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Agonist Concentration-response Models in Mouse and Rabbit Neocortex: Re-evaluation of Classical Models in Comparison with a Recently Developed General Response Function

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Abstract: In the present study concentration-response curves of four α_2-adrenoceptor agonists on the α_2-autoreceptor-mediated inhibition of [^{3}H]-noradrenaline release in mouse and rabbit neocortex slices were obtained in the absence of autoinhibition. Some of the experiments were performed after short-term presence of phenoxybenzamine to reduce the number and affinity of α_2-autoreceptors and after short-term presence of N-ethylmaleimide to destroy the intracellular coupling of α_2-autoreceptor-mediated events. Evaluation of the concentration-response curves was done with the double reciprocal method of Furchgott[4], with the operational model of Black *et al.*[5] and with the general response function of Feuerstein *et al.*[2]. It was found that both the methods of Furchgott[4] and the operational model of Black *et al.*[5] were inapplicable to quantify a receptor reserve. Only the general response function appropriately evaluated the receptor reserve in the case of the full agonists used, denied spare receptors for partial agonists and confirmed the absence of spare receptors after pretreatment of the neocortex slices with phenoxybenzamine or N-ethylmaleimide.

Key words: [^{3}H]-noradrenaline release, presynaptic autoinhibition, α_2-adrenoceptors, mathematical modeling, agonist concentration-response relationship

INTRODUCTION

Recently, a mathematical model of receptor agonism has been proposed which considered that semi-logarithmic concentration-response sigmoids may be asymmetric in their inflection points[1-3]. As a central assumption, the model implies that an asymmetric concentration-response curve reflects non-proportionality between agonist receptor occupation and the induced response, caused for example by the existence of a receptor reserve. So far, the new model - which was called the "general response function" - has not been tested in direct comparison with classical models of receptor agonism. In addition, only a limited number of full or partial agonists has been investigated previously by evaluation of their concentrations-response curves with the general response function.

In the present study, concentration-response curves of the α_2-adrenoceptor agonists, α-methylnoradrenaline, moxonidine, UK 14,304 and rilmenidine, for the α_2-autoreceptor-mediated inhibition of [^{3}H]-noradrenaline (NA) release were determined in mouse and rabbit neocortex and evaluated with the double reciprocal method of Furchgott[4], the operational model of Black *et al.*[5] and the general response function[2]. The alkylating agent phenoxybenzamine was used *in vitro* as an irreversible α-adrenoceptor antagonist in order to reduce the number of α_2-autoreceptors. However, alkylation by phenoxybenzamine may not only eliminate α-adrenoceptors in an all-or-none fashion, but may also reduce the agonist affinity of α-adrenoceptors which still remain functional[6]. Therefore, to eliminate α_2-autoreceptor-mediated events differently, N-ethylmaleimide was applied to disrupt the presynaptic signal transduction cascade by depleting the guanine nucleotide-binding protein[7]. All concentration-response curves were evaluated with the above mentioned agonist models to determine functionally the agonist-receptor dissociation constant K_a of agonists under different conditions of receptor density and affinity.

The α_2-autoreceptors are α_{2D} in the mouse neocortex[8] and α_{2A} in the rabbit neocortex[9], but this

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pharmacological difference was outside the scope of the present study.

MATERIALS AND METHODS

Male NMRI mice (25 to 35 g) and rabbits of either sex (1.8 to 2.4 kg) were killed by cervical dislocation. The brain was quickly removed and chilled in ice-cold medium. Round slices (mouse: 0.3 mm thick, 2 mm in diameter; rabbit: 0.4 mm thick, 3.5 mm in diameter) were cut from the cortex, parallel to the surface, after a superficial layer of 0.2 (mouse) or 0.3 mm (rabbit) had been removed. Eight or nine slices were incubated with (-)-[^{3}H]-NA (0.1 μM) in 2 mL medium for 30 min at 37°C. Six slices were then superfused in parallel with [^{3}H]-NA-free medium at 1.67 mL min^{-1}. With few exceptions, either solvent (tartaric acid; final concentration 7-17 μM) or phenoxybenzamine or N-ethylmaleimide was added to the superfusion medium at t = 10 min (t = 0 min being the start of superfusion), was present for the following 10 min and then washed out. Four periods of electrical stimulation were applied consisting of rectangular pulses of 2 ms width and 10.5 V/cm voltage drop, yielding a current strength of 18 mA. The first stimulation period (18 pulses, 1 Hz) was delivered at t = 30 min and was not used for determination of tritium overflow. The following periods (S_1-S_3) were applied at t = 56, 83 and 110 min and consisted of 4 pulses, 100 Hz. Successive 3 min samples of the superfusate were collected from t = 50 min onwards. Unless stated otherwise, the α_2-autoreceptor agonists α-methylnoradrenaline, moxonidine, rilmenidine and UK 14,304 were added at increasing concentrations 15 min before S_2 and S_3 (Fig. 1). At the end of the experiment, the tissue was dissolved and tritium was determined in superfusate samples and slices.

The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03 and desipramine 0.001 (0.005 in experiments with α-methylnoradrenaline). The medium for incubation with [^{3}H]-NA was desipramine-free. The outflow of tritium was calculated as a fraction of the tritium content of the tissue at the beginning of the respective collection period and expressed as fractional rate (min^{-1}). The electrically

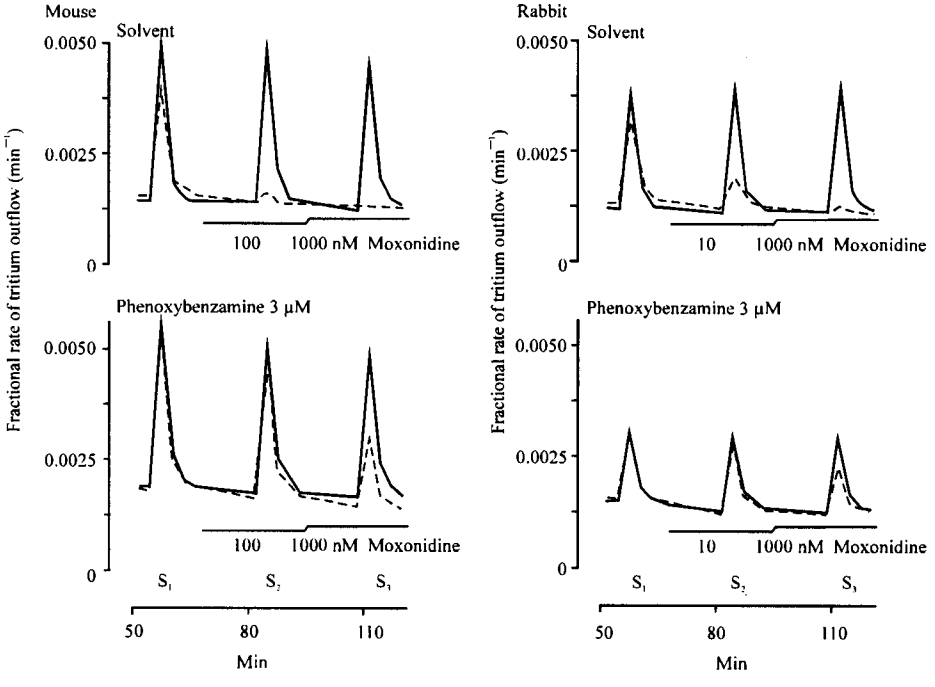

Fig. 1: Tritium outflow from mouse and rabbit brain cortex slices preincubated with [^{3}H]-NA, showing the effect of moxonidine and the influence of pretreatment with phenoxybenzamine
After preincubation, the slices were superfused in the presence of desipramine 1 μM and stimulated electrically three times with 4 pulses/100 Hz (S_1, S_2 and S_3). Solvent or phenoxybenzamine 3 μM was added to the superfusion medium from t = 10 to t = 20 min and then washed out. Solvent (solid lines) or increasing concentrations of moxonidine (dashed lines) were administered as indicated. Abscissae, minutes of superfusion. Each line represents the outflow of tritium from a single brain slice

evoked overflow of tritium was calculated by subtracting the estimated basal outflow from the total tritium outflow during the collection period in which the stimulation period was applied plus the two periods thereafter. The basal outflow of tritium was assumed to decline linearly from the collection period before to the collection period 9 to 12 min after stimulation. The overflow of tritium was then expressed as a percentage of the tritium content of the slice at the time of stimulation. For further evaluation of the stimulation-evoked overflow of tritium, ratios were calculated of the overflow evoked by S_2 and S_3 and the overflow evoked by S_1 (S_2/S_1 and S_3/S_1). Moreover, effects of α_2-autoreceptor agonists on the stimulation-evoked overflow were calculated, for each single slide, as percentage inhibition, using the corresponding mean average control S_2/S_1 and S_3/S_1 ratios (solvent-treated slices, no agonist, no antagonist) as a reference.

