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Enhanced α_{2A}-autoreceptor reserve for clonidine induced by reserpine and cholinomimetic agents in the rat vas deferens

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1 The adaptive changes in the functional parameters of the presynaptic α_{2A}-adrenoceptors in rat vas deferens were examined after treatments with the monoamine depleter reserpine or with the direct/indirect cholinomimetic agents pilocarpine and neostigmine.

2 For this purpose, we studied the inhibition induced by the α_2-adrenoceptor agonist clonidine on the twitch contraction of the vas deferens elicited by electrical field stimulation, in animals that had been treated with acute (single dose), short-term (for 4 days) and chronic (for 11 days) regimens of reserpine (0.25 mg kg^{-1}, s.c., every 48 h), pilocarpine (10 mg kg^{-1}, i.p., every 12 h) or neostigmine (0.1 mg kg^{-1}, i.p., every 12 h). The irreversible receptor alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 300 nM) was used to block partially the α_{2A}-adrenoceptor-mediated effect of clonidine.

3 In control (untreated) animals, clonidine inhibited concentration-dependently the twitch response of the vas deferens ($pEC_{50} = 8.66$) with a maximal effect near 100%. The apparent affinity constant for clonidine was estimated with the nested hyperbolic methodology ($pK_A = 7.10$). The analysis of the occupancy-effect relation for clonidine revealed a large receptor reserve at α_{2A}-adrenoceptors.

4 Acute, short-term and chronic treatments with reserpine increased the sensitivity of α_{2A}-adrenoceptors to clonidine (decreased the EC_{50}) by about 3, 4 and 9 fold, respectively, and also increased the pool of receptor reserve for this agonist (decreased the K_E) by 4, 10 and 10 fold, respectively. Receptor affinity values were not changed after treatments.

5 Short-term and chronic, but not acute, treatments with pilocarpine and neostigmine increased the sensitivity of α_{2A}-adrenoceptors to clonidine (decreased the EC_{50}) by about 3 and 2 fold, respectively, and also increased the pool of receptor reserve for this agonist (decreased the K_E) by 2 and 3 fold, respectively. Receptor affinity values were not changed after these treatments.

6 These results indicate that an enhancement of the receptor reserve for clonidine might account for the supersensitivity of α_{2A}-adrenoceptors induced by reserpine, pilocarpine or neostigmine treatments in the rat vas deferens.

Keywords: Presynaptic α_{2A}-adrenoceptors; clonidine; EEDQ; agonist affinities; receptor reserve; receptor occupancy; rat vas deferens; reserpine; pilocarpine; neostigmine

Introduction

Presynaptic inhibitory α_2-adrenoceptors play an important physiological role in the regulation of transmitter release from noradrenergic nerve terminals (Starke *et al*., 1989). Biochemical and functional studies have established that there is an enhanced sensitivity and an increased density of α_{2A}-adrenoceptors in major depression (García-Sevilla *et al*., 1986; Meana *et al*., 1992; González *et al*., 1994). Supersensitivity of α_2-adrenoceptors can also be induced after chronic treatments with the monoamine depleter reserpine (Grassby & Broadley, 1986; Estan *et al*., 1990; Ugedo *et al*., 1993). Prolonged activation of muscarinic acetylcholine receptors with cholinomimetic agents also causes the induction of supersensitive α_2-adrenoceptors (Olmos *et al*., 1993). These effects are consistent with the cholinergic/noradrenergic hypothesis of depression, which postulates that there is a relative predominance of the cholinergic system over the noradrenergic pathway in depressive illnesses (Wachtel, 1990; Janowsky & Overstreet, 1995).

The supersensitivity of α_2-adrenoceptors induced by reserpine has been postulated to be a homospecific adaptation of the noradrenergic pathway to the depletion of catecholamines (e.g., see Ugedo *et al*., 1993). In contrast, the supersensitivity of α_2-adrenoceptors induced by cholinomimetic agents would be a heterospecific adaptation of the noradrenergic system. The biochemical mechanisms underlying the supersensitivity of α_2-adrenoceptors induced by reserpine or cholinomimetic agents is yet a matter of controversy. Thus, modifications in receptor density (U'Prichard & Snyder, 1978; Bylund & Martínez, 1980) or receptor affinity (Hong *et al*., 1988; Giralt & García-Sevilla, 1989; Ugedo *et al*., 1993) have been demonstrated after reserpine treatments. Moreover, alterations in the density of receptors (Hollingsworth & Smith, 1989) or no change in receptor parameters (Olmos *et al*., 1993) have been described after administration of cholinomimetics.

The rat vas deferens has been commonly used in studies of noradrenergic mechanisms because this tissue is characerized by a predominantly sympathetic innervation. Stimulation of presynaptic α_2-adrenoceptors in this preparation inhibits the twitch response evoked by electrical stimulation, and thus it is a suitable model in which to study the activity and modulation of α_2-adrenoceptors (Drew, 1977; García-Sevilla & Zubieta, 1986). The α_2-autoreceptor present in the rat vas deferens belongs to the α_{2A} subtype (Connaughton & Docherty, 1990; Smith & Docherty, 1992). In addition, there is a large receptor reserve at presynaptic α_{2A}-adrenoceptors in rat vas deferens. This receptor reserve has been studied by using the irreversible α_2-adrenoceptor antagonist N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and the α_2-adrenoceptor agonist clonidine (Sallés *et al*., 1994). In the present study, the effect of EEDQ on the α_{2A}-adrenoceptor-mediated effect of clonidine in the stimulated rat vas deferens was analysed to characterize further the effects of prolonged treatments with reserpine, pilocarpine or neostigmine on the receptor affinity constant and on the pool of receptor reserve at α_{2A}-adrenoceptors.

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Methods

Animals and treatments

Male Sprague-Dawley rats (280–330 g) received a standard diet with water freely available and were housed at $22 \pm 2^{\circ}C$ with a 12 h light/dark cycle. The rats were injected either with reserpine (0.25 mg kg^{-1}, s.c., every 48 h), pilocarpine (10 mg kg^{-1}, i.p., every 12 h) or neostigmine (0.1 mg kg^{-1}, i.p., every 12 h) for 4 days (short-term regimens) or 11 days (chronic regimens) (Olmos *et al*., 1993; Ugedo *et al*., 1993). Acutely treated rats were injected with single doses of these drugs. Untreated rats were used as controls. Animals were killed by decapitation 48 h (reserpine) or 12 h (pilocarpine or neostigmine) after the last injection.

