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# Inhibition of adrenomedullary catecholamine release by propranolol isomers and clonidine involving mechanisms unrelated to adrenoceptors

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**1** Transmural electrical stimulation (10 Hz, 40 V, 1 ms for 60 s) increased total catecholamine secretion from perfused cat adrenal glands; this respnse was enhanced by neostigmine and inhibited by mecamylamine, suggesting that release of acetylcholine from splanchnic nerve terminals was stimulating nicotinic receptors and enhancing catecholamine secretion.

**2** Isoprenaline, (−)-propranolol and (+)-propranolol ($10^{-7}$–$10^{-5}$ M) inhibited the electrically-evoked secretory response by 40–70%; similar reductions were obtained with clonidine and yohimbine. Neither, (+)-propranolol nor (−)-propranolol inhibited K-evoked secretion from cat adrenals; in contrast, nimodipine potently inhibited it ($IC_{50}$ = 24 nM).

**3** Either, racemic propranolol or the (+)- or (−)-isomers (1–10 μM) equally inhibited [$^3$H]-noradrenaline release evoked by nicotine or acetylcholine from cultured bovine adrenal chromaffin cells; clonidine (10 μM) inhibited secretion by 50% and yohimbine or isoprenaline did not affect it.

**4** The results indicate that adrenomedullary catecholamine release evoked by splanchnic nerve stimulation is not modulated by α- or β-adrenoceptors and suggest that propranolol may inhibit secretion by blocking ion fluxes through the acetylcholine receptor ionophore. Clonidine may inhibit secretion by this same mechanism, and/or by interfering with some intracellular event in the secretory mechanism.

## Introduction

The concept of transmitter release modulation by so-called autoreceptors arose from the observation that certain α-adrenoceptor agonists inhibit, and the antagonists enhance noradrenaline release evoked by sympathetic nerve stimulation at different peripheral and central noradrenergic synapses; the converse occurs with β-agonists and antagonists. The hypothesis implies that α- and β-adrenoceptor sites, probably located at, or near the noradrenergic nerve terminal (but see Alonso *et al.*, 1982), modulate the release of the transmitter through a feed-back mechanism activated by the released noradrenaline (see reviews by Kirpekar, 1975; Stjärne, 1975; Westfall, 1977; Starke, 1977, Langer *et al.*, 1985).

Initially formulated on the basis of data obtained in peripheral sympathetic neurones, the hypothesis was soon extended to many other nerve cell types; the adrenal medulla was an obvious candidate since its chromaffin cells can be regarded as a highly specialized form of postganglionic sympathetic neurones. About 20 papers on the effects of adrenoceptor-acting drugs on adrenomedullary catecholamine release have provided conflicting results (see Discussion for references) in various preparations from different animal species. In those reports, exogenous secretogogues were used; therefore, physiological secretion evoked by splanchnic nerve stimulation was not studied.

The objective of this work was to measure total catecholamine release evoked by electrical stimulation of splanchnic nerves of perfused cat adrenal glands as well as [$^3$H]-noradrenaline release evoked by nicotine

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or acetylcholine (ACh) from cultured bovine adrenal chromaffin cells in an attempt to provide additional information on the effects of various α- and β-adrenoceptor agonists and antagonists on the nicotinic secretory response. Overall, the data are not consistent with the idea that α- or β-adrenoceptors modulate catecholamine release evoked by endogenous or by exogenous ACh in adrenal glands or isolated chromaffin cells. Alternative mechanisms to explain the inhibitory effects of propranolol and clonidine on secretion are discussed.

## Methods

### *Catecholamine release from perfused cat adrenals*

Cats of either sex weighing 2.5–4.0 kg were anaesthetized with sodium pentobarbitone (50 mg $kg^{-1}$). Both adrenal glands were isolated and prepared for retrograde perfusion as previously described (Garcia *et al.*, 1980). The perfusion rate was adjusted to 1 ml $min^{-1}$.