A logistic function, representing the (semi-logarithmic) log-concentration-response relationship:

$$\frac{E}{E_{max}} = \frac{10^{lg[A]c}}{10^{lg[EC_{50}]c} + 10^{lg[A]c}} \quad (1)$$

with E/E_{max} adapted to a concentration-inhibition relationship (here and in all following formulas[1,10]; for instance, function 7[10]), was fitted to each agonist concentration-inhibition data set. E was the effect (% inhibition, dependent variable) measured at the agonist concentration [A] (M, independent variable $lg[A] = \log_{10}[A]$). Parameters to be estimated were, E_{max} the (asymptotic) maximum percentage inhibition caused by the agonist, EC_{50} the location parameter, representing the concentration (M) at which the agonist causes half of its maximum inhibition and c the slope parameter of the sigmoid concentration-inhibition curve which is equal to the Hill coefficient. For a given agonist, single concentration-inhibition values entered into the fitting procedure. The EC_{50} is displayed as negative decadic logarithm, pEC_{50}.

Estimation of the dissociation constant K_a of the α_2-autoreceptor/agonist complex: Three methods were used to obtain K_a from the agonist concentration-inhibition data. One of them compares the agonist concentration-inhibition curve obtained in solvent-treated slices with that obtained in phenoxybenzamine-treated slices[4]. The other two methods are based on models which theoretically allow the estimation of an agonist's K_a by analyzing its concentration-inhibition curve in solvent-treated tissue. Two methods are well established[4,5] and have been described on various occasions; only a small account of the calculation procedure is given here. The general response function, has been introduced only recently[2].

Double reciprocal method of Furchgott[4]: This traditional method of K_a determination is based on the comparison of the agonist concentration-inhibition curve obtained in solvent-treated slices with the curve obtained in phenoxybenzamine-treated slices. The evaluation consisted of the following steps: (I) Fitting the logistic function to the agonist concentration-inhibition data set obtained in solvent-treated slices; (ii) calculating from this function the concentrations that were equieffective with agonist concentrations used in phenoxybenzamine-treated slices (only concentrations causing 15 to 85% of the maximum inhibition obtained in phenoxybenzamine-treated slices were used); (iii) calculation of a linear regression of the reciprocal values of the resulting pairs of equieffective concentrations according to equation 6 of Furchgott[4]; (iv) calculation of the receptor fraction still active in phenoxybenzamine-treated slices and of K_a from the slope and the ordinate intercept of the regression line.

Operational model of Black *et al.*[5]: For each agonist the model function, i.e. function (8) of Black *et al.*[5]:

$$\frac{E}{E_{max}} = \frac{\tau^n 10^{lg[A]n}}{(10^{lg[Ka]} + 10^{lg[A]})^n + \tau^n 10^{lg[A]n}}$$

in semilogarithmic form, was fitted to the concentration-inhibition data obtained in solvent-treated slices and, simultaneously, to the concentration-inhibition data set obtained in phenoxybenzamine-treated slices. The maximum possible response of the system (which is not the same as the maximum effect of a given agonist E_{max}) was constrained to 100% inhibition. With these restrictions, the model provides estimates of E_{max}, of K_a, of the 'transducer ratio' τ in solvent-treated slices (τ_{con}) and in phenoxybenzamine-treated slices (τ_{PBA}) and of a parameter (exponent n) that determines the "sensitivity" of the occupancy-effect relation. The ratio τ_{PBA}/τ_{con} indicates the receptor fraction still active in phenoxybenzamine-treated slices.

The general response function of Feuerstein *et al.*[2]: The following assumptions have to be made for this model. (I) Each functional unit in a tissue (in the present study each active site at which release, and inhibition of release, occurs) possesses a total of N receptors. (ii) The occupation of the receptors by an agonist is a bimolecular reaction according to the Law of Mass Action and is represented by the following equation, representing the (semi-logarithmic) log-concentration-binding relationship:

$$\frac{[AR]}{R_{tot}} = \frac{10^{lg[A]}}{10^{lg[Ka]} + 10^{lg[A]}} \quad (2)$$

with $[R]_{tot}$ being the total receptor concentration, [AR] the concentration of the agonist-receptor complex, [A] the free concentration of the agonist and K_a the dissociation constant of the agonist-receptor complex. (iii) For a given agonist, K receptors have to be occupied in order to obtain the maximum effect of the functional unit and N minus K receptors are spare receptors. (iv) For each functional unit a graded response from no effect to maximum effect is obtained when the agonist combines with none to K ('non-spare') receptors.

The overall response of the tissue is the sum of the responses of all functional units and depends on the probability that, at a given agonist concentration, a certain fraction (K/N for instance) of the receptors of a single functional unit is occupied. This probability is computed from the binomial distribution. By adding the frequency of the functional units with a smaller fraction (than K/N) of receptors occupied plus the frequency of units with fractions ≥ K/N ≤ 1 of receptors occupied the general response function of the (semi-logarithmic) form

$$\frac{E}{E_{max}} = 1 - \sum_{i=0}^{K-1} \left(1 - \frac{i}{K}\right)\binom{N}{i} \left(\frac{10^{lg[A]}}{10^{lg[Ka]} + 10^{lg[A]}}\right)^{i} \left(1 - \frac{10^{lg[A]}}{10^{lg[Ka]} + 10^{lg[A]}}\right)^{N-i} \quad (3)$$

is obtained[2]. This function is valid for all occupation ratios between zero and unity ([A], K_a, E_{max}, N and K as above) and was used in a form adapted to concentration-inhibition data[1].

The general response function permits the mechanistic interpretation of concentration-response relationships for agonists with a receptor reserve (rough estimation of the number of spare receptors). K and N are integer values. This makes a comparison of the 'parsimony of parameters' of the three models to estimate K_a difficult. For K = N, i.e. when spare receptors do not exist, the function reduces to the logistic function with the slope parameter c being unity. The concentration-response curve then is identical to the concentration-binding curve (function 2). For K = 1, the function describes an all-or-nothing response of each functional unit[1,11].

In practice, the general response function was fitted to each agonist concentration-inhibition data set with single concentration-inhibition values entering into the fitting procedure. In a superfusion system as it was used in the present study, in which a small tissue specimen is superfused with a large, theoretically infinite, quantity of medium containing the agonist at a fixed concentration, only a negligible fraction of the total ligand concentration [A] is bound to the receptor. Therefore, the (unknown) free agonist concentration $[A]_f$ was replaced by the (known) concentration [A]. Due to limited computer capacity, N and K were not allowed to exceed 50. As in the case of the logistic equation, K_a is expressed as negative decadic logarithm, pK_a.

Statistics and drugs: Results are given as arithmetic mean±standard error of the mean. Parameters from fitting procedures are given as estimate±SD or as estimate with the 95% confidence interval (CI_{95}; NLIN of the SAS system; SAS Institute, Heidelberg, Germany). N and K take on integers only and the fitting producer therefore did not allow to estimate their error. The stimulation-evoked tritium overflow was calculated as difference between the overall [^{3}H]-outflow due to the stimulation and the basal [^{3}H]-outflow and was expressed as fractional rate of the total radioactivity present in the tissue at the onset of stimulation.

Purchased drugs were (-)-[ring-2,5,6-^{3}H]-NA, specific activity 40.5 Ci/mmol (DuPont, Dreieich, Germany); desipramine HCl and N-ethylmaleimide (Sigma, Deisenhofen, Germany). The following drugs were kindly provided by the producer, moxonidine HCl (Beiersdorf, Hamburg, Germany); (-)-erythro-α-methylnoradrenaline HCl (Hoechst, Frankfurt am Main, Germany); 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline tartrate (UK 14,304; Pfizer, Sandwich, Kent, UK); phenoxybenzamine HCl (Röhm, Weiterstadt, Germany); rilmenidine H_3PO_4 (Servier, Courbevoie, France). Drugs were dissolved in distilled water except phenoxybenzamine (tartaric acid 10 mM) and α-methylnoradrenaline (HCl 1 mM). None of the solvents had any effect on the basal or the stimulation-evoked overflow of tritium.

RESULTS

After preincubation with [^{3}H]-NA, the slices were superfused with medium containing, unless stated otherwise, desipramine 1 μM. In solvent-treated slices, the basal outflow of tritium in the 3 min sample immediately before S_1 averaged 0.77±0.02 nCi in the mouse, corresponding to a fractional rate of 0.00204±0.00005 min^{-1} (n = 62) and 0.97±0.03 nCi in the rabbit, corresponding to a fractional rate of 0.00149±0.00003 min^{-1} (n = 65). Neither pretreatment with phenoxybenzamine nor an increase in the desipramine concentration to 5 μM caused any major (>15%) change in basal outflow of tritium. In N-ethylmaleimide-treated slices (mouse only) the basal efflux was slightly enhanced by about 30%.