The rat vas deferens preparation

Preparation of the vas deferens was done as described previously (García-Sevilla & Zubieta, 1986). Both vasa deferentia were removed and carefully cleaned. The tissues were set up between platinum electrodes in a 7 ml organ bath and incubated in Krebs bicarbonate solution of the following composition (in mM)): NaCl 112, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. The solution was maintained at $31 \pm 1^{\circ}C$ and gassed with 95% O_2/5% CO_2. Contractile responses were recorded by means of an isometric transducer attached to an OmniScribe pen recorder. At the beginning of each experiment, a 15 min stabilization period was allowed before a resting tension of 0.5 g was applied to each tissue (for an additional 15 min interval). Then, the tension was re-stated. Neurogenic contractions of the tissue were elicited by field stimulation at supra-maximal voltage (20–30 V) with square wave electrical pulses of 3 ms duration at a frequency of 0.1 Hz delivered via a Cibertec model CS-14 stimulator. Constant isometric tension changes of the vas deferens were recorded as individual contractile responses (twitches). The preparation was stimulated for 20–30 min to allow for equilibration of the twitch responses before the experiments were started.

Irreversible receptor inactivation

Experiments were conducted on both vasa deferentia from each treated or control rat. One vas deferens of the pair was treated with a single concentration of the irreversible antagonist EEDQ (300 nM) for 15 min immediately before the electrical stimulation of the preparation (Sallés *et al*., 1994). The other vas deferens was treated with the vehicle used to dissolve the alkylating agent. After this period of incubation with EEDQ the excess of the inhibitor was removed by several changes of the organ bath Krebs solution. Once reproducible responses to electrical stimulation were established, agonist concentration-effect curves were constructed by cumulative additions of clonidine at 0.5 log unit increments until a maximal effect was achieved. Twitch contractions were measured as the height of the twitch after each concentration of clonidine and expressed as the percentage reduction of the height of the basal twitch contraction induced by electrical stimulation. Only a single curve was made in each tissue because clonidine did not readily wash out.

Analysis of data

All mathematical calculations were done by use of nonlinear regression by the computer software GraFit (version 2.08, Leatherbarrow, Erithacus Software Ltd., 1990).

Analysis of agonist concentration-effect curves

All individual experiments of concentration-effect curves in each group were pooled and then fitted to a logistic equation of the following form (Parker & Waud, 1971; De Lean *et al*., 1978):

$$E = \frac{E_m \times [A]^n}{[A]^n + EC_{50}{}^n} \quad (1)$$

in which E and [A] are the observed effect and the concentration of the agonist (clonidine), respectively; E_m is the maximal inhibitory effect of clonidine, expressed in percentage values; EC_{50} is the concentration of the agonist required to promote, in each case, 50% of the maximal effect, and n represents the slope factor of the function. One estimate of E_m, EC_{50} and n was calculated in each EEDQ- and vehicle-treated group. EC_{50} values were finally expressed as negative logarithm (pEC_{50}).

Analysis of the affinity constant of the agonist-receptor complex

All individual sets of clonidine concentration-effect curves obtained in each experimental group were pooled and then analysed by the null method described by Furchgott (1966) and modified by James *et al*. (1989). This approach, which has been referred to as the 'nested hyperbolic method', is analytically simpler than the classical method of Furchgott and involves fitting the data from the concentration-effect curve of the vehicle-treated group to the eqauation described above (Parker & Waud, 1971), while simultaneously fitting the data from the concentration-effect curve of the EEDQ-treated group to the following equation:

$$E = \frac{E_m}{\left(\frac{EC_{50}}{q \times K_A \times [A']} \times (K_A + [A'] \times (1-q))\right)^n + 1} \quad (2)$$

where [A′] is the concentration of the agonist (clonidine) in the EEDQ-preincubated tissue; K_A is the dissociation constant of the agonist from the receptor (this was estimated as the negative logarithm, namely the pK_A); q is the fraction of functional receptors remaining (non-inactivated) after administration of EEDQ. Other parameters are as described above.

Analysis of the occupancy-effect relation

The fraction of receptor occupancy (R_A/P_T) for each concentration of the agonist ([A]) was calculated by substituting the value of K_A previously estimated (see above) in the following equation derived from the mass action law:

$$\frac{R_A}{R_T} = \frac{[A]}{[A] + K_A} \quad (3)$$

Thus, all experimental sets of data of the occupancy-effect relation in each group were pooled and then fitted by nonlinear regression to the hyperbolic equation described by Black & Leff (1983):

$$E = \frac{E'_m + (R_A/R_T)^{n'}}{(R_A/R_T)^{n'} + K_E{}^{n'}} \quad (4)$$

in which E'_m and n′ are the maximal effect and the slope factor, respectively, for this equation; and K_E is the fraction of receptors needed to be occupied to promote the 50% of the maximal effect (the maximal value of K_E would be 1). The K_E was expressed as the pK_E, that is the negative logarithm of the K_E.

Statistics

Values of basal twitch are expressed as mean ± s.e.mean. Statistical comparisons of basal twitches between groups were determined by one-way ANOVA followed by Tukey's test.

Values obtained from the fitting analyses are expressed as the best fit ± s.e. provided by the computer programme. These s.e. estimates are not real errors of the mean and, thus, were not used for further statistical evaluations. To evaluate statistically differences of the parameters of the fitting analyses between groups, we compared the goodness of fit of different models that shared one or more parameters of the function. If the goodness of fit of a more complex model (without constraint parameters) was significantly different from a simple model (with shared parameters) by an F test (Motulsky & Ransnas, 1987), then the parameters from the groups were considered as different. This approach is statistically optimal because experimental errors are not systematically distorted (Motulsky & Ransnas, 1987). $P=0.05$ was chosen as the level of significance.

