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INTERACTIONS OF ENDOGENOUS AND EXOGENOUS NOREPINEPHRINE WITH ALPHA$_2$ ADRENOCEPTOR BINDING SITES IN RAT CEREBRAL CORTEX

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Abstract—The specific binding of (^{3}H)yohimbine to alpha$_2$ adrenoceptors in rat cerebral cortex varied significantly with methods of membrane preparation. Membranes prepared in a sucrose containing buffer showed markedly lower B_{max} values than those prepared in hypotonic buffer without any change in affinity (K_d) for (^{3}H)yohimbine. Higher concentrations of residual endogenous norepinephrine were found in sucrose prepared membranes. In the presence of exogenous norepinephrine (10^{-8}, 10^{-7} M), membranes prepared in hypotonic buffer showed apparent reduced receptor densities similar to those observed in sucrose prepared cerebral membranes. The presence of exogenous norepinephrine did not produce any apparent change in the overall K_d for (^{3}H)yohimbine. The depression of (^{3}H)yohimbine binding capacities could, in all cases, be reversed by performing incubations in the presence of 200 mM NaCl and 10 μM Gpp(NH)p which synergistically dramatically reduce the affinity of norepinephrine for cerebral alpha$_2$ adrenoceptors. It is concluded, therefore, that the lower (^{3}H)yohimbine binding capacities in sucrose prepared membranes appears to be due to the occupancy of receptor sites by residual norepinephrine in a pseudo non competitive manner. The reduced affinity of retained endogenous norepinephrine for the alpha$_2$ adrenoceptor, in the presence of Na^+ and guanine nucleotides, would seem to be the cause of the apparant increas in (^{3}H)yohimbine binding sites produced by these modulators in sucrose prepared membranes.

The radioactively labelled selective antagonists (^{3}H) yohimbine and (^{3}H)rauwolscine have been used extensively to label alpha$_2$ adrenoceptors in a variety of tissues [1–6]. Using these radioligands, agonist interactions, qualitatively similar to that seen with other adenylate cyclase linked receptors, have been demonstrated. Thus, the alpha$_2$ adrenoceptor appears to exist in two affinity states modulated by the presence of guanine nucleotides and overall agonist affinity can be markedly reduced by Na^+ ions. In contrast antagonist binding to the alpha$_2$ adrenoceptor is little affected by these modulators [7, 8]. However, in a recent study using rat cerebral cortical membranes, Woodcock and Murley [9] reported a low maximal binding capacity of (^{3}H)-yohimbine, which could be markedly increased when incubations were performed in the presence of Na^+ and guanine nucleotides. In view of the quite different binding capacities reported by these workers compared to our own studies [2] in the same tissue, it remained possible that differences in membrane preparations could be particularly significant. Woodcock and Murley [9] used a hypertonic sucrose containing buffer for membrane preparation and it seemed possible that in this medium, intact synaptosomes containing endogenous norepinephrine could interfere with the subsequent alpha$_2$ adrenoceptor binding assay, and that these effects could be reversed by Na^+ ions and guanine nucleotides. In the present study, we examine these possibilities in detail and provide evidence for a pseudo non competitive interaction of alpha$_2$ adrenoceptors with norepinephrine. These results could have wide implications in the interpretation of apparent altered receptor site density in many systems.

MATERIALS AND METHODS

Preparation of rat cerebral cortical membranes. Adult male Sprague–Dawley rats (200–300 g) were decapitated and cerebral cortical membranes were prepared by one of the following methods: (A) Cerebral cortex was homogenised in 20 vol of sucrose buffer (50 mM Tris–HCl, 0.25 M sucrose, 10 mM $MgSO_4$, pH 7.7) using a motordriven glass–Teflon homogeniser as described by Woodcock and Murley [9]. Homogenates were filtered through a double-layer of cheesecloth and centrifuged at 27,000 *g* for 15 min. Pellets were subsequently washed three times with the same sucrose buffer, and finally resuspended in assay buffer (50 mM Tris–HCl, 10 mM $MgSO_4$, 0.5 mM EDTA, 1 mM mercaptoethanol pH 7.7) at a protein concentration of 4–6 mg/ml. (B) Cerebral cortex was homogenised and washed in hypotonic buffer (5 mM Tris, 5 mM EDTA pH 7.7) as previously described [2]. Final pellets were resuspended in the same assay buffer as in (A). (C) Cerebral cortex was initially homogenised and washed in sucrose buffer as in (A), but after the third washing, pellets were homogenised once more in the buffer (5 mM Tris, 5 mM EDTA) used in (B) before being resuspended in the assay buffer as in (A).

