SE) of triplicate determinations in a single experiment is shown.

prevent export of Na through the Na pump (100 μM), the major channel remaining in bovine chromaffin cell membranes that permits Na movement is that controlled by the nicotinic receptor (28). Fig. 9 shows that, in chromaffin cells pretreated as above, CCh (200 μM) increased by 151% the uptake of $^{22}$Na. Clonidine (10 μM) did not affect basal uptake of $^{22}$Na but reduced by 83% ($p < 0.0005$) the CCh-stimulated uptake of the isotope.

### Patch-Clamp Studies

Preliminary experiments using the technique of whole-cell voltage clamp (26, 27) have shown that, with ACh (30 μM), clonidine (5 μM) reduces the ACh-activated inward current; clonidine (30 μM) abolishes the current (Fig. 10). On washout of clonidine the ACh-activated current reappears.

### Ligand Binding Studies

Experiments were performed with $^{3}$H-clonidine and with $^{3}$H-DHE in an attempt to identify a saturable component of binding that might reflect the presence of α-adrenoceptors on the chromaffin cell membranes.

Over a range of concentrations of clonidine (0.001–100 μM) within which its pharmacological effects on catecholamine secretion are expressed, no saturable component of binding was observed in intact cells. Likewise, although the total amount of clonidine associated with the tissue was reduced (by ca. 65%) as a result of prior chromaffin cell lysis by osmotic shock or when using membrane-enriched preparations, still no saturable component could be demonstrated. In one experiment $^{3}$H-DHE binding to intact cells was measured; over the concentration range used (0.001–0.1 μM) its binding characteristics resembled those of clonidine.

## Discussion

**Functional α-adrenoceptors.** The experiments described give no substantive evidence for the presence on bovine chromaffin cells of α-adrenoceptors that regulate catecholamine secretion. If there were functional α-adrenoceptors then α-adrenoceptor agonists should have depressed secretion evoked by depolarizing stimuli (1–3). Of those agonists investigated only clonidine was effective in this regard, and even then this agonist depressed only that secretion evoked by nicotinic agonists; it did not reduce secretion evoked either by raised K or by veratridine. The physiological α-adrenoceptor agonists noradrenaline and adrenaline, even at 100 μM, were ineffective.

Likewise, if there were functional α-adrenoceptors, then α-adrenoceptor antagonists should have increased the secretion of catecholamines evoked by depolarizing stimuli. Over an extended concentration range none of the three antagonists tested consistently elevated secretion evoked either by CCh or by raised K.

The support obtained with the clonidine experiments for the hypothesis that there are functional α-adrenoceptors on bovine

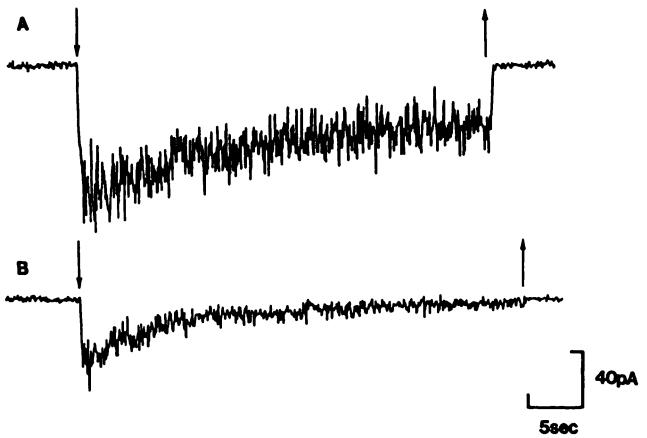


**Fig. 10.** Membrane currents produced by steady application of ACh to a chromaffin cell under whole-cell voltage-clamp. A. Membrane current change in response to 30 μM cACh applied between *arrows*. B. Membrane current change from the same cell in response to 30 μM ACh in the presence of 10 μM clonidine. Holding potential = −70 mV throughout. Note the reduction in B in both the mean current change and the membrane noise associated with the ACh-activated current.[2]

[2] S. G. Cull-Candy and A. Mathie, unpublished experiment.

chromaffin cells is weakened considerably by the observation that α-adrenoceptor antagonists do not prevent, or modify, the clonidine inhibition of evoked secretion.

It might be argued that α-adrenoceptors on bovine chromaffin cells disappear as a result of enzymic digestion and dissociation of the adrenal medulla. This is unlikely to be the explanation for the present findings since α-adrenoceptors cannot be demonstrated either in intact, perfused glands not subjected to enzymic digestion or in cells in culture which might be expected to have regenerated lost receptors (30).