Evoked tritium overflow: Electrical stimulation with 4 pulses at 100 Hz greatly accelerated the efflux of tritium (Fig. 1). In solvent-treated slices, the overflow of tritium elicited by S_1 averaged 1.56±0.06 nCi in the mouse, corresponding to 1.27±0.05% of the tritium content of the

Table 1: Electrically evoked overflow (S_1) of tritium from mouse and rabbit brain cortex slices preincubated with [^{3}H]-NA

		Mouse		Rabbit	
Drugs throughout superfusion	Pretreatment	S_1 (% of tissue tritium)	n	S_1 (% of tissue tritium)	n
Desipramine 1 μM	-	1.27±0.05	62	0.66±0.03	65
Desipramine 1 μM	PBA 0.5 μM	1.55±0.04**	25	0.79±0.03*	26
Desipramine 1 μM	PBA 2 μM	1.40±0.08	32	-	-
Desipramine 1 μM	PBA 3 μM	1.37±0.08	25	0.70±0.03	62
Desipramine 1 μM	NEM 30 μM	2.01±0.08**	48	-	-
Desipramine 5 μM	-	1.57±0.10**	18	0.73±0.03**	25
Desipramine 5 μM	PBA 0.5 μM	-	-	0.75±0.04	28
Desipramine 5 μM	PBA 5 μM	1.62±0.07**	24	0.78±0.05	29

After preincubation, the slices were superfused in the presence of the drugs indicated. Phenoxybenzamine (PBA) or N-ethylmaleimide (NEM), when used, were added to the superfusion medium from t = 10 to t = 20 min (i.e. 46 to 36 min before S_1) and then washed out. S_1 represents the overflow of tritium elicited by the first period of electrical stimulation with 4 pulses at 100 Hz and is expressed as a percentage of the tritium content of the tissue. Means±SEM of n brain slices. Significant differences from corresponding experiments with desipramine only: * $p<0.05$; ** $p<0.01$ (Mann-Whitney test and Bonferroni correction)

Table 2: Influence of the exposure time on the effects of rilmenidine and moxonidine on electrically evoked overflow of tritium in mouse and rabbit brain cortex slices preincubated with [^{3}H]-NA

	α_2-Agonist Present at	Mouse			Rabbit		
Pretreatment	S_2 and S_3	S_2/S_1	S_3/S_1	n	S_2/S_1	S_3/S_1	n
-	-	1.08±0.02	1.08±0.02	12	1.09±0.02	1.10±0.01	18
PBA 0.5 μM	-	1.07±0.02	1.09±0.03	5	1.06±0.02	1.07±0.03	5
PBA 0.5 μM	Rilm 0.3 μM	0.80±0.03	0.81±0.03	6	0.66±0.01	0.63±0.03	5
PBA 3 μM	-	1.07±0.05	1.04±0.04	5	1.08±0.03	1.12±0.06	10
PBA 3 μM	Mox 0.3 μM	0.77±0.03	0.75±0.03	6	-		
PBA 3 μM	Mox 0.1 μM	-			0.75±0.01	0.73±0.02	6

After preincubation, the slices were superfused in the presence of desipramine 1 μM and stimulated electrically three times with 4 pulses/100 Hz (S_1, S_2 and S_3). Either solvent or phenoxybenzamine (PBA) was added to the superfusion from t = 10 to t = 20 min (i.e. 46 to 36 min before S_1) and then washed out. Rilmenidine (Rilm) or moxonidine (Mox), when used, was present from 15 min before S_2 onwards at a constant concentration. S_n/S_1 values represent the ratios of the overflow elicited by S_2 and S_3 and the overflow elicited by S_1. Means±SEM of n brain slices

tissue (S_1 in Table 1; n = 62) and 1.43±0.07 nCi in the rabbit, corresponding to 0.66±0.03% of the tritium content of the tissue (n = 65). Pretreatment with phenoxybenzamine caused no concentration-dependent change in the electrically evoked overflow of tritium whereas pretreatment with N-ethylmaleimide (mouse only) enhanced it by about 60%. In the presence of desipramine 5 μM the evoked overflow at S_1 was increased by about 25%.

When no α_2-autoreceptor agonist was used, repeated stimulation with 4 pulses/100 Hz elicited a reproducible overflow of tritium. This was true in slices pretreated with either solvent or phenoxybenzamine (Fig. 1) or N-ethylmaleimide.

Evoked tritium overflow: effects of α_2-adrenoceptor agonists: α-Methylnoradrenaline, moxonidine, rilmendine and UK 14,304, when added before S_2 and S_3, all reduced the electrically evoked overflow of tritium in a concentration-dependent manner. Figure 1 illustrates representative experiments with moxonidine in solvent- and in phenoxybenzamine-treated slices. Concentration-inhibition relationships in mouse brain cortex slices are shown in Fig. 2 and those in rabbit brain cortex slices in Fig. 3. When, in phenoxybenzamine-treated slices, moxonidine 0.3 μM (mouse) or 0.1 μM (rabbit) was present from 15 min before S_2 and kept for the remainder of the experiment, the evoked overflow was reduced to the same extend at S_2 and at S_3 (Table 2). The same was true for rilmenidine 0.3 μM (mouse and rabbit, data not shown).

Results of the analysis of the concentration-inhibition data by fitting the logistic function are summarized in Table 3 for mouse and in Table 4 for rabbit brain cortex. In solvent-treated slices, the agonists reduced the electrically evoked overflow with an asymptotic maximum of 92-100%, except rilmenidine in mouse brain slices that caused maximally 83% inhibition. Moxonidine and UK 14,304 both were slightly less potent in the mouse than in the rabbit brain. α-Methylnoradrenaline and rilmenidine were equipotent in either species. In the mouse, α-methylnoradrenaline, moxonidine and UK 14,304 had steep concentration-inhibition curves and the slope parameter c of the logistic function was significantly greater than unity[12]. In the rabbit, c was greater than unity for moxonidine only. In all other cases, c did not significantly differ from unity.

Pretreatment with phenoxybenzamine markedly affected the concentration-inhibition relationships of the α_2-autoreceptor agonists (Fig. 2 and 3). For each of the agonists, the concentration of phenoxybenzamine was chosen as to reduce the maximum inhibition by 20-50%; the concentration was lowest for rilmenidine (0.5 μM) and highest for α-methylnoradrenaline (4 μM). Analysis

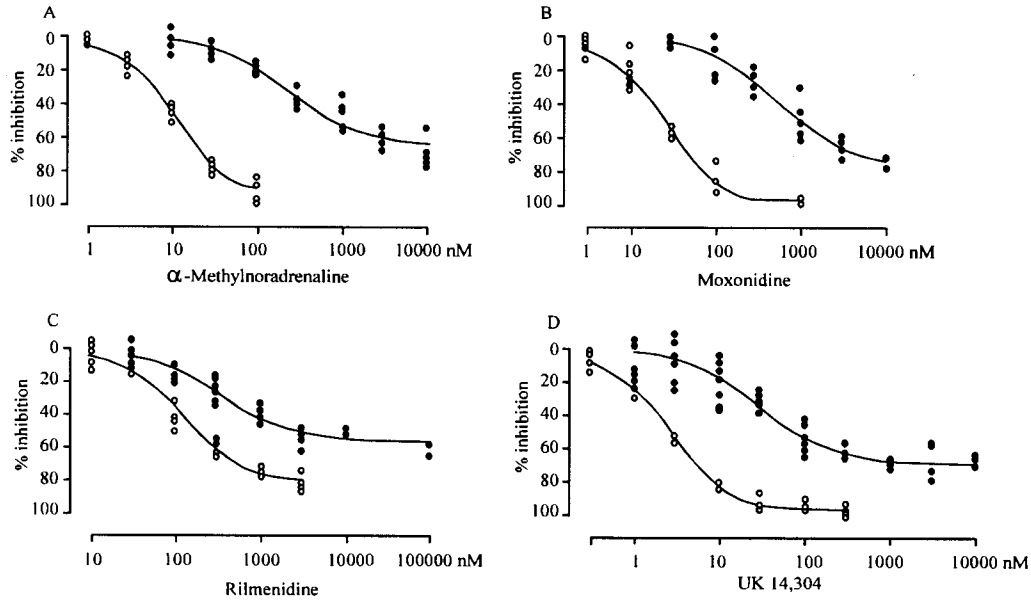

Fig. 2: Stimulation-evoked overflow of tritium from mouse brain cortex slices preincubated with [^{3}H]-NA: effects of α_2-adrenoceptor agonists and influence of pretreatment with phenoxybenzamine
After preincubation, the slices were superfused in the presence of desipramine 1 μM (5 μM in experiments with α-methylnoradrenaline) and stimulated electrically three times with 4 pulses/100 Hz (S_1, S_2 and S_3). Solvent (open circles) or phenoxybenzamine (4 μM in A, 3 μM in B, 0.5 μM in C, 2 μM in D; filled circles) was added to the superfusion medium from t = 10 to t = 20 min and then washed out. Agonists were administered at increasing concentrations (abscissae) before S_2 and S_3. Ordinates, percentage inhibition caused by the agonists, calculated from S_n/S_1 ratios. Lines are the results of non-linear regression analysis of the data with the general response function

with the logistic function showed that pretreatment of the slices with phenoxybenzamine shifted the concentration-inhibition curves to the right. The pEC_{50} values (negative logarithm of the agonist concentration causing half-maximum inhibition) were markedly decreased when compared with those in solvent-treated slices (Table 3 and 4). With three exceptions, the slope parameter c was always close to unity. Only for α-methylnoradrenaline c was estimated considerably smaller than unity in either species. In rabbit brain slices, even a low concentration of phenoxybenzamine (0.5 μM), which did not alter the maximum inhibition, significantly reduced the slope parameter c of the concentration-inhibition curve below unity. In the rabbit, pretreatment with phenoxybenzamine also decreased c of the concentration-response curve of UK 14,304.