The normal perfusion solution was Krebs-bicarbonate with the following composition (mM): NaCl 119; KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2 and glucose 11. The solution was equilibrated with 95% $O_2$ and 5% $CO_2$, the final pH being 7.4. High K solutions were made up by substituting appropriate amounts of NaCl with isoosmotic concentrations of KCl.

After 1 h of initial perfusion with normal Krebs solution, collection of perfusate samples at 2 min intervals was initiated and continued to the end of each experiment. The first two samples were collected in normal Krebs solution to determine the spontaneous catecholamine output. Then, evoked secretion was achieved by stimulating the gland electrically through two fine platinum wire electrodes inserted inside the medullary tissue, or with repeated K pulses (35 mM for 1 min) given at 30 min intervals. At the end of each experiment, a 2 min pulse with 120 mM K (low Na) was routinely given in order to ascertain the functional viability of the secretory machinery. Samples were collected continuously for 2 min each in acidified (0.05 N perchloric acid), chilled tubes. Total catecholamine content of perfusate samples (noradrenaline plus adrenaline) was determined according to Shellenberger & Gordon (1971) without further purification on alumina. Catecholamine values are expressed as μg per 2 min perfusion period.

### *[$^3$H]-noradrenaline release from cultured bovine adrenal chromaffin cells*

Chromaffin cells were prepared from bovine adrenal medullae (Almazan *et al.*, 1984) and plated on uncoated plastic culture wells containing 1 ml of Dulbecco's modified Eagle's medium (DMEM) ($5 \times 10^5$ cells per well).

Two to four day-old cells were incubated for 1 h in 1 ml fresh DMEM lacking amino acids and containing 125 nM (±)-[$^3$H]-noradrenaline (specific activity 27 Ci $mmol^{-1}$; Amersham). Then, cells were washed 3 times with fresh DMEM for 30 min in the incubator, followed by a further 2 h washout period at room temperature using 1 ml aliquots of oxygenated Krebs-HEPES (KH) solution of the following composition (mM): NaCl 140, KCl 4.7, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 1.2, glucose 11, EDTA, 0.01, ascorbic acid 0.56 and HEPES 15 at pH 7.4. High K KH solutions were prepared by substitution of NaCl with the desired KCl concentration.

[$^3$H]-noradrenaline release was studied by incubating cells of each individual well during different time periods in 0.5 ml aliquots of KH (spontaneous release), or KH containing increasing concentrations of nicotine or K (evoked release). To terminate the secretory process, test solutions were removed and transferred to vials containing 4 ml of scintillation fluid (Instagel, Packard); samples were counted in a Beckman scintillation β-counter, model 2800. The radioactivity remaining in the cells at the end of the experiment was determined by adding 0.5 ml of 10% trichloroacetic acid, transferring them to scintillation vials and counting as above. [$^3$H]-noradrenaline release was expressed as a percentage of the total radioactivity present in the cells before stimulation. Drugs were present 5 min before and during the stimulation period.

### *Drugs used*

ACh chloride, nicotine, isoprenaline bitartrate, clonidine hydrochloride, yohimbine hydrochloride, atropine sulphate, neostigmine methyl sulphate, mecamylamine hydrochloride, (±)-propranolol hydrochloride and (−)-phenylephrine hydrochloride were obtained from Sigma. Nimodipine was a gift from Prof. F. Hoffmeister, of Bayer AG, FRG, and the (+)- and (−)-forms of propranolol were gifts from ICI-FARMA, Spain. Drugs were dissolved in distilled water, and yohimbine in dimethyl sulphoxide; final dilutions were made in Krebs solution.

## Results

### *Effects of drugs acting on adrenoceptors on catecholamine release evoked by transmural electrical stimulation of perfused cat adrenals*

The rates of catecholamine release were dependent on voltage, frequency and the duration of each shock.