Receptor binding assays. All binding assays were performed in assay buffer (50 mM Tris–HCl, 10 mM

$MgSO_4$, 0.5 mM EDTA, 1 mM mercaptoethanol pH 7.7) in a final volume of 250 μl. Incubations were performed to equilibrium at room temperature (22–25°) for 30 min upon the addition of membranes. Subsequently, membranes were collected on Whatman GF/B filters under vacuum and rapidly washed with 3 × 5 ml ice-cold buffer. Filters were counted for radioactivity in FisoFlour 1 scintillation fluid at an efficiency of about 40%. Specific binding was assessed as that binding of (^{3}H)yohimbine which could be displaced by 5 μM phentolamine and represented about 60–70% of total binding at a concentration of 4–5 nM (^{3}H)yohimbine. Previous studies have indicated that this concentration of phentolamine clearly defined specific and non-specific components of (^{3}H)yohimbine binding in cerebral cortex [2]. Maximal binding capacities (B_{max}) and affinity (K_d) of (^{3}H)yohimbine were calculated from saturation experiments by Scatchard analysis. Hill slopes (nH) of displacement curves were obtained by graphical analysis. Protein was determined by the method of Lowry *et al.* [10].

Norepinephrine assays. Membranes prepared as above were homogenised with 0.1 N perchloric acid and following centrifugation, catecholamines were extracted with alumina and separated on a reverse phase-ion pair HPLC column (Ultrasphere 5 μ ODS) and assayed by electrochemical detection [11]. The limit of norepinephrine detection was 0.2 pmoles/mg membrane protein.

(^{3}H)yohimbine (New England Nuclear) used in this study had a specific activity of 89.7 Ci/mmole. (−)norepinephrine was obtained from Sigma Chemical Company; phentolamine was donated by Ciba–Geigy; Gpp(NH)p* was obtained from Boehringer.

RESULTS

Effect of methods of cortical membrane preparation on binding capacities of (^{3}H)yohimbine. Cerebral cortical tissue was divided, membranes prepared simultaneously by methods (A), (B) and (C) and assayed for (^{3}H)yohimbine binding. Significant differences in the binding capacities in these preparations were observed, even under identical assay conditions (Table 1, Fig. 1a and b). B_{max} values for (^{3}H)yohimbine were generally low in membranes prepared by method (A), which was similar to that adopted by Woodcock and Murley [9]. In contrast, the B_{max} of (^{3}H)yohimbine was significantly higher in membranes prepared by method (B), in which a hypotonic buffer instead of sucrose buffer was used in the homogenisation and washing process. When membranes prepared by method (C), (initial preparation by method A, followed by an additional homogenisation and washing in hypotonic buffer) were used for assays, (^{3}H)yohimbine B_{max} was also found to be higher than membranes prepared by method (A). The apparent K_d of (^{3}H)yohimbine binding, however, remained unchanged regardless of the method of membrane preparation.

Effect of NaCl *and guanine nucleotide on* (^{3}H) *yohimbine binding capacities in cortical membranes.* In agreement with the results reported by Woodcock and Murley [9], the apparent densities of alpha$_2$ adrenoceptors determined by (^{3}H)yohimbine binding in cortical membranes prepared by method (A) (sucrose containing buffer) could be increased more than 2-fold if assays were performed in the presence of NaCl (200 mM) and the hydrolysis resistant GTP analogue Gpp(NH)p (10 μM) (Table 1). There was a small increase in (^{3}H)yohimbine binding sites in the presence of NaCl and Gpp(NH)p in membranes prepared by method (C), though this did not reach statistical significance.

However, there were no significant differences in the apparent B_{max} values of (^{3}H)yohimbine binding in membranes prepared by method (B), (hypotonic buffer) in the absence or presence of NaCl and Gpp(NH)p.