Drugs

The following drugs were used: clonidine hydrochloride (Sigma Chemical Co.), N-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (EEDQ; Sigma), neostigmine methyl sulphate (Sigma), pilocarpine hydrochloride (Sigma) and reserpine (Ciba Geigy). EEDQ was dissolved initially in absolute ethanol and then diluted in propylene glycol and distilled water (final v/v/v ratio 1 : 1 : 2) for a final concentration of 100 μM. Further dilutions were made in Krebs solution. At final concentrations to which tissues were exposed, the vehicle of EEDQ did not reach a 0.3% (v/v) concentration in the bath medium. Reserpine was first dissolved in a tiny amount of glacial acetic acid and then diluted in 5.5% glucose for the s.c. injections. Clonidine was dissolved in distilled water and then diluted in Krebs solution. Neostigmine and pilocarpine were dissolved in 0.9% NaCl for the i.p. injections.

Results

α_{2A}-Adrenoceptor affinity constant and occupancy-effect relation for the inhibitory effect of clonidine in the rat vas deferens

In control preparations, basal twitch responses elicited by electrical stimulation developed a constant tension of 1150 ± 144 mg ($n=16$). The addition to the bath of clonidine (10^{-11}–10^{-6} M) inhibited in a concentration-dependent manner the twitch responses, with a pEC_{50} of 8.66 ± 0.02 ($n=16$) and a maximal response near 100% (Table 1; Figure 1). Preincubation of the vas deferens with EEDQ (300 nM) for 15 min reduced the maximal effect of clonidine by 53% ($P<0.001$) and shifted the concentration-effect curve to the right ($pEC_{50}=7.39$, $n=7$) ($P<0.001$) (Table 1; Figure 1). Next, the affinity constant of the agonist-receptor complex for clonidine (K_A) and the fraction of receptors remaining after EEDQ inactivation (q) were calculated. The pK_A was estimated to be 7.10 ± 0.12 ($n=7$), a value lower than that of the pEC_{50}. The amount of remaining receptors ($q \times 100$) after EEDQ was 2.4 ± 0.6% ($n=7$) (Table 2).

The plot of receptor occupancy against clonidine effect resulted in a hyperbolic relation. The analysis of this occupancy-effect curve allowed us to estimate that only a small fraction (0.03) of total receptors was needed to be occupied to yield a 50% of the maximal effect of clonidine ($pK_E=1.54\pm0.02$, $n=16$) (Table 2; Figure 2b). In addition, only a fraction (0.60) of total receptors was required for a submaximal effect (95%) of clonidine.

Effect of treatments with reserpine on α_{2A}-adrenoceptor sensitivity and the occupancy-effect relation for the inhibitory effect of clonidine in the rat vas deferens

There was a progressive decrease (ANOVA: $P<0.05$) in the strength of the twitch response of the vas deferens to electrical stimulation after the acute (2 days) (decreased by 43%) and short-term (4 days) (decreased by 59%, $P<0.05$) treatments with reserpine (0.25 mg kg^{-1}, s.c., every 48 h). After chronic

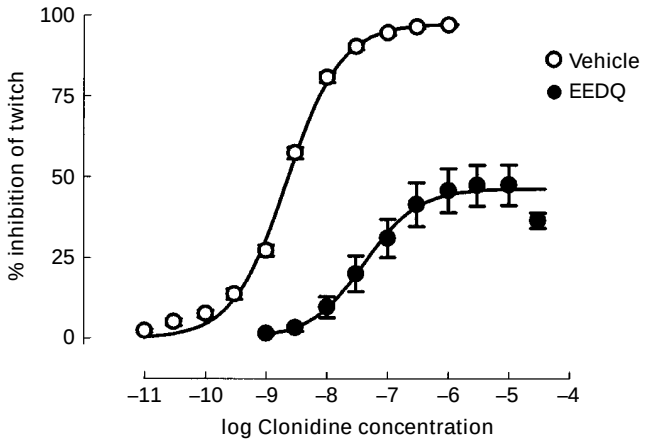

Figure 1 Concentration-effect curves for the inhibition induced by clonidine of twitch contractions of electrically stimulated vas deferens after vehicle or EEDQ (300 nM) preincubation. Symbols are mean, and vertical lines show s.e.mean, of at least 7 experiments. The vertical axis represents the percentage decrease from the basal twitch. The lines through the data are the theoretical curves calculated by nonlinear regressions to Eq. (1) in the text.

Table 1 Effect of treatments with reserpine on the basal twitch and the clonidine-induced inhibition of twitch in vehicle- or EEDQ-preincubated vas deferens

Treatments		*Basal twitch* (mg)	*Parameters of the clonidine concentration-effect curve* E_m (%)[b]	pEC_{50}[c]	n
Control	Vehicle	1150 ± 144	97 ± 1	8.66 ± 0.02	16
	EEDQ	907 ± 118	46 ± 3**	7.39 ± 0.18**	7
Reserpine[a]					
Acute	Vehicle	650 ± 120	95 ± 2	9.19 ± 0.06††	4
	EEDQ	667 ± 81	61 ± 4**,††	7.59 ± 0.17**	5
4 days	Vehicle	467 ± 106†	96 ± 2	9.24 ± 0.06††	5
	EEDQ	476 ± 78	75 ± 4**,††	7.76 ± 0.14**	6
11 days	Vehicle	1233 ± 287	94 ± 2	9.60 ± 0.06††	6
	EEDQ	1040 ± 254	66 ± 7*,††	7.46 ± 0.38**	3

[a]Dose given 0.25 mg kg^{-1}, s.c., every 48 h. [b]Maximal effect. [c]Negative logarithm of the clonidine concentration that elicits 50% of E_m. (Slope factor values ranged from 0.7 to 1.0). Basal twitches are given as the mean ± s.e.mean of n experiments. These values were compared by ANOVA followed by Tukey's test. Parameter values are the best fit ± s.e. of n experiments as obtained by the nonlinear regression. *$P<0.05$, **$P<0.001$ from vehicle-preincubated preparation within the same treatment group and †$P<0.05$, ††$P<0.005$ from vehicle- or EEDQ-preincubated control group.