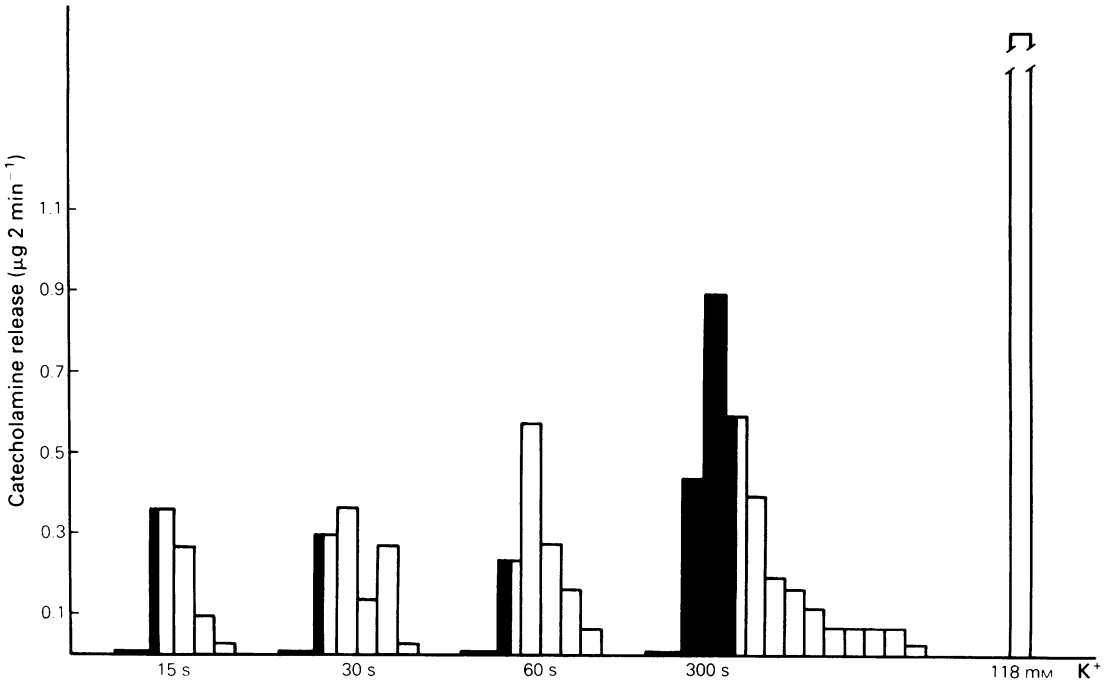


**Figure 1** Catecholamine release evoked by electrical field stimulation from perfused cat adrenal glands. A train of stimuli (10 Hz, 40 V, 1 ms duration) was applied during the length of time shown at the bottom (shaded part of the second bar in each group of columns). Stimuli were applied at 30 min intervals; the column on the right reflects catecholamine release evoked by a 2 min perfusion with Krebs-solution containing 118 mM K. Each individual column represents a collection sample and its catecholamine content in μg per 2 min (ordinate scale). Data are from an experiment made in duplicate.

Figure 1 shows the profile of catecholamine release evoked by trains of stimuli applied at 40 V, 1 ms and 10 Hz for different time periods. Usually, the peak secretory response was obtained during the second 2 min sample collected; then, the rate of catecholamine release declined to basal levels in 4–6 min. When repeated several times at 30 min intervals, the following parameters of stimulation gave the best reproducible secretory responses: 40 V, 1 ms duration, 10 Hz for 60 s; they were used for all the subsequent experiments. The fact that neostigmine (30 μM) enhanced 3 times the electrically-evoked catecholamine release and that mecamylamine (10 μM) greatly inhibited it, suggests that this response is mediated by the endogenous release of ACh from the splanchnic terminals innervating chromaffin cells.

To test the effects of drugs acting on adrenoceptors on this response, cat adrenal glands were stimulated several times by applying trains of stimuli (10 Hz, 40 V, 1 ms for 60 s) at 30 min intervals. The secretory response of the second stimulus was normalized to 100%. From the third stimulus onwards, increasing cumulative concentrations of various drugs were perfused through the glands and the stimulus repeated every 30 min. Figure 2 shows that isoprenaline, (+)-propranolol and (−)-propranolol reduced catecholamine release in a concentration-dependent manner. With isoprenaline, the inhibition was already apparent at $10^{-7}$ M; at $10^{-5}$ M, catecholamine release was reduced by 50%. At both 1 and 10 μM, (−)-propranolol and (+)-propranolol decreased the secretory response to 70 and 40% of control ($P<0.01$).