In all cortical preparations the apparent K_d of (^{3}H) yohimbine was not altered when incubations were carried out in the presence of NaCl and Gpp(NH)p. Furthermore, it should be noted that in the presence of these modulators the B_{max} was not significantly different between the three preparations.

Norepinephrine contamination of cortical membranes. Samples of membranes prepared by methods (A), (B) and (C) were analysed using HPLC/electrochemical detection for possible contamination with endogenous norepinephrine with the following results: membranes (A) 1.98 ± 0.24 pmoles/mg protein (mean ± S.E.M., N = 3); membranes (B) < 0.2 pmoles/mg protein; membranes (C) < 0.2 pmoles/mg protein.

In the final binding assay (assuming that all the catecholamine is free) the overall concentration of norepinephrine in assays containing membranes (A) would be 5 ± 0.4 nM (N = 3) whereas in membranes (B) or (C) this concentration is < 0.5 nM.

Effect of exogenous norepinephrine on (^{3}H)yohimbine binding capacities. In view of the possibility that the effects of Na^+ and Gpp(NH)p on the apparent binding capacity of (^{3}H)yohimbine to cerebral membranes could relate to retained endogenous norepinephrine, experiments were performed in which exogenous norepinephrine was added to membranes prepared in hypotonic buffer. The results are shown in Table 2 and Figs. 2(a) and (b).

When saturation experiments were performed using membranes prepared in the hypotonic buffer, (very low residual endogenous norepinephrine), the addition of 10 nM norepinephrine was found to cause an apparent large decrease in (^{3}H)yohimbine binding capacity. A further loss in sites was observed when a higher concentration (100 nM) of norepinephrine was added. However, these changes in B_{max} values were not accompanied by any change in the apparent affinity of (^{3}H)yohimbine. Furthermore, in the presence of 200 mM NaCl and 10 μM Gpp(NH)p, the "lost" binding sites could be recovered. Thus, in the presence of NaCl and Gpp(NH)p a similar binding capacity was observed regardless of the presence or absence of exogenous norepinephrine.

Effect of NaCl *and guanine nucleotide on norepinephrine displacement of (^{3}H)yohimbine binding to cortical membranes.* The data in Fig. 3 demonstrates the effect of NaCl and Gpp(NH)p alone or in combination on the ability of norepinephrine to displace

* The abbreviation used is Gpp(NH)p, 5′-guanylyl imidodiphosphate.

Table 1. Effect of methods of cortical membrane preparation on binding capacities of (^{3}H)yohimbine

Membrane preparation* method	Assay buffer		Assay buffer plus NaCl (200 mM), Gpp(NH)p (10 μM)	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
A	49 ± 5.94†	11.6 ± 1.2	125.7 ± 14.6‡	11.2 ± 1.0
B	124.3 ± 9.84	12.18 ± 0.99	138.3 ± 16.2	11.9 ± 0.9
C	105.4 ± 15.4	11.80 ± 0.89	141.08 ± 17.06	11.8 ± 1.1

* Membranes were prepared by methods (A), (B) and (C) and saturation binding assays were carried out in parallel experiments as described in the text. Values shown were obtained from Scatchard plots and are means ± S.E.M. of at least six separate experiments. Comparisons were made by Student's *t*-test.

† Indicates significantly different from membrane preparations (B) and (C) in assay buffer ($P < 0.05$).

‡ Indicates significantly different from membrane preparation (A) in assay buffer ($P < 0.05$).

All other comparisons were not significantly different.