Table 2 Effect of treatments with reserpine on the functional factors governing the clonidine-induced response in the vas deferens

Treatments	*Parameters derived from the nested hyperbolic function* pK_A[a]	$q \times 100$[b]	pK_E[c]	n
Control	7.10±0.12	2.4±0.6	1.54±0.02	7–16
Reserpine (acute)	7.01±0.21	1.3±0.4	2.17±0.06**	4–5
Reserpine (4 days)	6.71±0.18	1.7±0.4	2.52±0.06**	5–6
Reserpine (11 days)	7.08±0.26	0.6±0.2*	2.52±0.06**	3–6

[a]Negative logarithm of the dissociation constant of the agonist-receptor complex. [b]Percentage of receptors remaining after EEDQ inactivation. [c]Negative logarithm of the fractional receptor occupancy needed to acheive 50% of the maximal effect. (Maximal effect and slope factor values ranged from 95% to 100% and from 0.7 to 1.0, respectively). Parameter values are the best fit±s.e. of *n* experiments as obtained by the nonlinear regression. *$P<0.01$, *$P<0.001$ from control group.

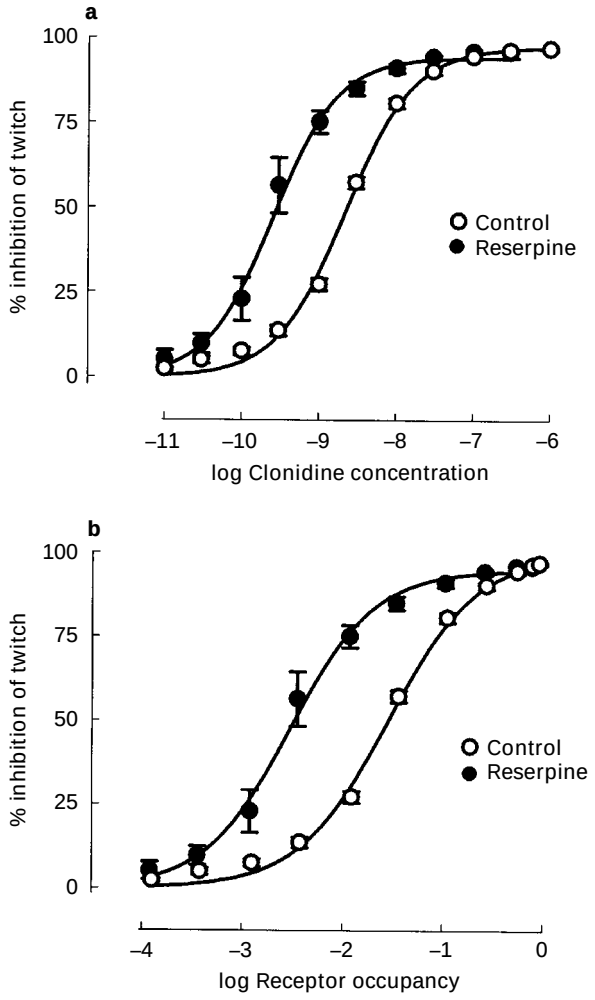

Figure 2 Concentration-effect curves (a) and occupancy-effect curves (b) for the inhibition induced by clonidine of twitch contractions of vas deferens in control or reserpine (0.25 mg kg^{-1}, s.c., every 48 h, for 11 days)-treated rats. Symbols are mean, and vertical lines show s.e.mean, of at least 6 experiments. The vertical axis represents the percentage decrease from the basal twitch. In (b), the horizontal axis is the $\log_{10}$ of the fractional receptor occupancy, which was calculated for each concentration of clonidine by substituting the estimated dissociation constant (K_A) in Eq. (4). (Note that the maximum value in this case would be: $\log_{10} 1 = 0$). The lines through the data are the theoretical curves calculated by nonlinear regressions to Eq. (1) (a) or to Eq. (4) (b) in the text.

(11 days) treatment with reserpine, basal twitch responses returned to basal values (Table 1).

Acute, short-term and chronic treatments with reserpine resulted in progressive shifts to the left of the concentration-effect curves for clonidine, with increases in the potency of clonidine (decreases in the EC_{50} values) of about 3 fold ($P<0.005$), 4 fold ($P<0.005$) and 9 fold ($P<0.005$) respectively (Table 1; Figure 2a). The maximal responses to clonidine were not changed after any of the reserpine treatments (Table 1; Figure 2a). As in control preparations, preincubation of vasa deferentia from reserpine-treated rats with EEDQ (300 nM) caused reductions in the maximal responses to clonidine (22–36%, $P<0.05$) and shifts to the right of the clonidine concentration-effect curves (EC_{50} increased by 30 to 139 fold, $P<0.001$) (Table 1). EEDQ was less effective in reducing maximal responses to clonidine in vasa deferentia from reserpine-treated groups (22–36%, $P<0.005$) than in preparations from control groups (53%). However, EEDQ did not alter basal twitch responses or the sensitivity of the agonist in reserpine-treated rats (Table 1).

Acute, short-term and chronic treatments with reserpine failed to change the K_A value for clonidine (Table 2). Chronic, but not acute or short-term, treatment with reserpine decreased by 75% the fraction of receptors remaining after EEDQ inactivation (q) ($P<0.01$) (Table 2). After acute, short-term and chronic administration of reserpine, the occupancy-effect curves were shifted to the left, leading to increases in the efficacy of clonidine (decreases in the K_E values) of about 4 fold ($P<0.001$), 10 fold ($P<0.001$) and 10 fold ($P<0.001$), respectively (Table 2; Figure 2b). Therefore, acute, short-term and chronic treatments with reserpine induced an increase in the sensitivity to clonidine, without altering the α_{2A}-adrenoceptor affinity constant for this agonist, which was associated with a decrease in the fraction of receptors needed to be occupied by clonidine to achieve 50% of the maximal effect (that is, reserpine increased receptor reserve at α_{2A}-adrenoceptors).

Effect of treatments with pilocarpine and neostigmine on α_{2A}-adrenoceptor sensitivity and the occupancy-effect relation for the inhibitory effect of clonidine in the rat vas deferens

There was no significant change in the strength of the basal twitch response of the vas deferens to electrical field stimulation after acute, short-term (4 days) or chronic (11 days) treatments with pilocarpine (10 mg kg^{-1}, i.p., every 12 h) or neostigmine (0.1 mg kg^{-1}, i.p., every 12 h) (Table 3).