In mouse brain slices, pretreatment with N-ethylmaleimide (30 μM) reduced the maximum inhibition caused by rilmenidine and UK 14,304 and significantly diminished the pEC_{50} value of UK 14,304 when compared with its pEC_{50} value in solvent-treated slices (Fig. 4). The slope parameter c of the two concentration-inhibition curves was not different from unity in N-ethylmaleimide-treated slices.

Estimation of K_a: Furchgott method[4]: The logistic function (1) was used to calculate agonist concentrations in solvent-treated slices (A_{con}) equieffective to the concentrations administered in phenoxybenzamine-treated slices (A_{PBA}). Figure 5 illustrates the plot of the pairs of reciprocals, $1/A_{PBA}$ and $1/A_{con}$ and the resulting straight line for α-methylnoradrenaline and rilmenidine in mouse and rabbit brain slices. K_a and the receptor fraction still active in phenoxybenzamine-treated slices were calculated from the slope and the Y-axis intercept of the straight line (Table 5). With K_a known, the Law of Mass Action was applied to compute the fraction of α_2-autoreceptors that was occupied when an agonist caused half-maximal inhibition (Table 5).

The corresponding logistic function that fitted best the concentration-inhibition data in phenoxybenzamine-treated slices was used to compute A_{BPA} values at 1%-inhibition steps ranging from 15-85% inhibition. Equieffective concentrations A_{con} were then calculated by

Table 3: Non-linear regression analysis of the concentration-inhibition curves of α_2-autoreceptor agonists in mouse brain cortex using the logistic function or the general response function; estimates of the function parameters and CI_{95}

		Analysis with the logistic function			Analysis with the general response function				
Agonist	Pretreatment	pEC_{50} (CI_{95})	E_{max} (%) (CI_{95})	c (CI_{95})	pK_a (CI_{95})	E_{max}(%) (CI_{95})	N	K	Receptor fraction occupied at the agonist's EC_{50}
α-Methylnoradrenaline	-	7.96	97.1	1.26*	7.42	91.7	5	2	0.22
		(7.88,8.05)	(90.1, 105.3)	(1.02, 1.50)	(7.35, 7.48)	(87.7, 96.2)			0.57
α-Methylnoradrenaline	PBA 4 μM	6.51	71.4	0.75*	6.64	65.4	K	N	
		(6.29, 6.72)	(63.7, 81.3)	(0.55, 0.94)	(6.52, 6.76)	(61.3, 69.9)			
Moxonidine		7.63	97.1	1.39**	7.17	96.2	4	2	0.26
		(7.57, 7.69)	(92.6, 102.0)	(1.18, 1.60)	(7.11, 7.23)	(92.6, 101.0)			
Moxonidine	PBA 3 μM	6.20	80.7	0.92	6.24	77.5	K	N	0.52
		(6.01, 6.39)	(70.4, 93.5)	(0.67, 1.16)	(6.17, 6.36)	(71.9, 84.8)			
Rilmenidine	-	6.91	83.3	1.13	6.76	81.3	6	5	0.42
		(6.80, 7.02)	(76.9, 90.1)	(0.87, 1.41)	(6.67, 6.86)	(76.9, 86.2)			
Rilmenidine	PBA 0.5 μM	6.47	58.1	0.92	6.49	57.1	K	N	0.51
		(6.32, 6.61)	(53.8, 63.3)	(0.68, 1.15)	(6.37, 6.61)	(53.8, 60.6)			
Rilmenidine	NEM 30 μM	6.74	82.6	0.87	6.80	62.9	K	N	0.53
		(6.54, 6.93)	(58.5, 76.3)	(0.64, 1.09)	(6.68, 6.90)	(58.8, 67.6)			
UK 14,304	-	8.61	96.2	1.23**	8.33	95.2	3	2	0.34
		(8.56, 8.67)	(93.5, 99.0)	(1.05, 1.41)	(8.28, 8.39)	(92.6, 98.0)			
UK 14,304	PBA 2 μM	7.53	69.0	0.90	7.52	66.7	20	19	0.49
		(7.38, 7.69)	(64.1, 74.6)	(0.66, 1.13)	(7.39, 7.64)	(62.9, 70.9)			
UK 14,304	NEM 30 μM	8.39	90.9	0.94	8.38	86.2	17	16	0.49
		(8.28, 8.50)	(84.7, 98.0)	(0.75, 1.13)	(8.30, 8.46)	(82.6, 90.9)			

Each agonist concentration-inhibition curve was analysed separately either with the logistic function or with the general response function. E_{max} indicates the maximum inhibition obtained with the agonist, pEC_{50} is the negative logarithm of the agonist concentration causing half-maximum inhibition, pK_a is the negative logarithm of the dissociation constant of the α_2-adrenoceptor/agonist complex. N indicates the total number of α_2-adrenoceptors per functional unit and K indicates the receptor number that have to be activated in order to obtain the maximum inhibition. If K appears in a row then it was estimated to equal N and vice versa. Function (2) was applied to calculate the receptor fraction occupied by the agonist at its EC_{50} (from logistic curve fitting). PBA: phenoxybenzamine; NEM: N-ethylmaleimide. Significant differences from unity: * $p<0.05$; ** $p<0.01$ (tested according to Mackay[12])

Table 4: Non-linear regression analysis of the concentration-inhibition curves of α_2-autoreceptor agonists in rabbit brain cortex using the logistic function or the general response function; estimates of the function parameters and CI_{95}

		Analysis with the logistic function			Analysis with the general response function				
Agonist	Pretreatment	pEC_{50} (CI_{95})	E_{max} (%) (CI_{95})	c (CI_{95})	pK_a (CI_{95})	E_{max}(%) (CI_{95})	N	K	Receptor fraction occupied at the agonist's EC_{50}
α-Methylnoradrenaline	-	8.05	100.0	0.99	8.00	97.1	16	15	0.47
		(7.98, 8.11)	(96.2, 104.2)	(0.86, 1.11)	(7.94, 8.06)	(94.3, 101.0)			
α-Methylnoradrenaline	PBA 0.5 μM	7.28	100.0	0.68**	7.48	88.5	K	N	0.61
		(7.14, 7.42)	(91.7, 109.9)	(0.62, 0.76)	(7.35, 7.62)	(81.3, 97.1)			
α-Methylnoradrenaline	PBA 4 μM	6.83	76.3	0.35**	7.80	50.3	K	N	0.90
		(6.15, 7.50)	(62.1, 100.0)	(0.25, 0.46)	(7.65, 7.94)	(47.2, 53.5)			
Moxonidine		8.10	95.2	1.47**	7.48	90.9	20	8	0.19
		(8.02, 8.17)	(89.3, 101.0)	(1.17, 1.77)	(7.43, 7.54)	(87.0, 94.3)			
Moxonidine	PBA 3 μM	7.00	68.5	0.84	7.04	65.8	K	N	0.52
		(6.85, 7.15)	(63.3, 74.6)	(0.63, 1.05)	(6.93, 7.16)	(62.1, 70.4)			
Rilmenidine	-	7.18	92.6	1.16	7.04	90.1	19	16	0.42
		(7.10, 7.26)	(87.7, 97.1)	(0.96, 1.36)	(6.97, 7.11)	(86.2, 93.5)			
Rilmenidine	PBA 0.5 μM	6.73	62.1	0.96	6.74	61.7	K	N	0.51
		(6.59, 6.87)	(57.5, 67.6)	(0.73, 1.19)	(6.63, 6.85)	(58.1, 65.4)			
UK 14,304	-	9.18	100.0	1.00	9.12	95.2	20	18	0.47
		(9.09, 9.27)	(94.3, 106.4)	(0.84, 1.15)	(9.05, 9.19)	(91.7, 99.0)			
UK 14,304	PBA 3 μM	8.44	65.8	0.69**	8.60	59.2	K	N	0.59
		(8.25, 8.64)	(59.9, 73.0)	(0.54, 0.84)	(8.47, 8.72)	(55.6, 63.3)			

Each agonist concentration-inhibition curve was analysed separately either with the logistic function or with the general response function. E_{max} indicates the maximum inhibition obtained with the agonist, pEC_{50} is the negative logarithm of the agonist concentration causing half-maximum inhibition, pK_a is the negative logarithm of the dissociation constant of the α_2-adrenoceptor/agonist complex. N indicates the total number of α_2-adrenoceptors per functional unit and K indicates the receptor number that have to be activated in order to obtain the maximum inhibition. If K appears in a row then it was estimated to equal N and vice versa. Function (2) was applied to calculate the receptor fraction occupied by the agonist at its EC_{50} (from logistic curve fitting). PBA: phenoxybenzamine. Significant differences from unity: * $p<0.05$; ** $p<0.01$ (tested according to Mackay[12])