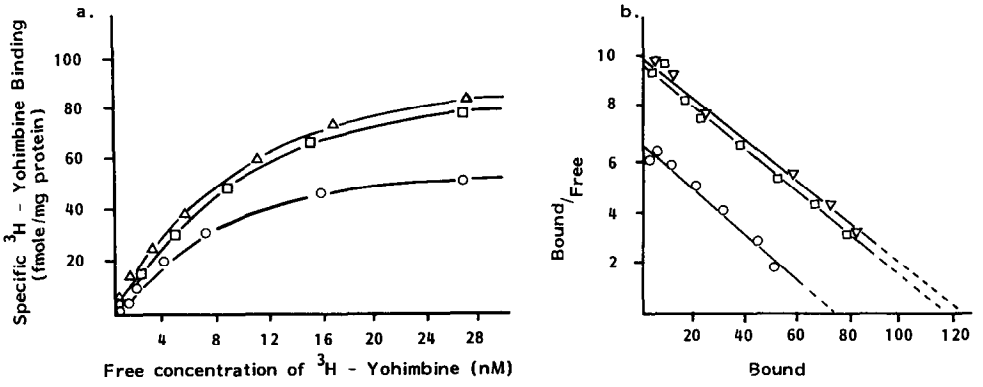

Fig. 1. Binding of (^{3}H)yohimbine to rat cerebral cortex membranes prepared by different methods. Membranes were prepared as described in the text using hypertonic sucrose buffer (method A ○—○); hypotonic Tris buffer (method B △—△); hypertonic sucrose buffer followed by a final wash in hypotonic Tris buffer (method C □—□). (a) Saturation binding assays were carried out as described in Materials and Methods with approximate concentrations of (^{3}H)yohimbine from 0 to 30 nM for each of the membrane preparations. (b) Scatchard analysis of saturation curves to assess total binding capacities (B_{max}) and dissociation constants (K_d) for each preparation. Data illustrated is from single representative parallel experiments performed in duplicate. B_{max} = 73, 124, 117 fmoles/mg protein for membrane preparation (A), (B) and (C) respectively. K_d = 10.23, 11.1, 10.95 nM for membrane preparation (A), (B) and (C) respectively.

Table 2. Effect of exogenous norepinephrine on (^{3}H)yohimbine binding capacities in cortical membranes prepared in hypotonic buffer

	Assay buffer		Assay buffer plus NaCl (200 mM), Gpp(NH)p (10 μM)	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_s (nM)
Control*	135.8 ± 29.9	11.5 ± 0.6	193.4 ± 25.3	11.6 ± 1.2
+ 10^{-8} M norepinephrine	70.3 ± 4.8†	11.7 ± 1.6	178.1 ± 36.5‡	12.8 ± 1.9
+ 10^{-7} M norepinephrine	49.2 ± 5.4†	10.9 ± 2.4	162.6 ± 32.3‡	13.7 ± 1.8

* Membranes were prepared in hypotonic buffer (method B) and assayed as described in the text. Saturation binding assays were carried out in parallel experiments without addition of agonist (control) or in the presence of norepinephrine in final concentrations of 10^{-8} or 10^{-7} M as listed, and with or without addition of NaCl (200 mM) and Gpp(NH)p (10 μM)

Values shown were obtained by Scatchard analysis and are means ± S.E.M. of at least three separate experiments. Comparisons were made using Student's *t*-test.

† Indicates significantly different from "control" in assay buffer ($P < 0.05$).

‡ Indicates significantly different from results in the absence of NaCl and Gpp(NH)p ($P < 0.05$).

All other comparisons were not significantly different.

