

# Ouabain distinguishes between nicotinic and muscarinic receptor-mediated catecholamine secretions in perfused adrenal glands of cat

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1 The effect of ouabain on catecholamine (adrenaline and noradrenaline) secretion induced by agents acting on cholinergic receptors was studied in perfused cat adrenal glands. Acetylcholine (ACh) ( $5 \times 10^{-7}$  to  $10^{-3}$  M), pilocarpine ( $10^{-5}$  to  $10^{-3}$  M) and nicotine ( $10^{-6}$  to  $5 \times 10^{-5}$  M) caused dose-dependent increases in catecholamine secretion. Both ACh and nicotine released more noradrenaline than adrenaline and the reverse was the case for pilocarpine.

2 Ouabain ( $10^{-5}$  M) enhanced catecholamine secretion induced by ACh ( $10^{-5}$  M), pilocarpine ( $10^{-3}$  M) and nicotine ( $3 \times 10^{-6}$  M) during perfusion with Locke solution. The ratio of adrenaline to noradrenaline was not affected by ouabain.

3 In the absence of extracellular  $\text{Ca}^{2+}$ , ACh and pilocarpine, but not nicotine, still caused a small increase in catecholamine secretions, which were enhanced by treatment with ouabain ( $10^{-5}$  M) plus  $\text{Ca}^{2+}$  (2.2 mM) for 25 min. The effect of ouabain was much more significant on noradrenaline secretion than on adrenaline secretion. The enhanced response was blocked by atropine ( $10^{-6}$  M) but not by hexamethonium ( $5 \times 10^{-4}$  M).

4 Nifedipine ( $2 \times 10^{-6}$  M) inhibited the responses to pilocarpine and nicotine. The treatment with ouabain ( $10^{-5}$  M) reversed only the response to pilocarpine and resulted in a significant increase in the proportion of noradrenaline released.

5 It is suggested that ouabain enhances evoked catecholamine secretions by facilitating  $\text{Ca}^{2+}$  entry through nicotinic receptor-linked  $\text{Ca}^{2+}$  channels and by increasing the intracellular  $\text{Ca}^{2+}$  pool linked to muscarinic receptors.

## Introduction

It has been reported that cardiac glycosides increase both spontaneous and evoked catecholamine secretions from perfused adrenal glands (Banks, 1967; 1970; García *et al.*, 1981b; Wakade, 1981; Nakazato *et al.*, 1986) and isolated adrenal chromaffin cells (Aunis & García, 1981; Sorimachi *et al.*, 1981; Pocock, 1983a,b). As is the case for the adrenergic nerve terminals (Nakazato *et al.*, 1978; 1980; 1983), these ouabain actions have been mainly explained by an increase in  $\text{Na}^+$ -dependent  $\text{Ca}^{2+}$  influx resulting from the inhibition of the  $\text{Na}^+$ - $\text{K}^+$  pump (García *et al.*, 1980; 1981a; Sorimachi *et al.*, 1981). We also assumed that ouabain increases  $\text{Ca}^{2+}$  entry through the ACh receptor-linked  $\text{Ca}^{2+}$  channels and voltage-dependent  $\text{Ca}^{2+}$  channels by  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange, of which the rate is accelerated by the reduction of  $\text{Na}^+$  electrochemical gradient resulting from the inhibition of the  $\text{Na}^+$ - $\text{K}^+$  pump, and causes the

enhancement in the evoked catecholamine secretions (Nakazato *et al.*, 1986).

We have argued that the mechanism underlying nicotinic and muscarinic receptor-mediated catecholamine secretions is different, as the former is exclusively mediated by voltage-dependent entry of extracellular  $\text{Ca}^{2+}$  but the latter may be caused by  $\text{Ca}^{2+}$  mobilized from intracellular storage sites (Nakazato *et al.*, 1984; 1988, Yamada *et al.*, 1988). The positive inotropic action of cardiotonic steroids is interpreted as an increased amount of  $\text{Ca}^{2+}$  resulting from  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange after inhibition of the  $\text{Na}^+$ - $\text{K}^+$  pump by cardiac glycoside. This  $\text{Ca}^{2+}$  is stored in the sarcoplasmic reticulum, from which more than normal amounts of  $\text{Ca}^{2+}$  are released when the cell is activated (Blaustein, 1985). It therefore seems possible that ouabain increases intracellularly stored  $\text{Ca}^{2+}$  and results in the enhancement of catecholamine secretion in response to the activation of muscarinic receptors. If this is

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**Table 1** Effects of atropine and hexamethonium on acetylcholine (ACh)-induced catecholamine secretion (nmol 5 min<sup>-1</sup>) from perfused cat adrenal glands

	n	Control		n	Atropine		n	Hexamethonium	
		Ad	NA		Ad	NA		Ad	NA
Resting release	36	1.7 ± 0.2	1.1 ± 0.1 (39.3 ± 0.6%)	4	0.5 ± 0.1**	0.6 ± 0.3 (44.9 ± 9.0%)	6	1.5 ± 0.3	0.5 ± 0.2* (27.4 ± 4.7%)
ACh									
10 <sup>-6</sup> M	5	2.0 ± 0.1	2.2 ± 0.6 (55.9 ± 7.9%)	4	0.3 ± 0.1***	0.4 ± 0.2* (59.8 ± 4.5%)	6	2.7 ± 0.7	1.9 ± 0.7 (42.3 ± 5.1%)
10 <sup>-5</sup> M	17	15.7 ± 1.9	37.6 ± 4.9 (69.5 ± 2.9%)	5	2.6 ± 0.5***	7.0 ± 1.1*** (73.7 ± 2.0%)	6	9.4 ± 2.1*	7.2 ± 1.7*** (43.1 ± 6.4%)
10 <sup>-4</sup> M	4	64.6 ± 11.9	159.5 ± 4.6 (70.0 ± 3.8%)	5	68.1 ± 3.9	113.6 ± 15.5* (61.9 ± 3.2%)	6	15.0 ± 2.9*	21.9 ± 5.0*** (51.5 ± 6.2%)