Clonidine also inhibited catecholamine release in a concentration-dependent manner (Figure 3); the inhibition was apparent at $10^{-6}$ M and was 60% at $10^{-5}$ M. In contrast, phenylephrine ($10^{-6}$ M) did not modify the release (data not shown). On the other hand, yohimbine decreased electrically-evoked secretion in a concentration-dependent manner; at $10^{-5}$ M, catecholamine release was blocked by 60%. Yohimbine ($10^{-6}$ M) did not modify the inhibitory effects of clonidine (data not shown).

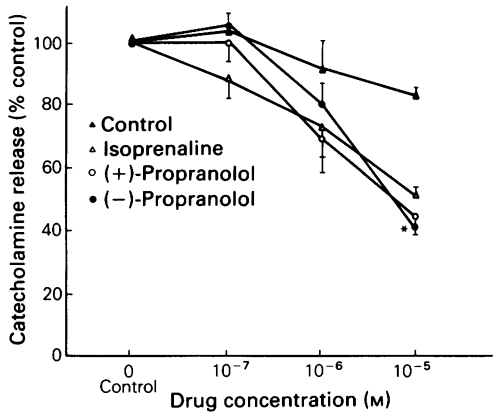


**Figure 2** Effects of isoprenaline, (+)-propranolol and (−)-propranolol on catecholamine release evoked by electrical field stimulation of perfused cat adrenals. Glands were stimulated 4 times at 40 V, 10 Hz, 1 ms for 60 s at 30 min intervals. The first control stimulation was taken as 100%; the release obtained in the presence of each drug concentration was expressed as % of control (ordinate scale). Data are means of 5 experiments; vertical lines show s.e.; $*P < 0.005$, compared to control.

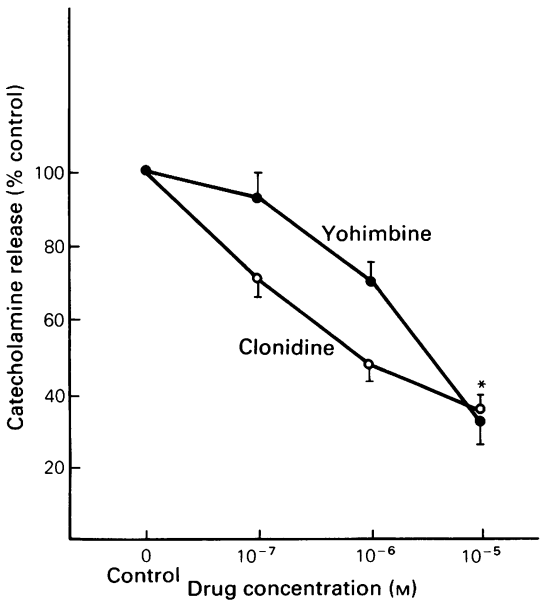


**Figure 3** Effects of increasing concentrations of clonidine or yohimbine (abscissa scale) on the release of catecholamines evoked by electrical field stimulation (40 V, 10 Hz, 1 ms for 60 s) of perfused cat adrenal glands. Drugs were present 10 min before and during the stimulation sample and 4 additional samples were subsequently collected. Data are means of 5 experiments with s.e. shown by vertical lines; $*P < 0.05$, compared to control.