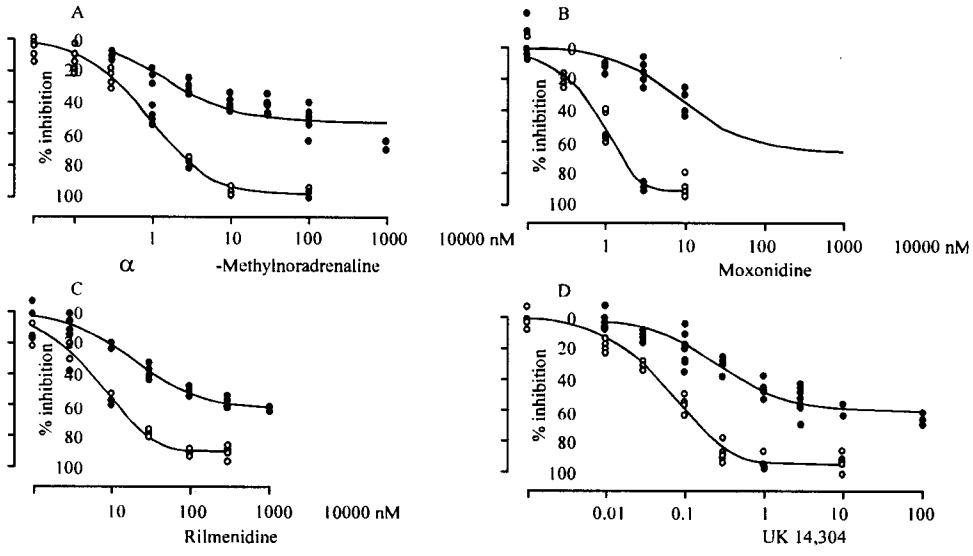

Fig. 3: Stimulation-evoked overflow of tritium from rabbit brain cortex slices preincubated with [^{3}H]-NA: effects of α_2-autoreceptor agonists and influence of pretreatment with phenoxybenzamine
After preincubation, the slices were superfused in the presence of desipramine 1 μM (5 μM in experiments with α-methylnoradrenaline) and stimulated electrically three times with 4 pulses/100 Hz (S_1, S_2 and S_3). Solvent (open circles) or phenoxybenzamine (4 μM in A, 3 μM in B and D, 0.5 μM in C; filled circles) was added to the superfusion medium from t = 10 to t = 20 min and then washed out. Agonists were administered at increasing concentrations (abscissae) before S_2 and S_3. Ordinates, percentage inhibition caused by the agonists, calculated from S_n/S_1 ratios. Lines are the results of non-linear regression analysis of the data with the general response function

means of the logistic function that was applied to the concentration-inhibition data in solvent-treated slices. Figure 5 also contains the pairs of reciprocals of the computed equieffective concentrations for α-methylnoradrenaline and rilmenidine. They clearly did not yield the requested straight line. Moreover, the relationship of the reciprocals of the computed equieffective concentrations is not linear at all, but rather curvilinear (Fig. 5). The same was true for moxonidine and UK 14,304 in either species.

Estimation of K_a: method of Black *et al.*[5]: The model function was initially fitted to the agonist concentration-inhibition data obtained in solvent-treated slices only: The fitting procedure did not converge. Next, the model function was fitted to the data obtained in solvent-treated slices and, simultaneously, to the data obtained in phenoxybenzamine-treated slices. The fitting procedure then either yielded meaningless estimates of the maximum possible response of the system (much more than 100% inhibition; according to the model, the maximum possible inhibition is not the same as E_{max} of a given agonist) or did still not converge. Only if the maximum response was constrained to 100% inhibition the model described the concentration-inhibition relationships (Table 6). The pK_a values thus obtained were in the same range as the pK_a values estimated with the null method of Furchgott[4], except for rilmenidine in the mouse and α-methylnoradrenaline in the rabbit. Moxonidine had the highest and rilmenidine the lowest efficacy (τ_{con}) in the mouse as well as in the rabbit. Table 6 also indicated the receptor fraction that was occupied by the agonist when it caused half its maximum inhibition.

Estimation of K_a: method of Feuerstein *et al.*[2]: The general response function estimates four parameters by analysing position, slope and shape of the concentration-inhibition curve of an agonist. Graphs of the best fits are represented by the lines in Fig. 2, 3 and 4. The function parameters are summarized in Table 3 for mouse and in Table 4 for rabbit brain slices. For those concentration-inhibition curves for which logistic curve fitting had indicated a slope parameter c of unity, pK_a (estimated by the general response function) was very similar to pEC_{50} (estimated by the logistic function). In addition, the number of receptors of a functional unit that must be activated for the maximum effect, K, was close (or equal) to the total number of receptors, N. For

Table 5: Estimation of the dissociation constant K_a of agonists at presynaptic α_2-autoreceptors in mouse and rabbit brain cortex slices with the double reciprocal method of Furchgott[4]

Agonist	Concentration of PBA	pK_a	Receptor fraction still active after PBA	Receptor fraction occupied at the agonist's EC_{50} in	
				solvent	PBA-treated slices
Mouse					
α-Methylnoradrenaline	4.0 μM	6.68	0.059	0.05	0.60
Moxonidine	3.0 μM	6.16	0.056	0.03	0.48
Rilmenidine	0.5 μM	5.61	0.449	0.05	0.12
UK 14,304	2.0 μM	7.13	0.062	0.03	0.29
Rabbit					
α-Methylnoradrenaline	4.0 μM	8.16	0.449	0.56	0.96
Moxonidine	3.0 μM	6.95	0.099	0.07	0.47
Rilmenidine	0.5 μM	6.65	0.315	0.23	0.45
UK 14,304	3.0 μM	7.91	0.100	0.05	0.23

For each agonist, the inhibition data obtained in phenoxybenzamine (PBA)-treated slices (concentrations A_{PBA}) were compared with equieffective concentrations in solvent-treated slices (A_{con}). Equieffective concentrations were calculated by means of function (1) that fitted best the concentration-inhibition data obtained in solvent-treated slices (see Table 3 and 4 for parameters of function (1)). A linear regression of $1/A_{con}$ on $1/A_{PBA}$ was calculated in order to estimate the agonist dissociation constant K_a according to Furchgott[4]. Function (2) was applied to calculate the receptor fraction occupied by the agonist at its EC_{50} (from logistic curve fitting)

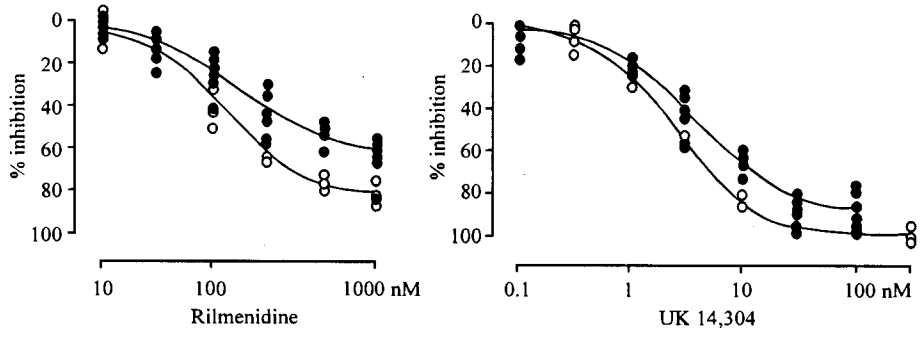

Fig. 4: Stimulation-evoked overflow of tritium from mouse brain cortex slices preincubated with [^{3}H]-NA: effects of rilmenidine and UK 14,304 and influence of pretreatment with N-ethylmaleimide
After preincubation, the slices were superfused in the presence of desipramine 1 μM and stimulated electrically three times with 4 pulses/100 Hz (S_1, S_2 and S_3). Solvent (open circles; same as in Fig. 2) or N-ethylmaleimide 30 μM (filled circles) was added to the superfusion medium from t = 10 to t = 20 min and then washed out. Rilmenidine or UK 14,304 was administered at increasing concentrations (abscissae) before S_2 and S_3. Ordinates, percentage inhibition caused by rilmenidine or UK 14,304, calculated from S_n/S_1 ratios. Lines are the results of non-linear regression analysis of the data with the general response function

instance, in mouse brain cortex c was not significantly different from unity for the concentration-inhibition curve of rilmenidine (1.13; Table 3). Thus, pK_a (= 6.76) closely resembled pEC_{50} (= 6.91) and K (= 5) was similar to N (= 6). In contrast, for those concentration-inhibition curves for which logistic curve fitting had indicated a c significantly greater than unity, pK_a was smaller than pEC_{50} and K was markedly lower than N. For instance, in rabbit brain cortex c of the concentration-inhibition curve of moxonidine was significantly greater than unity (1.47; Table 4). Then, pK_a (= 7.48) was 0.62 units lower than pEC_{50} (= 8.10) and only K = 8 of a total of N = 20 receptors had to be activated for the maximum inhibition. With one exception, the general response function estimated the maximum inhibition of the agonists similar as did the logistic function (close agreement of the parameters E_{max}). Only for the concentration-inhibition curve of α-methylnoradrenaline in phenoxybenzamine-treated slices of the rabbit, E_{max} was considerably smaller than with logistic curve fitting (see the flat concentration-response curve in Fig. 3A which did not appropriately reflect the data points at 10000 nM). For a given agonist, the general response function estimated a much higher pK_a value from the concentration-inhibition curve in solvent-treated slices than from the curve in phenoxybenzamine-treated slices (up to 0.93 units). The latter ones were in the range of the pK_a values estimated either with the null method of Furchgott[4] or with the operational model of Black *et al.*[5].