In this and the following tables, the numbers indicate the mean ( $\pm$ s.e.) of adrenaline (Ad) and noradrenaline (NA) released and the percentage of noradrenaline is given in parentheses under the various conditions indicated. The values of resting output were subtracted from each evoked response. The numbers of cats (*n*) are indicated. Concentrations of atropine and hexamethonium used were 10<sup>-5</sup> M and 5 × 10<sup>-4</sup> M, respectively. \* *P* < 0.05, \*\* *P* < 0.01 and \*\*\* *P* < 0.005 when compared with control values.

the case, ouabain is an interesting experimental tool for studying the differences between the mechanisms of nicotinic and muscarinic receptor-mediated catecholamine secretions.

The purpose of the present experiments is to investigate the effect of ouabain on catecholamine secretion evoked by nicotinic and muscarinic agonists in the presence and absence of extracellular Ca<sup>2+</sup> in isolated and perfused cat adrenal glands.

## Methods

### Preparations

Cats of either sex, weighing 1.5 to 3.5 kg, were anaesthetized with sodium pentobarbitone (40 mg kg<sup>-1</sup>) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Douglas & Rubin, 1961; Ito *et al.*, 1979). The glands were perfused at a flow rate of 0.6 to 0.8 ml min<sup>-1</sup> and maintained at room temperature (approximately 25°C). The adrenal effluent was collected continuously in 5 min aliquots into glass tubes kept on ice.

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, CaCl<sub>2</sub> 2.2, MgCl<sub>2</sub> 1.2, Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.0) 3 and glucose 10. In the Ca<sup>2+</sup>-free solutions, CaCl<sub>2</sub> was omitted and glycoetherdiaminetetraacetic acid (EGTA) (10<sup>-5</sup> M) was added. All solutions contained physostigmine (2 × 10<sup>-7</sup> M) to prevent hydrolysis of ACh and were bubbled with pure O<sub>2</sub>.

Experiments were started 40 to 60 min after isolation of the adrenal glands. Secretagogues were

administered for 1 min beginning 1 min before the following 5 min collection periods because of the 1 min dead time of the arterial cannula. Samples collected were acidified with 8 M perchloric acid to a final concentration of 0.4 M and stored on ice until assayed.

### Catecholamine assays

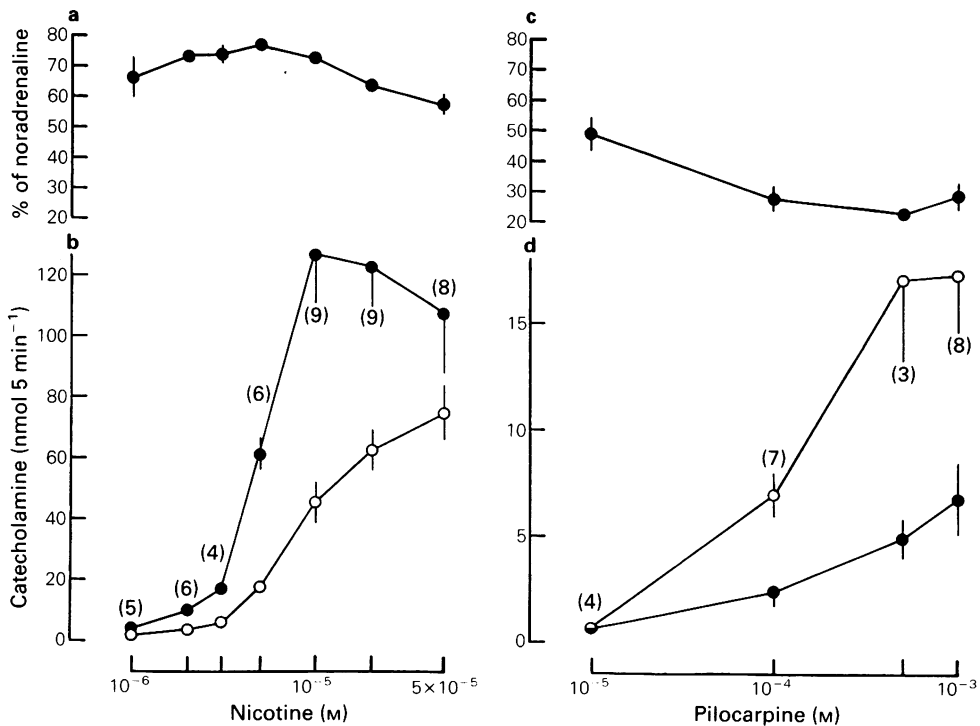
Adrenaline, noradrenaline and dopamine were separated by high performance liquid chromatography (h.p.l.c., Jasco) and detected by an electrochemical detector (LC-4B, BAS). The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982). Total catecholamines were also assayed by the fluorometric method of Anton & Sayre (1962).