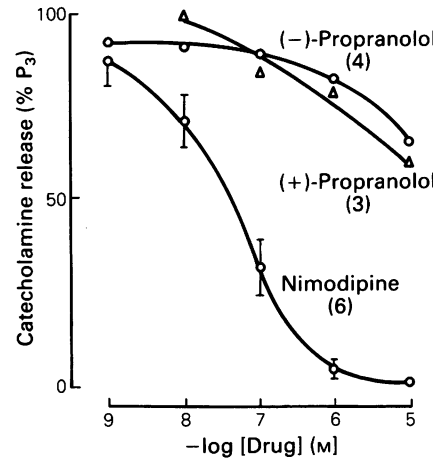


**Figure 4** Effects of nimodipine, (−)-propranolol and (+)-propranolol on catecholamine release evoked by high-K solutions. Sequential K pulses (35 mM for 1 min) were applied to each gland at 30 min intervals; secretion was stable from the third pulse ($P_3$) onwards; catecholamine release in the presence of drugs (each concentration was present from 20 min before and during the corresponding K pulse) were expressed as % of $P_3$. Data are means of the number of experiments shown in parentheses; vertical lines show s.e.

*Effects of propranolol isomers and nimodipine on potassium-evoked catecholamine release from perfused cat adrenals*

In these experiments, catecholamine release was evoked by applying 1 min pulses of Krebs solution containing 35 mM K at 30 min intervals. In 13 glands, the release was $3.1 \pm 0.23$ μg per pulse; with subsequent stimulations, this figure fell to $2.8 \pm 0.19$ μg at the eighth K pulse. Nimodipine inhibited secretion in a concentration-dependent manner; at 1 μM, secretion was abolished (Figure 4). The $IC_{50}$ for nimodipine (calculated with the method of least squares fitting to convert the sigmoid inhibition curve into a straight line) was 24 nM. In contrast, the (+)- and (−)-forms of propranolol did not substantially inhibit K-evoked secretion.

*Effects of drugs acting on adrenoceptors on the nicotinic-mediated secretory response in bovine adrenomedullary chromaffin cells*

In 4 experiments with cells from two different batches, the effects of isoprenaline or (±)-propranolol on nicotine-evoked (5 μM for 5 min) [$^3$H]-noradrenaline release were tested. Isoprenaline ($10^{-9}$–$10^{-5}$ M) did not affect significantly the secretory response (Figure 5).

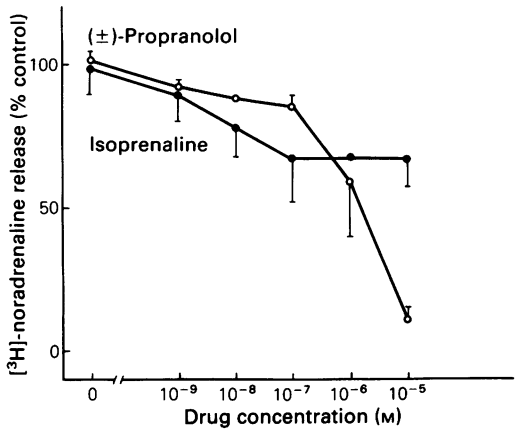


**Figure 5** Effects of increasing concentrations (abscissa scale) of isoprenaline or (±)-propranolol on the release of [$^3$H]-noradrenaline evoked by nicotine (5 μM for 5 min) from cultured bovine adrenal chromaffin cells. Drugs were present 10 min before and during nicotine stimulation. The amount of amine secreted in the abscence of drugs was calculated as fractional release and taken as 100%. Data are means of 4 experiments with s.e. shown by vertical lines.

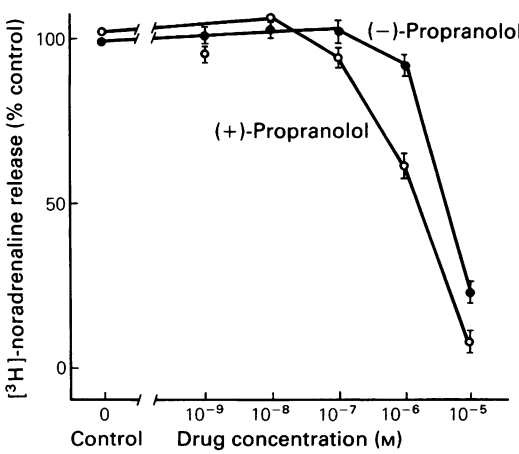


**Figure 6** Effects of (+)-propranolol and (−)-propranolol on the nicotinic-mediated [$^3$H]-noradrenaline release from cultured chromaffin cells. Experimental design as in Figure 5. Data are means of 4 experiments with s.e. shown by vertical lines.