Short-term treatment with pilocarpine and chronic treatment with neostigmine, but not acute treatments with these drugs, resulted in shifts to the left of the clonidine concentration-effect curves, with increases in the potency of clonidine concentration-effect curves, with increases in the potency of clonidine (decreases in the EC_{50} values) of about 3 fold ($P<0.001$) and 2 fold ($P<0.001$), respectively (Table 3; Figures 3a and 4a). The maximal effect of clonidine was not changed after the administration of pilocarpine or neostigmine (Table 3; Figures 3a and 4a). As in control preparations, preincubation of vasa deferentia from pilocarpine- or neostigmine-treated rats with EEDQ (300 nM) caused reductions in the maximal effects of clonidine (42–67%, $P<0.001$) and shifts to the right of the clonidine concentration-effect curves (EC_{50} increased by 20 to 55 fold, $P<0.001$) (Table 3). EEDQ was more effective in reducing maximal responses to clonidine

Table 3 Effect of treatments with cholinomimetic agents on the basal twitch and the clonidine-induced inhibition of twitch in vehicle- or EEDQ-preincubated vas deferens

Treatments		*Basal twitch* (mg)	*Parameters of the clonidine concentration-effect curve* E_m (%)[b]	pEC_{50}[c]	n
Control	Vehicle	1150±144	97±1	8.66±0.02	16
	EEDQ	907±118	46±3*	7.39±0.18*	7
Pilocarpine[a]					
Acute	Vehicle	847±98	100±2	8.71±0.04	4
	EEDQ	857±146	41±3*	7.22±0.19*	5
4 days	Vehicle	1053±185	98±2	9.07±0.04††	5
	EEDQ	861±58	32±2*,†	7.33±0.16*	7
Neostigmine[a]					
Acute	Vehicle	675±127	96±2	8.68±0.03	5
	EEDQ	875±163	48±3*	7.38±0.19*	5
11 days	Vehicle	893±119	95±1	8.99±0.03††	6
	EEDQ	832±92	55±3*	7.29±0.16*	8

[a]Pilocarpine: 10 mg kg^{-1}, i.p., every 12 h; neostigmine: 0.1 mg kg^{-1}, i.p., every 12 h. [b]Maximal effect. [c]Negative logarithm of the clonidine concentration that elicits 50% of E_m. (Slope factor values ranged from 0.6 to 1.1). Basal twitches are given as the mean±s.e.mean of *n* experiments. These values were compared by ANOVA followed by Tukey's test. Parameter values are the best fit±s.e. of *n* experiments as obtained by the nonlinear regression. *$P<0.001$ from vehicle-preincubated preparation within the same treatment group and †$P<0.05$, ††$P<0.001$ from vehicle- or EEDQ-preincubated control group.

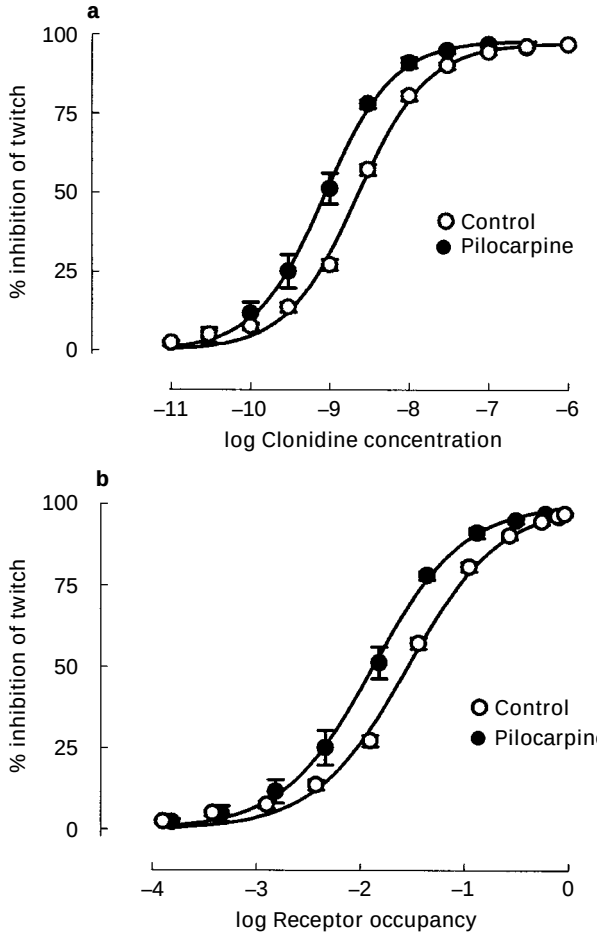

Figure 3 Concentration-effect curves (a) and occupancy-effect relations (b) for the inhibition induced by clonidine of twitch contractions of vas deferens in control or pilocarpine (10 mg kg^{-1}, i.p., every 12 h, for 4 days)-treated rats. Symbols are mean and vertical lines show s.e.mean of at least 5 experiments. The vertical axis represents the percentage decrease from the basal twitch. In (b), the horizontal axis is the $\log_{10}$ of the fractional receptor occupancy, which was calculated for each concentration of clonidine by substituting the estimated dissociation constant (K_A) in Eq. (4). (Note that the maximum value in this case would be: $\log_{10} 1 = 0$). The lines through the data are the theoretical curves calculated by nonlinear regressions to Eq. (1) (a) or to Eq. (4) (b) in the text.