Table 6: Estimation of the dissociation constant K_a of α_2-adrenoceptor agonists at presynaptic α_2-autoreceptors in mouse and rabbit brain cortex slices with the operational model of Black *et al.*[5]

Agonist	Concentration of PBA	pK_a	τ_{con}	τ_{PBA}	Sensitivity parameter "n"	Receptor fraction occupied at the agonist's EC_{50} in solvent	Receptor fraction occupied at the agonist's EC_{50} in PBA-treated slices
Mouse							
α-Methylnoradrenaline	4.0 μM	6.35±0.11	42.2±9.7	1.7±0.2	1.22±0.09	0.02	0.33
Moxonidine	3.0 μM	5.93±0.13	51.1±12.9	2.4±0.3	1.36±0.09	0.02	0.29
Rilmenidine	0.5 μM	6.19±0.16	4.9±1.1	1.4±1.1	1.14±0.13	0.16	0.34
UK 14,304	2.0 μM	7.18±0.12	28.4±7.2	2.0±0.2	1.18±0.10	0.03	0.29
Rabbit							
α-Methylnoradrenaline	4.0 μM	7.21±0.14	9.1±2.3	1.2±0.1	1.03±0.10	0.11	0.24
Moxonidine	3.0 μM	6.85±0.11	17.4±3.9	1.5±0.1	1.35±0.11	0.06	0.18
Rilmenidine	0.5 μM	6.47±0.14	5.8±1.2	1.5±0.1	1.27±0.12	0.15	0.36
UK 14,304	3.0 μM	8.12±0.13	13.4±3.4	1.6±0.2	1.03±0.08	0.07	0.29

For each agonist, the concentration-response curve in solvent-treated slices and that in phenoxybenzamine (PBA)-treated slices were fitted simultaneously to function (8) of Black *et al.*[5]. The maximum response of the system (maximum possible percentage inhibition of a full agonist) was fixed to 100 % inhibition. The model provides estimates of K_a and of the efficacy of the agonist in solvent- (τ_{con}) and phenoxybenzamine-treated slices (τ_{PBA}). K_a values are expressed as negative decadic logarithm, pK_a. Function (2) was applied to calculate the receptor fraction occupied by the agonist at its EC_{50} {$= K / ([2 + \tau_{con}]^{1/n} - 1)$}. According to the model, the sensitivity parameter "n" determines the sensitivity of the occupancy-effect relationship

In N-ethylmaleimide-treated mouse brain slices the pK_a of rilmenidine and of UK 14,304 agreed well with their pK_a in solvent-treated slices.

Note that the residual sums of squares of the fitting procedures were always lower with the general response function (3) than with the logistic function (1) in the case of Table 3: α-methylnoradrenaline, moxonidine, UK 14,304 and Table 4: moxonidine (not shown), proving the goodness-of-fit.

DISCUSSION

The present study was devised to determine functionally the agonist-receptor dissociation constant K_a of four chemically different agonists at presynaptic release-inhibiting α_2-autoreceptors in mouse and rabbit brain cortex. In order to obtain valid agonist pK_a values at α_2-autoreceptors, it is necessary to evoke NA release free from autoinhibition[13]. In the present study this was achieved by stimulating the brain slices with 4 pulses at 100 Hz.

Beyond the important exclusion of autoinhibition, in the present study all concentration-inhibition curves were determined under equilibrium conditions for the agonists: (I) The high affinity uptake of NA and α-methylnoradrenaline was blocked by desipramine. (ii) When no agonist was added, the S_n/S_1 ratios were close to unity and did not change over the course of the experiment, irrespective of whether the slices were pretreated with solvent or phenoxybenzamine or with N-ethylmaleimide. (iii) The inhibitory effect of the agonists did not change with prolonged exposure time[8,14]. (iv) Pretreatment of the slices with phenoxybenzamine caused a stable inactivation of some fraction of the autoreceptors over the course of the experiment, since the inhibitory effect of the agonists after prolonged exposure time remained constant. (v) The latter two observations also indicated that there was no desensitization to the agonists.

In order to determine the dissociation constant of a receptor agonist from functional data, it is necessary to deduce from the concentration-response curve of the agonist to its concentration-binding curve. This, however, may be difficult since the relationship between receptor occupation by the agonist and the transformation into effect is usually not known. In the most simple case a proportionality exists between receptor occupation and effect. Then, the concentration-response curve of the agonist matches its concentration-binding curve and EC_{50} corresponds to K_a and the concentration-binding curve of the agonist reflects a logistic function with the slope parameter c (equivalent to the Hill coefficient being unity)[1]. Hence, in case the concentration-response curve of a full agonist fits a logistic function with c equating unity this suggests direct proportionality between binding and evoked effect[1-3]. For a partial agonist with reduced maximum effect, the evoked response has to be proportional to the receptor occupation. Only the probability is less than unity that occupation of a receptor by such a partial agonist initiates a (fractional) response (conditional probability of the action of a partial agonist)[2].

In solvent-treated slices of the mouse brain cortex, rilmenidine acted as a partial agonist. It inhibited the release of [^{3}H]-NA by maximally 83%, whereas the other three agonists reduced it by more than 95%. Analysis of the concentration-response relationship of rilmenidine with the logistic function yielded a slope parameter c not significantly different from unity. In contrast, analysis of the concentration-inhibition data of α-methylnoradrenaline, moxonidine and UK 14,304 with the logistic function yielded steep concentration-response

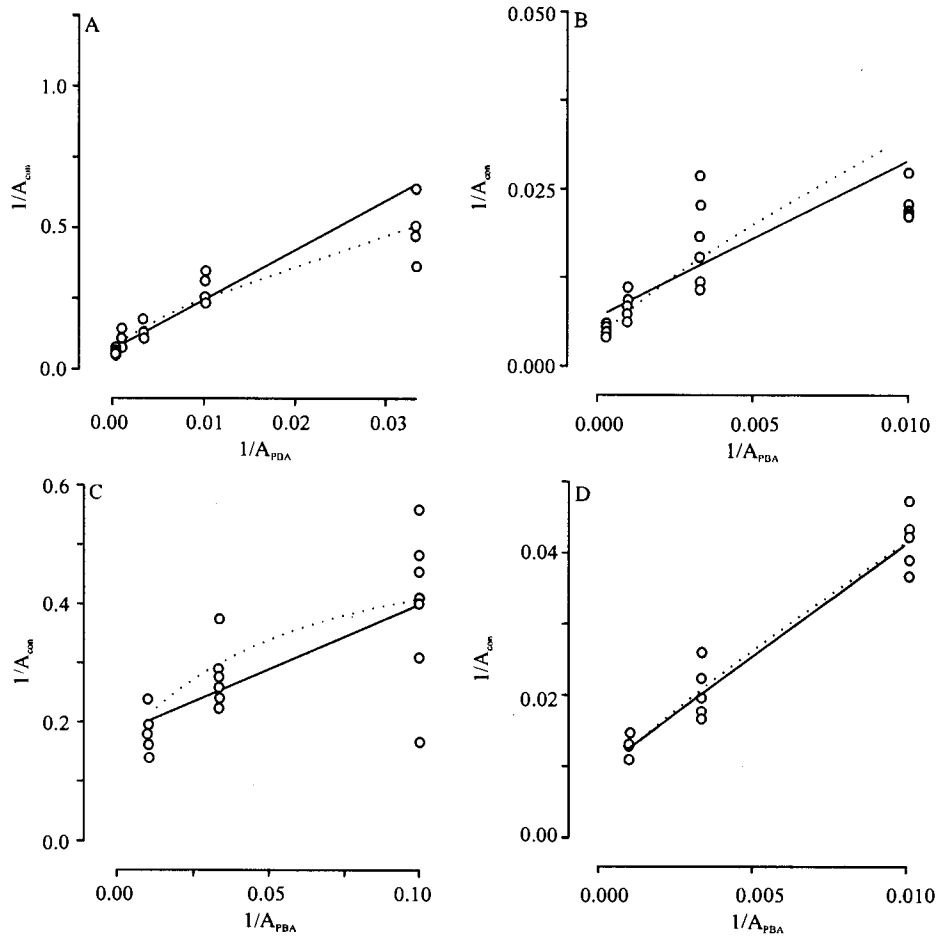

Fig. 5: Estimation of K_a of the presynaptic α_2-autoreceptor/agonist complex in mouse and rabbit brain cortex slices by elimination of a fraction of the receptor population with phenoxybenzamine: double-reciprocal plots for equieffective agonist concentrations in phenoxybenzamine-treated slices and in solvent-treated slices
Circles correspond to the inhibition data of α-methylnoradrenaline (A, C) and rilmenidine (B, D) obtained in phenoxybenzamine-treated slices (A_{PBA}; range: 15 to 85% of maximum inhibition obtained in phenoxybenzamine-treated slices; see Fig. 2A, C and 3A, C) with the equieffective concentrations (A_{Con}) calculated by means of the logistic functions that fitted best the concentration-inhibition data of the agonists in solvent-treated slices (see Table 3 and 4 for the parameters of the functions). Solid lines represent the regression lines of these values according to equation 6 of Furchgott[4]. Dots represent equieffective concentrations calculated at 1%-inhibition steps by means of the logistic functions that fitted best the concentration-inhibition data obtained in solvent-treated and in phenoxybenzamine-treated slices, respectively