Several other groups have investigated the possibility that adrenal chromaffin cells possess α-adrenoceptors that function to regulate evoked catecholamine release. Collett *et al.* (12, 13) conclude that, at least in rabbit adrenals, there is no evidence for α-adrenoceptors. Four other groups contend that there are (4–10) or might be (11) such α-adrenoceptors in other species. However, none of these present solid evidence in support of their claims. Three groups of investigators reported that in the rat (4–7) and in bovine adrenal glands (8–10) α-adrenoceptor agonists depress secretion of catecholamines evoked by depolarizing stimuli but only one of these groups (10) attempted to strengthen the support for the hypothesis of α-adrenoceptor involvement by the appropriate use of α-adrenoceptor antagonists. It is noteworthy that in this attempt they were unsuccessful. Sakurai *et al.* (10) used the relatively specific $\alpha_2$-adrenoceptor antagonist yohimbine to modify the depression of CCh-evoked secretion by clonidine and found it to be ineffective. As in the present experiments they found that yohimbine itself depressed the evoked secretion.

Gutman and Boonyaviroj (5) and Starke *et al.* (11) present data obtained with antagonists alone to provide support for the presence of α-adrenoceptors on chromaffin cells. Once again the data do not give unconditional support. Gutman and Boonyaviroj (5) found that both phentolamine and phenoxybenzamine increased catecholamine release from the rat unstimulated adrenal gland. Starke *et al.* (11) found that only phenoxybenzamine was effective at increasing the catecholamine release caused by elevated K in the bovine adrenal gland; phentolamine and oxymetazoline were ineffective.

Finally, Wada *et al.* (9) postulated the presence of α-adrenoceptors on bovine adrenal medullary cells solely on the basis of data obtained with clonidine. They did not attempt to block the effect with α-adrenoceptor antagonists. Their data can be reconciled in the same way as the present data: that they reflect an effect of clonidine independent of α-adrenoceptors.

Overall, the evidence for the presence of functional α-adrenoceptors on bovine adrenal medullary chromaffin cells is weak.

**Silent α-adrenoceptors.** It is possible still that there are α-adrenoceptors on bovine chromaffin cells but these are silent and have no function. Such receptors should be demonstrable by ligand binding methods, but in our experiments using either $^3$H-clonidine or $^3$H-DHE with intact chromaffin cells, with lysed cells or with preparations of chromaffin cell membranes, the nonspecific binding of ligand was, in every instance, too high to permit detection of α-adrenoceptors in densities that could reasonably be expected. It is likely that any specific binding that may have occurred was obscured by accumulation of the (lipophilic) ligands by the chromaffin cells or cell membranes.

In conclusion, the data obtained both with $^3$H-clonidine and with $^3$H-DHE cannot deny the contention that there are silent α-adrenoceptors on the bovine chromaffin cell membrane.

**How does clonidine depress catecholamine secretion?** If it is accepted that there are no functional α-adrenoceptors on bovine adrenal medullary chromaffin cells but that clonidine is highly effective at reducing catecholamine secretion, the question that remains to be answered is, "How is this achieved?"

One possibility might be that clonidine indiscriminately poisons the secretory mechanism of the adrenal chromaffin cell. This explanation can be discarded: clonidine did not depress secretion of catecholamines evoked either by raised K or by veratridine.

The available data suggest a direct effect upon the nicotinic cholinergic receptor. Inhibition by clonidine was observed upon that secretion evoked not only by CCh but also by ACh and by nicotine itself. Additional support is afforded by the observation that clonidine reduced the influx of Na caused by CCh and presumed to be through the nicotinic channel since it occurred when other routes of Na transport were blocked. Furthermore, whole-cell patch-clamp showed that ACh-evoked inward current was prevented by clonidine which itself did not affect current movement. The blockade by clonidine was reversible.

**How does clonidine affect the nicotinic receptor?** It is unlikely that clonidine binds specifically to the receptor; as noted above no component of saturable binding could be demonstrated. In addition, the nicotinic receptor antagonist hexamethonium, even at 100 μM, did not alter the binding curve for clonidine (data not shown). It is possible that clonidine, being strongly lipophilic, enters the chromaffin cell membrane and interferes with the nicotinic receptor channel by physical means. This is very likely; there are many reports in the literature of lipophilic compounds such as alcohols and inhalational anesthetic agents that also interfere with catecholamine secretion evoked by nicotinic receptor stimulation (see, for example, Ref. 31). However, as noted above, the existence of specific clonidine binding sites cannot yet be completely excluded.

**Consequences.** Clonidine is used as an antihypertensive agent. Its main mode of action has been considered to be via central $\alpha_2$-adrenoceptors to depress evoked secretion of neurotransmitter. The present study indicates that clonidine has an additional action in blocking very effectively, and over an equivalent concentration range, the nicotinic receptor of chromaffin cells. This itself might contribute in an important way to its antihypertensive action by reducing the level of circulating catecholamines. However, there may be further important ramifications. Sympathetic neurons are purported to bear on their terminals and varicosities nicotinic receptors, stimulation of which facilitates neurotransmitter release (1, 2). Clonidine might interfere with this neurotransmitter release pathway. Similarly, ganglionic transmission in the autonomic nervous system depends upon the nicotinic receptor which likewise might be affected by the presence of clonidine.

Clearly, further experiments are indicated to evaluate these possibilities.

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**Send reprint requests to:** Dr. David A. Powis, Neuroscience Group, Faculty of Medicine, The University of Newcastle, N.S.W. 2308, Australia.