In contrast, (±)-propranolol ($10^{-6}$–$10^{-5}$ M) markedly inhibited [$^3$H]-noradrenaline release.

In Figure 6, the effects of (−)-propranolol and (+)-propranolol on ACh-evoked (100 μM for 5 min) catecholamine release are shown. At $10^{-5}$ M, both isomers

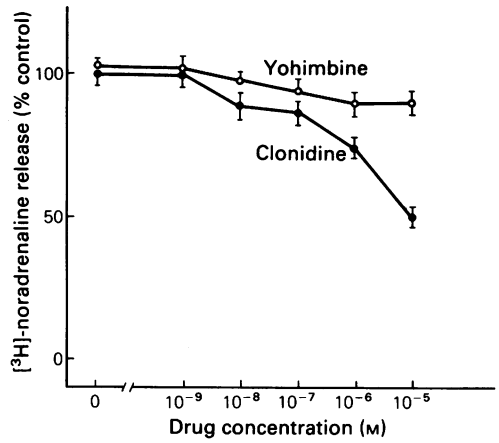


**Figure 7** Effects of increasing concentrations (abscissa scale) of clonidine or yohimbine on the release of [$^3$H]-noradrenaline (ordinate scale) evoked by nicotine (5 μM for 5 min) from cultured chromaffin cells. Experimental design as in Figure 5. Data are means of 5 experiments with s.e. shown by vertical lines.

decreased secretion by 80–90%. (+)-Propranolol caused a 50% inhibition at the concentration of $10^{-6}$ M; at this and lower concentrations, the (−)-isomer did not affect the release of [$^3$H]-noradrenaline.

Figure 7 shows an experiment in which the release of tritium evoked by 5 μM nicotine was tested in the presence of increasing concentrations of clonidine or yohimbine. It can be seen that the rate of secretion was not affected by these drugs; only at a concentration of $10^{-5}$ M did clonidine depress the response by 50% ($P < 0.01$ compared with control release).

## Discussion

The hypothesis of the modulation by α- and β-adrenoceptors of catecholamine release at various neuroeffector sympathetic junctions is based on the fact that α- and β-adrenoceptor-acting drugs enhance or decrease catecholamine release. Similar experiments performed in intact adrenal glands or in isolated chromaffin cells have provided many conflicting results. They will be discussed and compared with the results obtained in the present work.

### *Drugs acting on α-adrenoceptors*

Various agents known to block α-adrenoceptors have been tested to see their effects on adrenomedullary catecholamine release evoked by nicotinic stimulation, K-induced depolarization or splanchnic nerve stimulation. Phentolamine has been shown to be

devoid of effects on secretion (Kirpekar 6 Cervoni, 1963; Starke *et al.*, 1974a) or to decrease it (Serck-Hanssen, 1974; Collett & Story, 1984; Sharma *et al.*, 1986). The same applies to phenoxybenzamine; some authors report an increase of release (Kirpekar & Cervoni, 1963; Starke *et al.*, 1974a), others see no changes (Wakade, 1981) and some appreciable inhibition (Khan & Furchgott, 1972; Collett & Story, 1982; 1983; 1984). Finally, yohimbine either inhibits secretion (Sakurai *et al.*, 1983) or has no effect (Collett & Story, 1984; Sharma *et al.*, 1986).

Conflicting reports appeared also on the effects of α-adrenoceptor agonists. While phenylephrine has no effect (Sakurai *et al.*, 1983; Sharma *et al.*, 1986) or reduces secretion (Boonyaviroj *et al.*, 1977; Boonyaviroj & Gutman, 1979), oxymetazoline does not affect it (Starke *et al.*, 1974a). Clonidine has been found to have no effect (Wakade, 1981; Sharma *et al.*, 1986) or inhibit release (Wada *et al.*, 1982; Greenberg & Zinder, 1982; Sakurai *et al.*, 1983; Sharma *et al.*, 1986), yet yohimbine does not prevent such an inhibitory effect (Sakurai *et al.*, 1983).