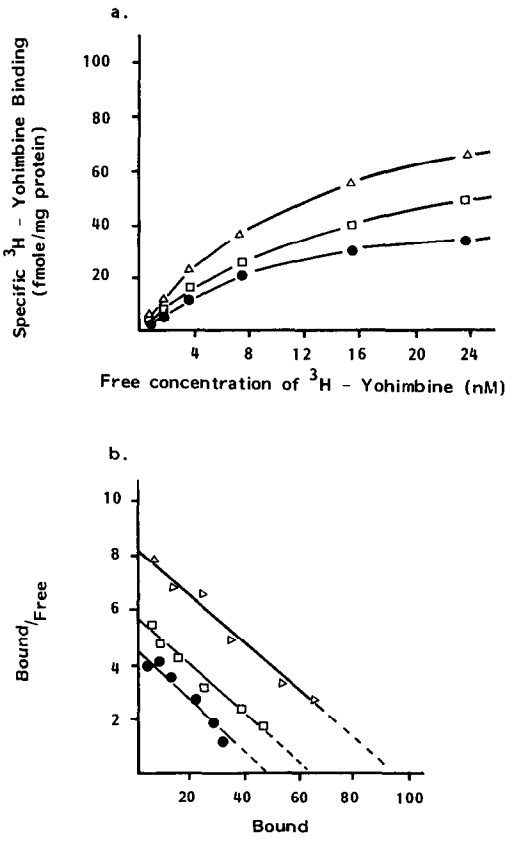

Fig. 2. Effect of exogenous norepinephrine on (^{3}H)yohimbine binding to rat cerebral cortical membranes. Membranes were prepared as described in the text by method (B) (i.e. in hypotonic Tris buffer only). (a) Saturation binding assays were carried out as described in the methods section with approximate concentrations of (^{3}H)yohimbine 0–30 nM under control conditions in assay buffer. (△—△) and in the presence of norepinephrine in final concentrations of 10^{-8} M (□—□) or 10^{-7} M (●—●). (b) Scatchard analysis of the saturation curves to assess total binding capacities (B_{max}) and dissociation constants (K_d) under the three different conditions. Data illustrated is from single representative parallel experiments performed in duplicate. B_{max} = 97, 60.38 and 42.8 fmoles/mg protein. K_d = 11.1, 10.52 and 9.07 nM—both for control assay and in the presence of 10^{-8} M and 10^{-7} M norepinephrine respectively.

specific (^{3}H)yohimbine binding to alpha$_2$ adrenoceptors in rat cerebral cortical membranes, prepared in hypotonic buffer. Norepinephrine characteristically displaces the labelled antagonist with a curve of low Hill slope (nH = 0.58). In the presence of Gpp(NH)p there is a small shift of the curve to the right with some steepening (nH = 0.74). NaCl, however, more effectively reduced the affinity of norepinephrine and the effect of NaCl and Gpp(NH)p together appeared to be synergistic. In the presence of both of these modulators, norepinephrine displaced (^{3}H) yohimbine with low affinity and with a steep displacement curve (nH = 0.91).

Note that neither NaCl nor Gpp(NH)p influence the capacity of (^{3}H)yohimbine binding to these membranes (Table 1).

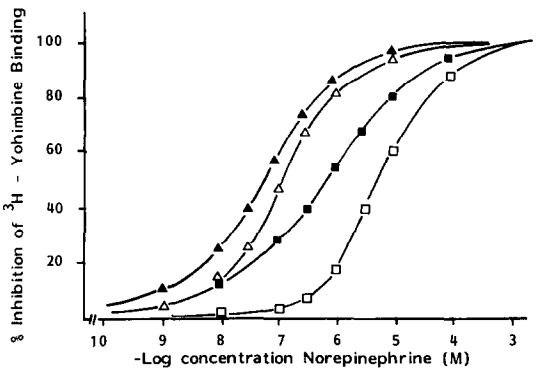

Fig. 3. Effect of Na^+ and Gpp(NH)p on norepinephrine displacement of (^{3}H)yohimbine binding to cortical membranes. Rat cerebral cortical membranes were prepared by method (B) as described in the text. Incubations were carried out with 4 nM (^{3}H)yohimbine in assay buffer as described in the methods section in the presence of increasing concentrations of norepinephrine in the presence or absence of 10 μM Gpp(NH)p, 200 mM NaCl or both. ▲—▲, norepinephrine alone; △—△, norepinephrine + 10 μM Gpp(NH)p; ■—■, norepinephrine + 200 mM NaCl; □—□, norepinephrine + 10 μM Gpp(NH)p and 200 mM NaCl. Data represent the means of four experiments performed in duplicate. S.E.M. for each point is less than ± 5%.

DISCUSSION

Agonists, but not antagonists, have been found to induce or stabilise high affinity guanine nucleotide sensitive states at catecholamine receptors coupled either positively [12, 13] or negatively [7, 8, 14, 15] to adenylate cyclase. Divalent cations, such as Mg^{2+} and Mn^{2+} have also been shown to promote the formation of high affinity agonist states [16–18]. Potentially, therefore, the presence of divalent cations and endogenous retained agonists in membrane preparations could result in problems with radio labelled ligand binding assays to receptors linked to adenylate cyclase. In view of this it was felt that the interesting report by Woodcock and Murley [9] that Na^+ ions and Gpp(NH)p increase the apparent density of cerebral alpha$_2$ adrenoceptors, could relate to the problems of retained norepinephrine. The present experiments provide very strong evidence for this interpretation.