### Materials

The following drugs were used: acetylcholine chloride (Ovisort; Daiichi), atropine sulphate (K & K), glycoetherdiaminetetraacetic acid (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), nicotine bitartrate (Tokyo Kasei), nifedipine (Bayer), g-strophanthin (Ouabain; Tokyo Kasei), pilocarpine hydrochloride (Tokyo Kasei), physostigmine sulphate (Wako Pure Chem.). All compounds were dissolved in Locke solution, except nifedipine, which was first dissolved in dimethylsulphoxide and then diluted with Locke solution.

### Statistics

The data are presented as arithmetic means  $\pm$  s.e. mean. Significance tests were performed by



**Figure 1** Dose-response curves for catecholamine secretion induced by nicotine (b) and pilocarpine (d) and the percentage of noradrenaline in each evoked response (a,c). Two or three different concentrations of each agonist were infused sequentially for 1 min into single preparations of adrenal glands during perfusion with Locke solution. Means, with s.e.mean indicated by vertical lines (if it exceeds the size of the symbols), of the percentage of noradrenaline (●) in (a) and (c), of the absolute amounts of adrenaline (○) and noradrenaline (●) released in (b) and (d) were plotted against the concentrations of agonists. Values of the resting output were subtracted from the evoked responses. The numbers in parentheses represent the number of experiments. The ordinate scale is the amount of catecholamines released and the abscissa scale the concentration of agonists on a logarithmic scale.

Student's *t* test. Statistical significance was assumed when  $P < 0.05$ .

## Results

### *Catecholamine secretions induced by acetylcholine, nicotine and pilocarpine*

The resting catecholamine secretion from perfused cat adrenal glands attained a steady level 40 to 60 min after the start of perfusion with Locke solution. The resting secretion consisted of adrenaline and noradrenaline, of which the ratio was approximately 6:4. No dopamine was detectable. When ACh ( $5 \times 10^{-7}$  M– $10^{-3}$  M) was infused for 1 min into the adrenal glands, catecholamine secretion was increased in a dose-dependent manner. The ED<sub>50</sub> values for ACh to release adrenaline and noradrenaline were  $2.1 \times 10^{-5}$  M and  $2.6 \times 10^{-5}$  M, respec-

tively. The dose-response relationships for ACh are partly shown in Table 1. A nicotinic agonist, nicotine ( $10^{-6}$  M– $5 \times 10^{-5}$  M), and a muscarinic agonist, pilocarpine ( $10^{-5}$  M– $10^{-3}$  M), also caused dose-dependent increases in adrenaline and noradrenaline secretions (Figure 1). The ED<sub>50</sub> values required for nicotine to release adrenaline and noradrenaline were  $8.7 \times 10^{-6}$  M and  $6.7 \times 10^{-6}$  M, and for pilocarpine they were  $9.2 \times 10^{-5}$  M and  $1.5 \times 10^{-4}$  M, respectively. Dopamine release was also increased by these agonists in parallel with the other two catecholamines, but its percentage of the total catecholamine released was only 0.4 to 2.0%, regardless of either the concentration or the kind of agonist applied.

ACh and nicotine released more noradrenaline than adrenaline and the reverse was the case for the response to pilocarpine. The percentage of noradrenaline released varied a little between different concentrations of each agonist. In the responses to ACh, the percentage of noradrenaline was

**Table 2** Effects of ouabain on catecholamine secretions (nmol 5 min<sup>-1</sup>) induced by acetylcholine (ACh), pilocarpine and nicotine from perfused cat adrenal glands

	n	S <sub>1</sub>		S <sub>2</sub>		Ratios of S <sub>2</sub> /S <sub>1</sub>	
		Ad	NA	Ad	NA	Ad	NA
Resting release	7	1.4 ± 0.5	1.2 ± 0.4 (46.7 ± 4.8%)	1.9 ± 0.3	2.0 ± 0.4 (49.8 ± 2.9%)	1.4	1.7
ACh (10 <sup>-5</sup> M)	3	17.2 ± 0.3	30.4 ± 6.1 (64.7 ± 3.4%)	33.6 ± 4.2	54.4 ± 11.7 (60.6 ± 7.0%)	1.9	1.8
Pilocarpine (10 <sup>-3</sup> M)	4	8.1 ± 0.8	5.0 ± 0.7 (38.0 ± 3.3%)	13.8 ± 1.2**	8.9 ± 1.8 (38.1 ± 3.0%)	1.7	1.8
Nicotine (3 × 10 <sup>-6</sup> M)	4	6.1 ± 1.2	17.0 ± 1.6 (74.1 ± 2.7%)	13.8 ± 2.4*	30.9 ± 4.3* (69.2 ± 2.5%)	2.3	1.8

Secretagogues were applied for 1 min before (S<sub>1</sub>) and during (S<sub>2</sub>) exposure to ouabain (10<sup>-5</sup> M). The numbers of cats (n) are indicated. \* *P* < 0.05 and \*\* *P* < 0.01 when compared with S<sub>1</sub> values.