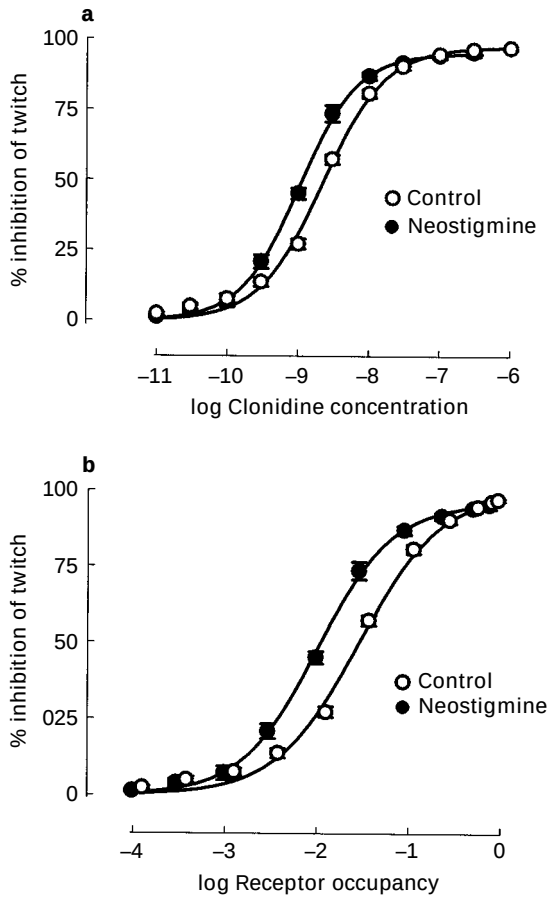

Figure 4 Concentration-effect curves (a) and occupancy-effect relations (b) for the inhibition induced by clonidine of twitch contractions of vas deferens in control or neostigmine (0.1 mg kg^{-1}, i.p., every 12 h, for 11 days)-treated rats. Symbols are mean, and vertical lines show s.e.mean, of at least 6 experiments. The vertical axis represents the percentage decrease from the basal twitch. In (b), the horizontal axis is the $\log_{10}$ of the fractional receptor occupancy, which was calculated for each concentration of clonidine by substituting the estimated dissociation constant (K_A) in Eq. (4). (Note that the maximum value in this case would be: $\log_{10} 1 = 0$). The lines through the data are the theoretical curves calculated by nonlinear regressions to Eq. (1) (a) or to Eq. (4) (b) in the text.

in vasa deferentia from pilocarpine (4 days)-treated rats (67%, $P<0.05$) than in preparations from control rats (53%). However, EEDQ did not alter basal twitch responses or the sensitivity of the agonist in either neostigmine- or pilocarpine-treated groups (Table 3).

Acute, short-term or chronic treatments with pilocarpine or neostigmine failed to change the K_A value for clonidine with respect to control (Table 4). Short-term treatment with pilocarpine, but not acute treatment with pilocarpine or acute and chronic treatments with neostigmine, reduced by 75% the fraction of receptors remaining after EEDQ inactivation (q) ($P<0.005$) (Table 4). After short-term treatment with pilocarpine and chronic treatment with neostigmine, but not after acute treatments with these drugs, the occupancy-effect curves were shifted to the left, leading to increases in the efficacy of clonidine (decreases in the K_E values) of about 2 fold ($P<0.005$) and 3 fold ($P<0.005$), respectively (Table 4; Figures 3b and 4b). Therefore, short-term and chronic, but not acute, treatments with pilocarpine or neostigmine induced an increase in the sensitivity to clonidine, without altering the α_{2A}-adrenoceptor affinity constant for this agonist, which was associated with a decrease in the fraction of total receptors needed to be occupied by clonidine to achieve 50% of maximal effect (that is, pilocarpine and neostigmine increased receptor reserve at α_{2A}-adrenoceptors).

Discussion

The results of this study confirm that treatments with reserpine, pilocarpine and neostigmine cause supersensitivity of α_{2A}-adrenoceptors modulating the twitch response of the rat vas deferens (Olmos *et al*., 1993; Ugedo *et al*., 1993). In addition, it was demonstrated that this drug-induced supersensitivity of α_{2A}-adrenoceptors is not related to a change in the apparent affinity of the agonist-receptor complex (K_A), but it is linked to an increase in the efficiency of clonidine to elicit the effect (decreased K_E); this change is commonly referred to as an increase in the receptor reserve for the agonist.

Recently, Sallés *et al*. (1994) have used receptor alkylation by EEDQ to study the clonidine-induced inhibition of the prostatic portion of the rat vas deferens, concluding that the estimation of agonist affinity is independent of whether the result is obtained by null methodology (i.e., the nested hyperbolic method) or operational analysis (Black & Leff, 1983). The nested hyperbolic method in combination with the use of the receptor antagonist EEDQ and the α_2-adrenoceptor agonist clonidine is a suitable approach to obtain accurate estimates of the affinity constant of α_2-adrenoceptors (Sallés *et al*., 1994; Pineda *et al*., 1997). Thus, the pK_A calculated in the present study for the effect of clonidine at α_{2A}-adrenoceptors in the entire vas deferens (7.10) was equivalent to the estimated pK_A for this agonist in the prostatic portion of the vas deferens (6.92) (Sallés *et al*., 1994). In addition, similar affinity constant values for clonidine at α_{2A}-adrenoceptors have been obtained *in vitro* buy using functional techniques in the CNS (pK_A = 7.14; Agneter *et al*., 1993).

The fraction of α_{2A}-adrenoceptors that was blocked by a single concentration of EEDQ (300 nM) was higher than the fractional reduction in the maximal response to clonidine induced by the blocker. This indicates that a fraction of α_{2A}-adrenoceptors can be blocked with only a slight modification in the intrinsic activity of the pathway (Kenakin, 1993). Moreover, the sensitivity of α_{2A}-adrenoceptors to clonidine in the rat vas deferens was estimated to be higher than the affinity of α_{2A}-adrenoceptors for this agonist, which suggests that an occupancy lower than 50% of total receptors is able to produce 50% of the maximal effect (Kenakin, 1993). The receptor occupancy-effect relation for clonidine was shown to be hyperbolic, which is typical for systems with large fractions of receptor reserve (Black & Leff, 1983). In fact, only 2.9% of total receptors were needed to be occupied to elicit 50% of the maximal effect (pK_E = 1.5). The prostatic portion of the rat vas deferens has a similar fraction of reserve at α_{2A}-adrenoceptors for clonidine (p$K_E \approx 1.8$) (Sallés *et al*., 1994).