curves with c's significantly greater than unity. As c>1 is not compatible with binding being proportional to evoked effect, the concentration-response curves no longer reflect the concentration-binding curves[1]. For these agonists, an amplification occurs somewhere in the chain of signal transduction from receptor occupation to inhibition of NA release. Such an amplification results in spare receptors that do not have to be activated in order to evoke maximum inhibition.

Irreversible elimination of receptors or receptor-mediated effects: The idea that a slope parameter c greater than unity of the logistic function, describing the concentration-response curve of an agonist, may indicate the existence of a receptor reserve can be proved by means of an irreversible receptor antagonist. Note that the asymmetry due to spare receptors is insufficiently reflected by the inherently symmetric logistic function[1]. The knockout of a sufficiently great fraction of receptors

converts a full agonist with a receptor reserve into a partial agonist: its concentration-response curve then fits a logistic function with a reduced maximum effect and a c of unity. A partial agonist, however, intensifies its partial agonist property: It displays a further reduced maximum effect. The logistic function should indicate an unchanged c equating unity in this case. The results obtained clearly support these considerations. In phenoxybenzamine-treated slices of the mouse, the maximum inhibition was diminished for all agonists. The slope parameter c of the concentration-inhibition curves of the full agonists moxonidine and UK 14,304 was no longer different from unity. In case of the partial agonist rilmenidine, c was not significantly changed by phenoxybenzamine and still close to unity. The findings in slices from rabbit brain cortex were similar to those in the mouse. In solvent-treated slices, all four agonists acted as quasi-full agonists with E_{max} = 92% inhibition. For moxonidine, logistic curve fitting yielded a slope parameter c greater than unity suggesting the existence of spare receptors for this agonist. In contrast, for α-methylnoradrenaline, rilmenidine and UK 14,304, c was not different from unity, compatible with the view that receptor occupation is proportional to effect. After pretreatment with phenoxybenzamine, E_{max} of all four agonists was diminished. Moxonidine now showed a concentration-inhibition curve with the slope parameter c not deviating from unity; the full agonist with a receptor reserve was changed into a partial agonist for which the evoked effect was proportional to receptor occupation. For rilmenidine, c (≈ 1) was not altered when compared to solvent-treated slices.

In phenoxybenzamine-treated slices the concentration-inhibition curves of α-methylnoradrenaline (mouse and rabbit) and of UK 14,304 (rabbit only) were shallow with c significantly lower than unity. However, a c<1 does not seem possible when a bimolecular agonist-receptor reaction with a directly proportional receptor-effect relation is assumed. The molecular mechanism of the phenoxybenzamine action offers an explanation for the unexpected finding. Phenoxybenzamine probably alkylated the α_2-autoreceptor proteins not always at exactly the same locus (amino acid) and, therefore, the receptor molecules may have been affected in different ways by irreversible alkylation: Some of the receptors were completely blocked, but others may have shown a reduced affinity for agonists only[6] while still mediating inhibition of release. The fully blocked fraction of the α_2-autoreceptors then was responsible for the diminished maximum effect of the agonists, whereas autoreceptors with a decreased agonist affinity shifted the concentration-response curves to the right. Moreover, the α_2-autoreceptors with reduced agonist affinity made up a second autoreceptor population besides the native autoreceptors, causing shallow concentration-inhibition curves of the agonists with a slope parameter c (or Hill coefficient) below unity. The effect of phenoxybenzamine to separate α_2-autoreceptors into three groups - native, alkylated with altered affinity, irreversibly blocked- may vary to some extent for different agonists. The phenylethanolamine agonist α-methylnoradrenaline seemed especially sensitive for this effect of phenoxybenzamine. In the rabbit, even a low concentration of the alkylating agent that did not reduce the maximum response diminished the slope of the concentration-inhibition curve of α-methylnoradrenaline. The extremely shallow concentration-inhibition curve of α-methylnoradrenaline after a high concentration of phenoxybenzamine may point to a biphasic curve in support of two populations of receptors (Fig. 3A). However, in general the data obtained in phenoxybenzamine-treated slices did not demonstrate clear biphasic concentration-inhibition relationships. Browne *et al.*[16] suggested that phenoxybenzamine may also inactivate guanine nucleotid-binding proteins sensitive to N-ethylmaleimide in concentrations at which they bind to α_2-adrenoceptors.

Double reciprocal method of Furchgott[4]: Being aware of possible adverse effects of phenoxybenzamine, the K_a of the agonist/α_2-autoreceptor complex was estimated from the concentration-inhibition curves before and after phenoxybenzamine according to Furchgott[4]. The logistic function that described the concentration-inhibition curve of a given agonist in solvent-treated slices was used to compute agonist concentrations A_{con} equieffective to the concentrations A_{PBA} administered in phenoxybenzamine-treated slices. The pairs of reciprocals of the equieffective concentrations fitted a straight line. In a second step, the logistic function that described best the concentration-inhibition curve in phenoxybenzamine-treated slices was used to create a data set at 1%-inhibition steps and equieffective concentrations were computed as above. In the double reciprocal plot, these pairs of equieffective concentrations did not yield a straight line at all (Fig. 5). For this reason, the regression line which is based on the reciprocals of equieffective concentrations actually used in the experiments was not functional for a correct estimation of the agonist's K_a. By generating systematically double reciprocal plots from pairs of theoretical concentration-response curves with the logistic function (Fig. 5) it was demonstrated that the linear relationship between equieffective concentrations holds true only when both the curve without

phenoxybenzamine and that with phenoxybenzamine had a slope parameter c = 1. Whenever one or both agonist concentration-response curves had a c different from unity, the relationship between $1/A_{con}$ and $1/A_{PBA}$ was not linear and the more the two c´s were different from each other the more was the line curvilinear. This was also true when the analysis was based on A_{PBA} values causing between 40 and 90% of the maximal effect in phenoxybenzamine-treated slices[17]. Similar results have been reported previously when K_a of isoprenaline was determined at atrial β-adrenoceptors)[18]. The systematic deviation from linearity renders the general use of Furchgott's method questionable. It seems suitable only for agonists without spare receptors and for partial agonists, i.e. when the pre- and the postinactivation concentration-response curves follow exactly so-called rectangular hyperbolas (slope parameter c of the logistic function = 1)[5]. The observed non-linearity for the partial agonist rilmenidine in mouse brain slices (Fig. 5B) is due to the slightly different values of c in solvent- and in phenoxybenzamine-treated slices (Table 3). The relationship between equieffective concentrations has also been found non-linear when, at presynaptic muscarine receptors, the concentration-response curve of the partial agonist pilocarpine was compared with that of the full agonist methacholine[19] and the same result was obtained when, at presynaptic α_2-autoreceptors, the full agonist oxymetazoline was compared with the partial agonist xylometazoline[20]. In both cases equieffective concentrations were determined graphically.

The adverse effects of phenoxybenzamine, i.e. its property to decrease receptor affinities[6], as well as the inconsistencies in Furchgott's method[4] are reflected by the resulting functional parameters of the agonist-α_2-autoreceptor system: Interpreting the estimated K_a of the Furchgott method[4] according to the Law of Mass Action in mouse brain cortex, the partial agonist rilmenidine occupied only 5% of the presynaptic autoreceptors in solvent-treated slices when inhibiting NA release half-maximally, a fraction almost identical to that occupied by the full agonists α-methylnoradrenaline, moxonidine and UK 14,304 at their EC_{50}s (Table 5). In phenoxybenzamine-treated mouse slices only 12% of the receptors were thought to be occupied by rilmenidine at it's EC_{50}. This suggested a large receptor reserve for the agonists, hardly diminished by phenoxybenzamine. But at least for the partial agonist rilmenidine and in phenoxybenzamine-treated slices, one would not expect any spare receptors at all. In contrast and most surprisingly, in phenoxybenzamine-treated slices of rabbit brain α-methylnoradrenaline at its EC_{50} occupied 96% of the receptor fraction still active. A second unreasonable result was obtained when the receptor fraction still active in phenoxybenzamine-treated rabbit slices was estimated higher from α-methylnoradrenaline experiments than from rileminidine experiments although the concentration of phenoxybenzamine was 4 μM in the former and only 0.5 μM in the latter.