41.3 ± 6.2% at 5 × 10<sup>-7</sup> M and 55.9 ± 7.9% at 10<sup>-6</sup> M. Then it increased to 69.9 ± 5.6% at 2 × 10<sup>-6</sup> M and was maintained at a nearly constant level up to 10<sup>-3</sup> M, the maximum concentration of ACh tested. The value of the noradrenaline percent is partly given in Table 1. In the response to nicotine, the percentage of noradrenaline was 66.5 ± 6.7% at 10<sup>-6</sup> M. It was increased with increasing concentrations of nicotine until it attained a maximum 77.5 ± 1.7% at 5 × 10<sup>-6</sup> M and then declined, attaining a minimum 58.3 ± 3.0% at 5 × 10<sup>-5</sup> M. On the other hand, the ratio of nor-

adrenaline to adrenaline in the response to pilocarpine was decreased in a monophasic way as the concentration of pilocarpine was increased. These changes in the percentage of noradrenaline in the responses to nicotine and pilocarpine are plotted in Figures 1a and 1c, respectively.

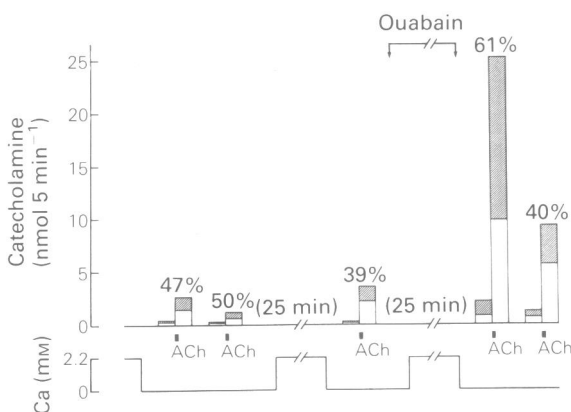
#### Effects of cholinceptor blocking agents on evoked catecholamine secretions

Atropine (10<sup>-5</sup> M) significantly inhibited the responses to 10<sup>-6</sup> M and 10<sup>-5</sup> M ACh by more than 80% of the control regardless of adrenaline or noradrenaline secretion. The response at 10<sup>-4</sup> M ACh was relatively resistant to atropine, which failed to inhibit adrenaline secretion and reduced noradrenaline secretion by only 30% of the control. In the presence of atropine, noradrenaline was still preferentially released by ACh, though the percentage of noradrenaline was increased by about 4% in the responses to 10<sup>-6</sup> M and 10<sup>-5</sup> M ACh and was reduced by about 10% in the response to 10<sup>-4</sup> M ACh.

Hexamethonium (5 × 10<sup>-4</sup> M) did not affect the response to 10<sup>-6</sup> M ACh, but inhibited adrenaline and noradrenaline secretions evoked by 10<sup>-5</sup> M ACh by about 40% and 80%, and those induced by 10<sup>-4</sup> M ACh by 77% and 86%, respectively. The preferential ratios of noradrenaline to adrenaline in the responses to ACh were significantly reduced or actually reversed by hexamethonium. These data are summarized in Table 1. Atropine and hexamethonium in the concentrations mentioned above completely blocked the secretory responses to pilocarpine (10<sup>-3</sup> M) and nicotine (10<sup>-5</sup> M), respectively.

#### Effects of ouabain on evoked catecholamine secretions

The effect of ouabain was studied on the catecholamine secretions induced by ACh, pilocarpine and



**Figure 2** Catecholamine secretion induced by acetylcholine (ACh) during perfusion with Ca<sup>2+</sup>-free solution before and after readmission of Ca<sup>2+</sup> with and without ouabain. ACh (10<sup>-4</sup> M) was applied for 1 min during perfusion with Ca<sup>2+</sup>-free Locke solution, 10 min after readmission of Ca<sup>2+</sup> (2.2 mM) for 25 min and 10 min after infusion of ouabain (10<sup>-5</sup> M) with Ca<sup>2+</sup> (2.2 mM) for 25 min. In this and the following two figures, the columns represent adrenaline (open) and noradrenaline (hatched) secretion, respectively and the percentage of noradrenaline is given by the numbers above columns.

**Table 3** Acetylcholine (ACh)-induced catecholamine secretion (nmol 5 min<sup>-1</sup>) from perfused cat adrenal glands in the absence of extracellular Ca<sup>2+</sup> before and after treatment with ouabain

	n	S <sub>1</sub>		S <sub>2</sub>		Ratios of S <sub>2</sub> /S <sub>1</sub>	
		Ad	NA	Ad	NA	Ad	NA
Resting release	10	0.9 ± 0.2	1.4 ± 0.3 (56.5 ± 4.5%)	1.3 ± 0.3	2.0 ± 0.2 (63.0 ± 3.8%)	1.4	1.4
ACh							
10 <sup>-5</sup> M	4	3.0 ± 1.1	2.5 ± 0.6 (49.7 ± 2.6%)	4.6 ± 1.6	9.2 ± 2.4 (72.0 ± 4.8%)	1.6	3.7
10 <sup>-4</sup> M	3	2.4 ± 0.7	5.4 ± 1.1 (69.4 ± 1.6%)	6.0 ± 0.7*	23.8 ± 5.4* (79.1 ± 2.0%)	2.5	4.4
10 <sup>-3</sup> M	3	2.9 ± 1.2	7.7 ± 1.9 (74.7 ± 3.3%)	7.4 ± 2.1	23.0 ± 3.5* (76.4 ± 3.5%)	2.6	3.0

ACh was applied for 1 min before (S<sub>1</sub>) and after (S<sub>2</sub>) exposure to ouabain (10<sup>-5</sup> M) with Ca<sup>2+</sup> (2.2 mM) for 25 min. The numbers of cats (n) are indicated. \* *P* < 0.05 when compared with S<sub>1</sub> values.