Repeated administration of reserpine causes a supersensitivity of presynaptic α_{2A}-adrenoceptors in the rat vas deferens (Ugedo *et al*., 1993). Other peripheral and central responses mediated by α_2-adrenoceptors are also supersensitive after chronic treatments with low doses of reserpine (Grassby & Broadley, 1986; Estan *et al*., 1990; Ugedo *et al*., 1993). In the present study, the enhanced functionality of α_{2A}-adrenoceptors after reserpine was found to be associated with an increase in the efficiency of clonidine to produce a response, but not linked to a change in the receptor affinity for clonidine. In contrast, previous biochemical studies have shown an increased affinity of [^{3}H]-clonidine binding for brain α_2-adrenoceptors after chronic treatment with low doses of reserpine (Hong *et al*., 1988; Giralt & García-Sevilla, 1989; Ugedo *et al*., 1993). The reason for this discrepancy is, as yet, unknown, although it might be related to differences between functional and biochemical methodologies or to tissue peculiarities (Kenakin, 1993). The increase in the efficiency of clonidine to produce a response suggests that there is an increase in the receptor reserve at α_{2A}-adrenoceptors in reserpine-treated animals. The enhanced α_{2A}-adrenoceptor reserve would explain the increase in the sensitivity of this receptor, because both parameters changed in parallel. According to the occupational theory of the receptor, an increase in the receptor reserve is secondary to an increase in the density of total receptors and/or in the efficiency of the stimulus-response coupling (Kenakin, 1993). In this regard, biochemical studies have shown that reserpine increases the density of α_2-adrenoceptor binding sites (U'Prichard & Snyder, 1978; Bylund & Martínez, 1980). An enhancement in the expression of receptor-coupled inhibitory G proteins has been also described after reserpine administration (Butkerait & Friedman, 1993), which would lead to an improvement in the efficiency of the stimulus-response coupling (Kenakin, 1993).

Table 4 Effect of treatments with cholinomimetic agents on the functional factors governing the clonidine-induced response in the vas deferens

	Parameters derived from the nested hyperbolic function			
Treatments	pK_A[a]	$q \times 100$[b]	pK_E[c]	n
Control	7.10±0.12	2.4±0.6	1.54±0.02	7–16
Pilocarpine (acute)	7.04±0.13	1.2±0.3	1.64±0.04	4–5
Pilocarpine (4 days)	7.19±0.16	0.6±0.2*	1.88±0.05*	5–7
Neostigmine (acute)	7.16±0.14	2.7±0.7	1.52±0.04	5
Neostigmine (11 days)	6.99±0.15	1.3±0.3	2.00±0.03*	6–8

[a]Negative logarithm of the dissociation constant of the agonist-receptor complex. [b]Percentage of receptors remaining after EEDQ inactivation. [c]Negative logarithm of the fractional receptor occupancy needed to acheive 50% of the maximal effect. (Maximal effect and slope factor values ranged from 96% to 100% and from 0.9 to 1.1, respectively). Parameter values are the best fit±s.e.of *n* experiments as obtained by the nonlinear regression. *$P<0.005$ from control group.

Interactions between the cholinergic system and presynaptic α_2-adrenoceptors have been recognized in peripheral tissues (Loiacono *et al*., 1985). The rat vas deferens is a suitable model to study this type of cholinergic-noradrenergic interaction, because in addition to the existence of presynaptic inhibitory α_{2A}-adrenoceptors, there are inhibitory presynaptic M_1-muscarinic ACh receptors and facilitatory postsynaptic M_2-muscarinic ACh receptors (Eltze, 1988). Repeated treatments with the muscarinic ACh receptor agonist pilocarpine or with the acetylcholinesterase inhibitor neostigmine have been shown to cause supersensitivity of α_{2A}-adrenoceptors to clonidine in the rat vas deferens (Olmos *et al*., 1993). This heterospecific supersensitivity of α_2-adrenoceptors after treatments with direct/indirect cholinomimetic agents has been also shown for other responses mediated by these receptors (Olmos *et al*., 1993). The present results confirm the induction of supersensitive α_{2A}-adrenoceptors in rat vas deferens after short-term and chronic treatments with cholinomimetic agents. In addition, this enhanced functionality of α_{2A}-adrenoceptors was linked to an elevation in the fraction of reserve receptors for clonidine, but not to a change in the receptor affinity for the agonist. In this sense, the density or affinity of [^{3}H]-clonidine binding to α_2-adrenoceptors do not appear to be affected by treatments with cholinomimetic agents (Olmos *et al*., 1993). Therefore, the prolonged activation of the muscarinic pathway with cholinomimetic agents induces a supersensitivity of α_{2A}-adrenoceptors by a mechanism distal to the receptor, probably related to an enhanced stimulus-response coupling. In contrast, the enhancement induced by pilocarpine in the efficacy of EEDQ at blocking the receptors and reducing the maximal effect of clonidine would be compatible with an increase in the affinity of EEDQ for α_{2A}-adrenoceptors.

In conclusion, the present study demonstrates that depletion of monoamines with reserpine and activation of muscarinic ACh receptors with cholinomimetic agents induce an increase in the α_{2A}-adrenoceptor reserve in rat vas deferens; this mechanism might underlie the α_{2A}-adrenoceptor supersensitivity caused by these drugs in this preparation. A better understanding of the mechanisms associated with the supersensitivity of α_{2A}-adrenoceptors (García-Sevilla *et al*., 1986; Meana *et al*., 1992) could be relevant to the pathophysiology of depressive disorders.

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References

AGNETER, E., DROBNY, H. & SINGER, E.A. (1993). Central α_2-autoreceptors: agonist dissociation constants and recovery after irreversible inactivation. *Br. J. Pharmacol*., **108,** 370–375.

BLACK, J.W. & LEFF, P. (1983). Operational models of pharmacological agonism. *Proc. R. Soc*., **B220,** 141–162.

BUTKERAIT, P. & FRIEDMAN, E. (1993). Repeated reserpine increases striatal dopamine receptor and guanine nucleotide binding protein RNA. *J. Neurochem*., **60,** 566–571.

BYLUND, D.B. & MARTÍNEZ, J.R. (1980). α_2-Adrenergic receptors appear in rat salivary glands after reserpine treatment. *Nature*, **285,** 229–230.

CONNAUGHTON, S. & DOCHERTY, J.R. (1990). Functional evidence for heterogeneity of peripheral prejunctional α_2-adrenoceptors. *Br. J. Pharmacol*., **101,** 285–290.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol*., **235,** E97–E102.

DREW, G.M. (1977). Pharmacological characterization of the presynaptic α-adrenoceptor in the rat vas deferens. *Eur. J. Pharmacol*., **42,** 123–130.

ELTZE, M. (1988). Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol*., **151,** 205–221.