With the perfused cat adrenal gland we observed that both clonidine and yohimbine inhibited catecholamine release evoked by splanchnic nerve stimulation in a concentration-dependent manner. Yohimbine lacked any effect on the release of [$^3$H]-noradrenaline evoked by nicotine from cultured bovine adrenal chromaffin cells, and clonidine was not a potent inhibitor; neither phentolamine nor yohimbine reversed the inhibitory effects of clonidine. The fact that these drugs are more potent on intact adrenal glands electrically stimulated might suggest an additional effect on ACh release from splanchnic nerve terminals. However, it is worth comparing this effect with that observed in sympathetic neurones. At a concentration of only 37 nM (about 1000 times lower than the effective concentrations seen in this paper and in those used by Wada *et al.* (1982) and Sakurai *et al.* (1983) on chromaffin cells), clonidine inhibits by 50% [$^3$H]-noradrenaline release evoked by transmural sympathetic nerve stimulation of rabbit pulmonary artery strips (Starke *et al.*, 1974b).

Since clonidine is considered to be a highly potent and selective agonist for $\alpha_2$-adrenoceptors (Kobinger, 1981) and yohimbine did not antagonize its effects, it is clear that its inhibitory actions on adrenomedullary catecholamine release are unrelated to such receptors. It may be that the drug (that readily crosses cell membranes) is acting at some step on the plasma membrane (i.e., inhibiting the entry of ions through the ACh receptor ionophore) or beyond it on the secretory mechanism, i.e., interferring with Ca binding to some specific protein receptor as do some neuroleptic sedative drugs. In a recent paper, Powis & Baker (1986) also suggest that clonidine probably acts at the nicotinic receptor to inhibit secretion.

*Drugs acting on β-adrenoceptors*

Conflicting data on the effects of drugs acting on β-receptors on secretion have also been reported. Isoprenaline increases secretion (Serck-Hassen, 1974) or does not affect it (Wakade, 1981; Collett *et al.*, 1984). In our experiments, isoprenaline inhibited release.

More consistent data are available with β-blockers. Except in one report where an effect was not observed (Wakade, 1981), the racemic form of propranolol has been found to inhibit catecholamine release in various preparations (Jaanus *et al.*, 1967; Khan & Furchgott, 1972; Serck-Hanssen, 1974; Collett & Story, 1983; Collett *et al.*, 1984).

It is interesting that the stereoisomer (+)-propranolol was as potent as the racemic mixture in inhibiting secretion from perfused bovine (Serck-Hanssen, 1974) and rabbit adrenals (Collett *et al.*, 1984). We have also demonstrated that (+)-propranolol and (−)-propranolol are equipotent in inhibiting nicotinic-mediated catecholamine release from perfused cat adrenals (Figure 3) and cultured bovine adrenal chromaffin cells (Figure 6). These effects of propranolol were attributed by some authors to its local anaesthetic activity since (+)-propranolol, which is equipotent with (−)-propranolol in local anaesthetic activity but possesses only one sixtieth of the activity of racemic propranolol as a β-adrenoceptor antagonist (Howe & Shanks, 1966; Barret & Cullum, 1968), produced a similar degree of inhibition of catecholamine release. However, the fact that in our hands propranolol did not inhibit K-evoked secretion, but markedly decreased nicotinic-mediated catecholamine release speaks in favour of an action beyond Ca or Na channels and suggest that, as imipramine and cocaine do (Ceña *et al.*, 1983), propranolol may be acting on the ACh ionophore to block ion fluxes through it.

In conclusion, our results demonstrate a concentration-dependent inhibition of nicotine-mediated catecholamine release from cat and bovine chromaffin cells evoked by clonidine and propranolol; this observation is consistent with other data from the literature. However, we believe that such inhibitory effects are unrelated to the well established $\alpha_2$-adrenoceptor stimulating action of clonidine and the β-blocking effects of propranolol.

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