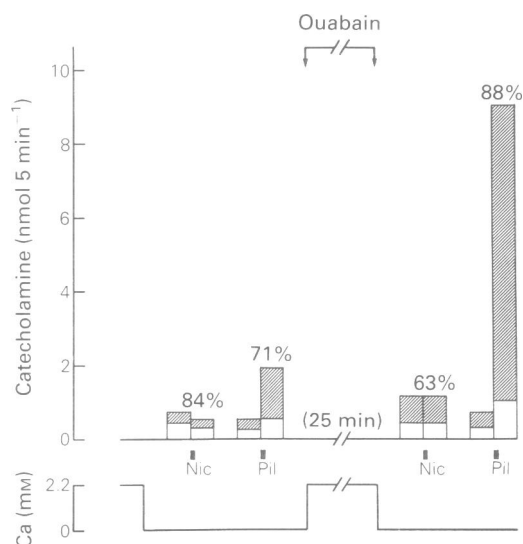
nicotine during perfusion with Locke solution. ACh (10<sup>-5</sup> M) was applied repeatedly and pilocarpine (10<sup>-3</sup> M) and nicotine (3 × 10<sup>-6</sup> M) were also infused sequentially for 1 min at 15-min intervals before and 15 min after exposure to ouabain (10<sup>-5</sup> M) for 25 min. As indicated in Table 2, all evoked catecholamine secretions were enhanced by ouabain by about 2 fold, regardless of adrenaline or noradrenaline secretion. Thus, ouabain did not change the proportion of the two catecholamines released by the three agonists. Ouabain also increased the resting release to an extent equal to or less than the evoked catecholamine secretions (Table 2).

#### Role of extracellular Ca<sup>2+</sup> in ouabain action

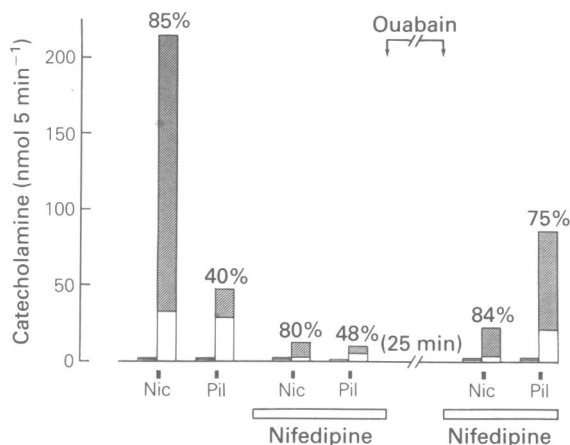
The results of experiments on the dependency of ouabain action on extracellular Ca<sup>2+</sup> are described in this and the following sections. ACh (10<sup>-5</sup> M) was infused repeatedly and nicotine (3 × 10<sup>-6</sup> M) and pilocarpine (10<sup>-3</sup> M) were applied sequentially for 1 min at 15 min-intervals in the absence of extracellular Ca<sup>2+</sup>. As reported previously (Nakazato *et al.*, 1984; 1988), ACh and pilocarpine, but not nicotine, caused small increases in catecholamine secretion in the absence of Ca<sup>2+</sup> (plus EGTA 10<sup>-5</sup> M) (Figures 2 and 3). The magnitude of residual responses in the absence of Ca<sup>2+</sup> varied from 5 to 25% of the control response in the presence of Ca<sup>2+</sup> in both ACh- and pilocarpine-induced catecholamine secretion.

As in the previous results (Nakazato *et al.*, 1984), the residual responses declined with repetition of stimulation, but were restored after readmission of Ca<sup>2+</sup> for 25 min (Figure 2). After exposure to ouabain (10<sup>-5</sup> M) plus Ca<sup>2+</sup> (2.2 mM) for 25 min, the responses to ACh (10<sup>-5</sup> M) and pilocarpine (10<sup>-3</sup> M) were increased significantly, but nicotine still

remained ineffective (Figures 2 and 3). The same results were obtained with differing concentrations of ACh as summarized in Table 3. The resting release was also enhanced after exposure to ouabain with Ca<sup>2+</sup>, though the enhancement was less than in the case of evoked responses (Table 3). These data indicate that treatment with ouabain plus Ca<sup>2+</sup>



**Figure 3** Catecholamine secretion induced by nicotine and pilocarpine during perfusion with Ca<sup>2+</sup>-free solution before and after exposure to ouabain with Ca<sup>2+</sup>. Nicotine (Nic, 3 × 10<sup>-6</sup> M) and pilocarpine (Pil, 10<sup>-3</sup> M) were applied sequentially for 1 min at 15 min intervals during perfusion with Ca<sup>2+</sup>-free Locke solution and 10 min after infusion of ouabain (10<sup>-5</sup> M) with Ca<sup>2+</sup> (2.2 mM) for 25 min.