ESTAN, L., SENARD, J.M., TRAN, M.A., MONTASTRUC, J.L. & BERLAN, M. (1990). Reserpine induces vascular α_2-adrenergic supersensitivity and platelet α_2-adrenoceptor up-regulation in dog. *Br. J. Pharmacol*., **101,** 329–336.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissocation constants of receptor-agonist complexes. In *Advances in Drug Research*. ed. Harper, N.J. & Simmonds, A.B. pp. 21–55. New York: Academic Press.

GARCÍA-SEVILLA, J.A., GUIMON, J., GARCÍA-VALLEJO, P. & FUSTER, M.J. (1986). Biochemical and functional evidence of supersensitive platelet α_2-adrenoceptors in major affective disorder. *Arch. Gen. Psychiat*., **43,** 51–57.

GARCÍA-SEVILLA, J.A. & ZUBIETA, J.K. (1986). Activation and desensitization of presynaptic α_2-adrenoceptors after inhibition of neuronal uptake by antidepressant drugs in the rat vas deferens. *Br. J. Pharmacol*., **89,** 673–683.

GIRALT, M.T. & GARCÍA-SEVILLA, J.A. (1989). Acute and long-term regulation of brain α_2-adrenoceptors after manipulation of noradrenergic transmission in the rat. *Eur. J. Pharmacol*., **164,** 455–466.

GONZÁLEZ, A.M., PASCUAL, J., MEANA, J.J., BARTUREN, F., DEL ARCO, C., PAZOS, A. & GARCÍA-SEVILLA, J.A. (1994). Autoradiographic demonstration of increased α_2-adrenoceptor agonist binding sites in the hippocampus and frontal cortex of depressed suicide victims. *J. Neurochem*., **63,** 256–265.

GRASSBY, P.F. & BROADLEY, K.J. (1986). Responses mediated via beta-1 adrenoceptors but not beta-2 adrenoceptors exhibit supersensitivity after chronic reserpine pretreatment. *J. Pharmacol. Exp. Ther*., **237,** 950–958.

HOLLINGSWORTH, P.J. & SMITH, C.B. (1989). Effects of muscarinic receptor agonists and antagonists on α_2-adrenoceptors in rat brain. *Eur. J. Pharmacol*., **168,** 159–167.

HONG, M., ROOTS, E.J., JENNER, P. & MARSDEN, C.D. (1988). The effect of long-term treatment with amine-depleting drugs or chlorpromazine on alpha-adrenoreceptors and 5-HT_2 receptors in the brain of the rat. *Neuropharmacology*, **27,** 519–527.

JAMES, M.K., MORGAN, P.H. & LEIGHTON, H.J. (1989). A new method for estimation of agonist dissociation constants (K_A): directly fitting of the postinactivation concentration-response curve to a nested hyperbolic equation. *J. Pharmacol. Exp. Ther*., **249,** 61–69.

JANOWSKY, D.S. & OVERSTREET, D.H. (1995). The role of acetylcholine mechanisms in mood disorders. In *Psychopharmacology: The Fourth Generation of Progress*. ed. Bloom, F.E. & Kupfer, D.J. pp. 945–956. New York: Raven Press.

KENAKIN, T.P. (1993). *Pharmacological Analysis of Drug-Receptor Interaction*. New York: Raven Press.

LOIACONO, R.E., RAND, M.J. & STORY, D.F. (1985). Interaction between the inhibitory action of acetylcholine and the alpha-adrenoceptor autoinhibitory feedback system on release of [^{3}H]-noradrenaline from rat atria and rabbit ear artery. *Br. J. Pharmacol*., **84,** 697–705.

MEANA, J.J., BARTUREN, F. & GARCÍA-SEVILLA, J.A. (1992). α_2-Adrenoceptors in the brain of suicide victims: increased receptor density associated with major depression. *Biol. Psychiat*., **31,** 471–490.

MOTULSKY, H.J. & RANSNAS, L.A. (1987). Fitting curves to data by nonlinear regression: a practical and nonmathematical review. *FASEB J*., **1,** 101–107.

OLMOS, G., MIRALLES, A., GARCÍA-SEVILLA, J.A., GIRALT, M.T., PINEDA, J., GARRO, M.A., UGEDO, L., MENARGUES, A. & OBACH, R. (1993). Acute and chronic effects of cholinesterase inhibitors and pilocarpine on the density of central and peripheral α_2-adrenoceptors. *Eur. J. Pharmacol*., **236,** 467–476.

PARKER, R.B & WAUD, D.R. (1971). Pharmacological estimation of drug-receptor dissociation constants. Statistical evaluation. I. Agonists. *J. Pharmacol. Exp. Ther*., **177,** 1–12.

PINEDA, J., RUIZ-ORTEGA, J.A. & UGEDO, L. (1997). Receptor reserve and turnover of α_2-adrenoceptors which mediate the clonidine-induced inhibition of rat locus coeruleus neurons *in vivo*. *J. Pharmacol. Exp. Ther*., **281,** 690–698.

SALLÉS, J., GIRALDO, J. & BADÍA, A. (1994). Analysis of agonism at functional prejunctional α_2-adrenoceptors of rat vas deferens using operational and null approaches. *Eur. J. Pharmacol*., **258,** 229–238.

SMITH, K. & DOCHERTY, J.R. (1992). Are the prejunctional α_2-adrenoceptors of the rat vas deferens and submandibular gland of the α_{2A}- or α_{2D}-subtype. *Eur. J. Pharmacol.*, **219,** 203–210.

STARKE, K., GÖTHER, M. & KILBINGER, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69,** 864–989.

UGEDO, L., GARRO, M.A., PINEDA, J., GIRALT, M.T., MIRALLES, A., OLMOS, G., GARCÍA-SEVILLA, J.A., MENARGUES, A. & OBACH, R. (1993). Acute and chronic effects of reserpine on biochemical and functional parameters of central and peripheral α_2-adrenoceptors. *Eur. J. Pharmacol.*, **239,** 149–157.

U'PRICHARD, D.C. & SNYDER, S.H. (1978). ^{3}H-Catecholamine binding to alpha-receptors in rat brain: enhancement by reserpine. *Eur. J. Pharmacol.*, **51,** 145–155.

WACHTEL, H. (1990). The second-messenger disbalance hypothesis of affective disorders. *Pharmacopsychiatry*, **23,** 27–32.

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