Operational model of Black *et al.*[5]: An alternative method for the determination of agonist affinity (and efficacy) from functional data is the application of a so-called operational model. Such a model may have the advantage that it theoretically allows the estimation of the (operational) K_a by evaluating just the concentration-inhibition curve of the agonist since no transformation of the data is necessary. However, with the operational model of Black *et al.*[5] it was not possible to estimate the dissociation constant and the (operational) efficacy τ from any of the concentration-inhibition data sets obtained in solvent-treated slices (the fitting procedures did not converge). Only when the model was used for the comparative analysis of concentration-inhibition curves in solvent- and in phenoxybenzamine-treated slices (with the maximum possible system response constrained to 100% inhibition) the fitting procedure yielded estimates of K_a and τ. In the comparative mode, the operational model can be regarded equivalent to Furchgott's method[4] with the concentration-inhibition curve described by the logistic function[21,22]. The similarity of the results - K_a values, the ratio $(\tau_{PBA}/(\tau_{con})$ corresponding to the receptor fraction still active in phenoxybenzamine-treated slices and the receptor fraction occupied at the agonist's EC_{50} in solvent-treated slices- is due to the equality of the two methods[23]. Salls *et al.*[23] obtained similar results with null methods and the operational model as well.

The aim of evaluating the relationship between the concentration of an agonist and its effect with a mathematical model certainly is to describe experimental data as close as possible. However, a mathematical model also should try to explain the physico-chemical mechanism of the interaction between agonist and receptor and of the biological transduction into the effect. Preferably, such a model is based on a physical law and its parameters should have a simple physico-chemical meaning. At first glance, this seems to be true in case of the operational model of Black *et al.*[5] describing hyperbolic concentration-response curves. However, the model holds a grave gap. It does not mechanistically delineate its 'transducer function'. Although the response is assumed to be a function of the number of receptors occupied by the agonist it does not allow the condition of a full agonist acting in a system without spare receptors,

i.e. when the agonist-induced effect is proportional to agonist-receptor binding[11]. The extended version of the model for non-hyperbolic concentration-response curves contains a parameter that determines the sensitivity of the occupancy-effect relationship. This parameter, the power to which the concentration of agonist-receptor complex is raised, is unlikely to have any simple physical meaning[22]. For a more detailed critique of the operational model[3].

General response function of Feuerstein *et al.*[2]: This model hypothetically divides the tissue into functional units and considers the degree of receptor occupation and, hence, the fractional effect at each unit as a statistical phenomenon depending on the concentration of the agonist. The overall response of the tissue (the percentage inhibition of [^{3}H]-NA release in a brain slice) is the sum of the responses of all individual functional units. Each single functional unit is assumed to possess a total of N α_2-autoreceptors and K of these receptors have to be occupied by an agonist in order to obtain its maximum response. The general response function estimates of K and N, however, may differ for each agonist (discussion of the K/N ratio below). For a partial agonist with reduced maximal effect, K equates N whereas, for a full agonist, K may be equal to N or, if an amplification occurs somewhere in the chain of signal transduction from receptor occupation to effect, K is smaller than N[1,2]. The general response function excellently fitted the concentration-inhibition data obtained in the present study. K was close to, or equated N in solvent-treated slices in case of the partial agonist rilmenidine in the mouse brain and in case of the agonists α-methylnoradrenaline, rilmenidine and UK 14,304 in the rabbit brain indicating that no spare receptors exist for these agonists. In contrast, for moxinidine in both species and for α-methylnoradrenaline and UK 14,304 in the mouse brain, K was considerably smaller than N indicating the existence of spare receptors. The spare receptors characteristically affected the shape of the concentration-inhibition curves. They were no longer symmetrical with respect to their inflection point (Fig. 2 and 3). In phenoxybenzamine-treated slices, when a receptor reserve was destroyed and full agonists were converted into quasi-partial agonists, K equated N, as expected. It should be pointed out that the general response function detected a receptor reserve only for those agonists for which non-linear regression analysis of the concentration-inhibition data with the logistic function had indicated a slope parameter c greater than unity. If c was not different from unity, then the EC_{50} estimated by the logistic function was almost identical to K_a estimated by the general response function and K equated N. Note that a c not significantly higher than unity precludes the interpretation of K<N as receptor reserve[1]. The significance of the slope parameter c of an agonist concentration-response curve as touchstone for the occurence of a receptor reserve has been discussed previously[2] and is confirmed in this study.

From the results obtained with the general response function it was computed that in solvent-treated slices of mouse brain the receptor fraction occupied by the partial agonist rilmenidine at its EC_{50} was close to 50%. Similarly, in phenoxybenzamine-treated slices, when all spare receptors had been knocked out, the agonists occupied about 50% of the α_2-autoreceptors at their EC_{50}. Only in case of α-methylnoradrenaline in mouse and rabbit and UK 14,304 in the rabbit the fractional occupation was estimated greater than 50% pointing to the adverse effects of phenoxybenzamine. The fraction of alkylated α_2-autoreceptors with reduced affinity for the agonists also explains that, for a given agonist, the general response function estimated a markedly higher pK_a from solvent-treated than from phenoxybenzamine-treated slices.

The α_2-adrenoceptor is a guanine nucleotide-binding protein-coupled receptor. The ternary complex model predicts that the depletion of receptors, i.e. reduction of the receptor density (or concentration), or depletion of the guanine nucleotide-binding protein causes a shift to the right of an agonist concentration-response curve[24,25]. In agreement with this model, in mouse brain slices both pretreatment with phenoxybenzamine (which reduced the α_2-autoreceptors and/or their affinity) and pretreatment with N-ethylmaleimide (which reduced the guanine nucleotide-binding protein) shifted the concentration-inhibition curves of rilmenidine and UK 14,304 to the right. Although N-ethylmaleimide principally can alkylate each protein that contains cystein residues it probably does not affect the agonist binding site of the α_2-autoreceptor[7], leaving the K_a unchanged. When the concentration-inhibition data of rileminidine and UK 14,304 in N-ethylmaleimide-treated slices were analysed with the general response function, the K_a was almost identical to the K_a estimated from solvent-treated slices. Moreover, for UK 14,304 the spare receptors detected in solvent-treated slices had disappeared: K almost equated N and the slope parameter c was no longer different from unity when the concentration-inhibition data were analysed with the logistic function. For the partial agonist rilmenidine, both pretreatments caused a comparable reduction of E_{max}, but the shift to the right of the concentration-response curve was more pronounced in phenoxybenzamine-treated slices than in N-ethylmaleimide-treated slices indicating again that

phenoxybenzamine did not only cause an irreversible blockade of the α_2-autoreceptors, but also decreased their affinity.

For a full agonist with a receptor reserve, the general response function yields an estimate of the total number N of α_2-autoreceptors at a single active site at which release occurs. Here, however, a word of caution seems necessary. N is evaluated from an asymmetry of the concentration-response curve of the agonist which is induced by spare receptors. The degree of asymmetry depends mainly on the absolute values of N and K[2]. In the present study, the agonist concentrations were increased in steps of half a logarithmic unit (or more) and, therefore, the asymmetry of the agonist concentration-inhibition curves was not optimally determined. Consequently the assessment of absolute values of N and K may have been inaccurate. This adds to the limitations of the method to estimate absolute numbers[1]. N was estimated to be (about) 5 in mouse and (about) 19 in rabbit brain with all four agonists (Table 3 and 4). On the other hand, the ratio K/N depends mainly on the asymmetry of the concentration-response curve that was indirectly determined by the present method. So the ratio K/N may be considered more reliable than the absolute values of N and K. This does not affect at all the general interpretation of the results on the agonist-α_2-autoreceptor interaction in terms of a receptor reserve.

The use of the ratio K/N offers a preferable interpretation. The guanine nucleotid-binding protein links the α_2-autoreceptor to a further unknown mechanism which induces inhibition of release. In such a complex system it is not known at which link the proportionality between receptor occupation and evoked effect disappears leading to the existence of 'spare receptors'. It seems therefore to be more appropriate to talk about a system reserve instead of a receptor reserve. The ratio K/N then indicates the degree of the system reserve. A guanine nucleotide-binding protein depleting agent like N-ethylmaleimide destroys the system reserve as does phenoxybenzamine when blocking α_2-autoreceptors irreversibly. A receptor reserve in the true sense of the word can be assumed only in a system that consists of a receptor and a single, directly coupled transducer[26].

In conclusion, the general response function appropriately reflected the existence or the removal of a receptor (or system) reserve by phenoxybenzamine or N-ethylmaleimide. The apparent absence of such a reserve due to the use of a partial (instead of a full) agonist was also correctly evaluated. In addition, the general response function quantified the receptor (or system) reserve as ratio K/N. Both the method of Furchgott[4] and that of Black *et al.*[5] were inapplicable to evaluate a receptor reserve of to detect its absence.

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