**Figure 4** Effect of  $\text{Ca}^{2+}$  antagonist on catecholamine secretion induced by nicotine and pilocarpine in the presence of  $\text{Ca}^{2+}$  before and after exposure to ouabain. Nicotine (Nic,  $10^{-5}$  M) and pilocarpine (Pil,  $10^{-3}$  M) were applied sequentially for 1 min at 15 min intervals during perfusion with Locke solution and during infusion of nifedipine ( $2 \times 10^{-6}$  M) before and after treatment with ouabain ( $10^{-5}$  M) for 25 min.

enhanced noradrenaline secretion more than adrenaline secretion in either the responses to ACh or pilocarpine. Atropine ( $10^{-6}$  M) blocked ACh- and pilocarpine-induced responses enhanced by treatment with ouabain, but hexamethonium ( $5 \times 10^{-4}$  M) did not.

Next, we determined whether ouabain was effective in enhancing the evoked catecholamine secretion when applied after the removal of extracellular  $\text{Ca}^{2+}$ . During perfusion with  $\text{Ca}^{2+}$ -free Locke solution, ACh ( $10^{-5}$  M) or pilocarpine ( $10^{-3}$  M) was applied for 1 min and then  $\text{Ca}^{2+}$  (2.2 mM) was reintroduced for 25 min. Five min after the end of readmission of  $\text{Ca}^{2+}$ , ouabain ( $10^{-5}$  M) was added, and another 10 min after the start of ouabain infusion,

the adrenal glands were again stimulated with ACh or pilocarpine. There was no enhancement of evoked adrenaline secretions, while noradrenaline secretions were about 1.4 and 2.2 fold of those evoked by ACh and pilocarpine, respectively, before exposure to ouabain (data not shown).

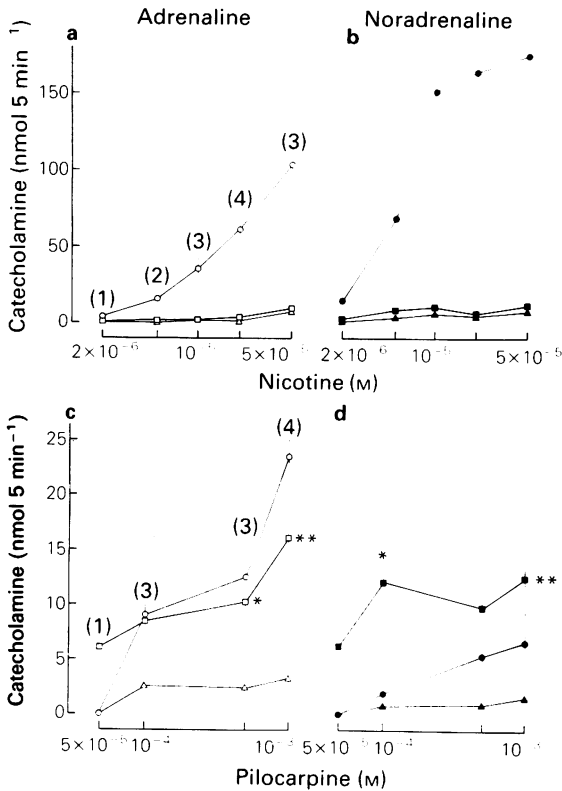
#### *Effects of $\text{Ca}^{2+}$ antagonist on evoked catecholamine secretion before and after exposure to ouabain*

Nicotine ( $10^{-5}$  M) and pilocarpine ( $10^{-3}$  M) were infused sequentially for 1 min at 15-min intervals before and during exposure to a dihydropyridine  $\text{Ca}^{2+}$  antagonist, nifedipine ( $2 \times 10^{-6}$  M). Then nifedipine was replaced by ouabain ( $10^{-5}$  M) for 25 min, after which this was again replaced by nifedipine, and the glands were stimulated with nicotine and pilocarpine. As shown in Figure 4, nifedipine inhibited the responses to nicotine and pilocarpine before exposure to ouabain. After treatment with ouabain, pilocarpine caused a secretory response almost the same as or greater than that of the control obtained in the absence of the  $\text{Ca}^{2+}$  antagonist, but nicotine failed to restore the catecholamine secretion to the degree that pilocarpine did. The resting adrenaline and noradrenaline secretions were slightly decreased by nifedipine, the effect of which was practically unaffected by the treatment with ouabain (Table 4). The experiment illustrated in Figure 4 was performed with different concentrations of agonists and the results are plotted in Figure 5a,b for nicotine and 5c,d for pilocarpine. Treatment with ouabain not only reversed the inhibitory effect of nifedipine on adrenaline secretion but also enhanced noradrenaline secretion induced by pilocarpine. Thus, the percentage of noradrenaline was increased significantly in the response to pilocarpine after exposure to ouabain, while that in the response to nicotine remained unchanged. The values for percentage of noradrenaline were calculated from the data indicated in Figure 5 and replotted in Figure 6a,b.

**Table 4** Effects of nifedipine on the resting catecholamine release ( $\text{nmol } 5 \text{ min}^{-1}$ ) before and after treatment with ouabain

	Control		Nifedipine		Nifedipine after ouabain	
	Ad	NA	Ad	NA	Ad	NA
	$1.3 \pm 0.2$	$0.9 \pm 0.2$ (40.8 $\pm$ 3.1%)	$1.1 \pm 0.2$	$0.5 \pm 0.1^*$ (35.2 $\pm$ 2.7%)	$1.1 \pm 0.2$	$0.7 \pm 0.1$ (40.3 $\pm$ 3.3%)

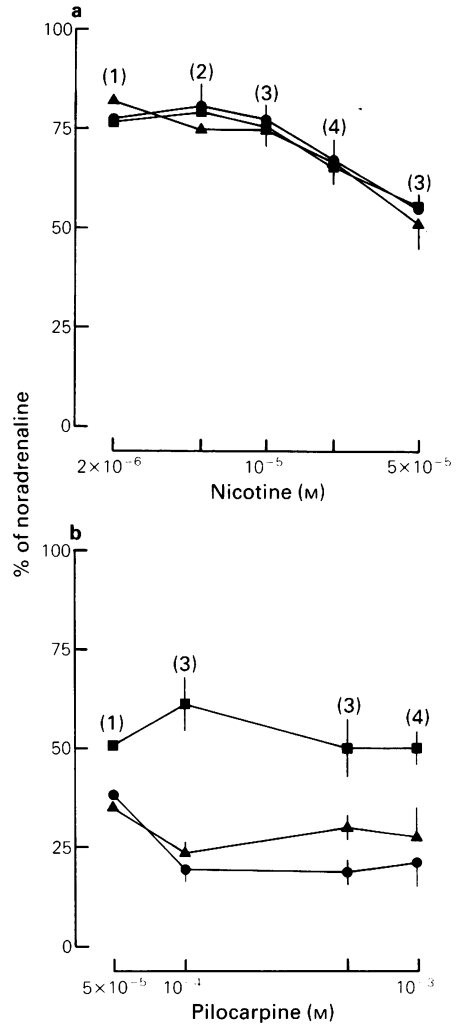
The numbers indicate the mean ( $\pm$ s.e.) of adrenaline (Ad) and noradrenaline (NA) obtained from 12 cats in the presence of nifedipine ( $2 \times 10^{-6}$  M) before and after exposure to ouabain ( $10^{-5}$  M) for 25 min. \*  $P < 0.05$  when compared with control values.



**Figure 5** Effect of a  $\text{Ca}^{2+}$  antagonist (nifedipine) on catecholamine secretion induced by various concentrations of nicotine (a,b) and pilocarpine (c,d) before and after treatment with ouabain. Protocol of the experiment is shown in Figure 4. Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbols, of adrenaline (a,c) and noradrenaline (b,d) released were plotted against the concentration of agonists. Values of resting output were subtracted from the evoked responses. Symbols indicate the control response ( $\circ, \bullet$ ) and the response in the presence of nifedipine ( $2 \times 10^{-6} \text{ M}$ ) before ( $\triangle, \blacktriangle$ ) and after ( $\square, \blacksquare$ ) treatment with ouabain ( $10^{-5} \text{ M}$ ) for 25 min. The numbers in parentheses represent the number of experiments. \*  $P < 0.05$ , \*\*  $P < 0.005$  when compared with the values obtained before treatment with ouabain ( $10^{-5} \text{ M}$ ).

**Discussion**

The following results were obtained: (1) ACh in lower concentrations increased catecholamine secretion from perfused cat adrenal glands mainly through the activation of muscarinic receptors, and the contribution of nicotinic receptors to ACh-induced response increased with increasing concentrations of ACh, as was reported for guinea-pig



**Figure 6** The percentage of noradrenaline in catecholamine secretions induced by various concentrations of nicotine (a) and pilocarpine (b) illustrated in Figure 5. Symbols indicate the percentages of noradrenaline in the control response ( $\bullet$ ) and in the response in the presence of nifedipine ( $2 \times 10^{-6} \text{ M}$ ) before ( $\blacktriangle$ ) and after ( $\blacksquare$ ) treatment with ouabain ( $10^{-5} \text{ M}$ ) for 25 min. The numbers in parentheses represent the number of experiments.

adrenal glands (Nakazato *et al.*, 1988). (2) Ouabain increased catecholamine secretions evoked by both nicotinic and muscarinic receptor activation through different mechanisms. (3) Pretreatment with ouabain plus  $\text{Ca}^{2+}$  enhanced noradrenaline secretion more than adrenaline secretion in response to muscarinic, but not nicotinic, receptor activation in either the

absence of extracellular  $\text{Ca}^{2+}$  or the presence of the  $\text{Ca}^{2+}$  antagonist, nifedipine.

Ouabain has been reported to enhance adrenal catecholamine secretion induced by various secretagogues such as  $\text{Ca}^{2+}$  reintroduced after exposure to  $\text{Ca}^{2+}$ -free environment (Esquerro *et al.*, 1980; Garcia *et al.*, 1980; 1981a), veratridine (Ito *et al.*, 1979; Wada *et al.*, 1985), nerve stimulation and ACh (Wakade, 1981; Nakazato *et al.*, 1986) and high  $\text{K}^+$  (Garcia *et al.*, 1981b; Nakazato *et al.*, 1986). It is known that the activation of nicotinic receptors stimulates catecholamine secretion by increasing  $\text{Ca}^{2+}$  entry through receptor-linked and/or voltage-dependent  $\text{Ca}^{2+}$  channels in both perfused rat adrenal glands (Wakade & Wakade, 1983) and bovine isolated adrenal chromaffin cells (Kilpatrick *et al.*, 1981; 1982; Knight & Kesteven, 1983). Thus, we previously suggested that ouabain enhances catecholamine secretion evoked by ACh and high  $\text{K}^+$  by increasing the rate of  $\text{Ca}^{2+}$  influx through the ACh receptor-linked  $\text{Ca}^{2+}$  channel and/or voltage-dependent  $\text{Ca}^{2+}$  channels on adrenal chromaffin cells as a result of the inhibition of the  $\text{Na}^+$ - $\text{K}^+$  pump (Nakazato *et al.*, 1986).

These experiments showed that ouabain was effective in enhancing catecholamine secretion induced by nicotinic receptor activation only in the presence of extracellular  $\text{Ca}^{2+}$ . One of the most plausible explanations for this ouabain action is the increase in  $\text{Ca}^{2+}$  entry through either receptor-linked or voltage-dependent  $\text{Ca}^{2+}$  channels during stimulation with nicotine as suggested previously (Nakazato *et al.*, 1986). However, both resting and muscarinic receptor-mediated catecholamine secretions were increased in either the presence or the absence of extracellular  $\text{Ca}^{2+}$  if the glands were exposed to ouabain prior to the removal of  $\text{Ca}^{2+}$ . Furthermore, pretreatment with ouabain enhanced muscarinic receptor-mediated response in the presence of the  $\text{Ca}^{2+}$  antagonist, nifedipine, leaving the resting secretion unchanged. In addition, even when applied after the removal of  $\text{Ca}^{2+}$ , ouabain enhanced the muscarinic response, though the enhancement was less than when applied with  $\text{Ca}^{2+}$ . These results agree with the view that the increase in the resting catecholamine secretion by ouabain may be mediated by the increase in resting  $\text{Ca}^{2+}$  influx (Garcia *et al.*, 1980; 1981a; Sorimachi *et al.*, 1981) and/or by inhibition of  $\text{Ca}^{2+}$  efflux (Pocock, 1983b). It has been reported that muscarinic receptor activation causes an increase in adrenal catecholamine secretion independent of extracellular  $\text{Ca}^{2+}$  in various species (Nakazato *et al.*, 1984; 1988; Wakade *et al.*, 1986; Harish *et al.*, 1987) and in cytosolic free  $\text{Ca}^{2+}$  in bovine isolated adrenal chromaffin cells without associated catecholamine secretion (Cheek & Burgoyne, 1985; Kao & Schneider, 1985; 1986; Mis-

bahuddin *et al.*, 1985). Furthermore, we recently found that catecholamine secretions induced by ACh as well as caffeine in the absence of extracellular  $\text{Ca}^{2+}$  were reversibly blocked by the intracellular  $\text{Ca}^{2+}$  antagonist, TMB-8 (Yamada *et al.*, 1988). It appears, therefore, that ouabain enhances the response to muscarinic stimulation by increasing  $\text{Ca}^{2+}$  entry, which in turn increases the capacity of the intracellular  $\text{Ca}^{2+}$  pool linked to muscarinic receptors.

As reported by Douglas & Poisner (1965), nicotine released more noradrenaline than adrenaline, while pilocarpine released more adrenaline than noradrenaline during perfusion with Locke solution. The proportions of these two catecholamines released were not significantly changed over a wide range of concentrations of each agonist. The ratio of noradrenaline to adrenaline released by ACh was basically of the nicotinic type and was not affected by atropine, but was altered to the muscarinic type by hexamethonium. Such dual control of adrenaline and noradrenaline could be peculiar to cats (Ungar & Phillips, 1983). However, Marley & Livett (1987) recently found that in cultured bovine adrenal medullary chromaffin cells, stimulation with nicotine sensitized adrenaline cells, but desensitized noradrenaline cells to subsequent high- $\text{K}^+$  stimulation. Therefore, as they concluded, the differential release of adrenaline and noradrenaline may be generated by different properties of the chromaffin cells themselves. This agrees with the view that adrenaline and noradrenaline cells of cat adrenal medulla possess certain distinguishable characteristics (Rubin & Miele, 1968).

In these experiments, we found that ouabain enhanced adrenaline and noradrenaline secretions evoked by three secretagogues equally (about 2 fold) in the presence of extracellular  $\text{Ca}^{2+}$ . However, the pretreatment with ouabain plus  $\text{Ca}^{2+}$  augmented noradrenaline secretion much more than adrenaline secretion in response to subsequent stimulation with pilocarpine in either the absence of extracellular  $\text{Ca}^{2+}$  or the presence of nifedipine, while nicotine was ineffective. The exact reason for this discrepancy of ouabain action between adrenaline and noradrenaline cells in the absence of  $\text{Ca}^{2+}$  is not clear at the present time, but the following are possible explanations: (1) Ouabain sensitizes noradrenaline cells much more than adrenaline cells to muscarinic agonists, releasing  $\text{Ca}^{2+}$  from intracellular pools. (2) Ouabain increases the capacity of the intracellular  $\text{Ca}^{2+}$  pool much more in noradrenaline cells than in adrenaline cells. (3) The density of membrane  $\text{Na}^+$ - $\text{K}^+$  ATPase is higher in noradrenaline cells than in adrenaline cells. In any case, ouabain seems to be an interesting agent for the study of the difference in receptor mechanisms and the  $\text{Ca}^{2+}$  require-



ment for catecholamine secretion in adrenaline and noradrenaline cells.

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