Preparation of cerebral homogenates in hypertonic sucrose solutions is known classically to produce a high proportion of "pinched off" nerve endings or synaptosomes [19]. As expected, such preparations contained a relatively high content of retained norepinephrine when compared to preparations that had been washed thoroughly in hypotonic buffers. In the present study, we have demonstrated an inverse relationship between the quantity of endogenous or added exogenous norepinephrine and the apparent binding capacity of (^{3}H)yohimbine to cerebral cortical alpha$_2$ adrenoceptors.

The presence of agonist in membrane preparations might merely be expected to reduce the affinity of the radio labelled ligand for the receptor sites. However, the apparent reduction in binding capacity is pre-

dicted in view of the behaviour of agonists and antagonists at adenylate cyclase linked receptors. Even present at low concentrations in membranes, catecholamine agonists can essentially occupy receptor sites by inducing or stabilising the formation of high affinity states of the alpha$_2$ adrenoceptor [7, 8, 14, 15]. The formation of such high affinity states, which are regulated by ions and guanine nucleotides, appears to involve interactions between the receptor and a guanine nucleotide binding protein [20] to form a "ternary complex" in a manner similar to that described for the beta adrenoceptor agonist/receptor interaction [13]. In contrast, the alpha$_2$ adrenergic antagonists appear to bind to the receptors with homogeneous affinity without the involvement of guanine nucleotide binding proteins. This is supported by the finding that only agonist but not antagonist binding to alpha$_2$ adrenoceptors could be significantly regulated by guanine nucleotides [7, 14]. Furthermore, since yohimbine (and also other alpha$_2$ antagonists) display binding curves of slope close to unity, it is likely that antagonists bind only to a single homogeneous affinity state of the receptor. Therefore, low but significant concentrations of residual endogenous agonist could preferentially occupy a proportion of receptor sites in the high affinity state and inhibit the binding of the (^{3}H) antagonist in an apparent non competitive fashion. Only unoccupied sites, which would be predominantly of the low agonist affinity form, could be labelled by the antagonist. The overall result would be a decrease in the apparent maximal binding capacity of the (^{3}H)antagonist, with no change in its affinity at the receptor. Of course, the correct interpretation is that agonists *competitively* displace (^{3}H)antagonist from two sites, one of high and the other of low agonist affinity. It should be noted that this "pseudo non competitive" interaction of agonists has been observed at dopamine D$_2$ receptors that like alpha$_2$ adrenoceptors, are negatively coupled to adenylate cyclase at least in the pituitary [21].

The modulatory effects of Na^+ and guanine nucleotides on agonist affinity at alpha$_2$ adrenoceptors have been widely reported [7, 8, 16, 22–24]. The results in this study have also illustrated the ability of these two modulators to restore the binding capacities of (^{3}H)yohimbine in cortical membranes previously depressed by the presence of endogenous or exogenous norepinephrine. In the presence of high concentrations of Na^+ and guanine nucleotides, agonist affinity can be markedly decreased as shown in the experiments illustrated in Fig. 3. As a result, the entire population of receptors is available for labelling by the (^{3}H)antagonist. Therefore, a consistent range of receptor concentrations can be obtained in the presence of high concentrations of both Na^+ and guanine nucleotides, regardless of membrane preparation methods or the presence of low concentrations of agonist.

In this study, exogenous norepinephrine apparently competes for (^{3}H)yohimbine binding less potently than retained endogenous agonist. Thus, in competition experiments (Fig. 3) 5 nM norepinephrine displaces approximately 25% of the binding, compared to the apparent 50% reduction in binding capacity in the synaptosomal cerebral membranes (A). This may relate to difference of access to the receptor site between exogenous and endogenous agonist and requires further investigation.

However, results from this study have indeed demonstrated the inverse relationship between the binding capacity of (^{3}H)yohimbine to alpha$_2$ adrenoceptors and the norepinephrine content in cerebral cortical membrane preparations. The inhibition of (^{3}H)yohimbine labelled sites by endogenous or exogenous norepinephrine can be readily reversed by high concentrations of NaCl and guanine nucleotide. The increases in binding capacities of (^{3}H) yohimbine in rat cerebral cortex by these modulators, as previously reported [9], might not necessarily represent a unique molecular behaviour of the cortical alpha$_2$ adrenoceptors compared with other alpha$_2$ adrenoceptors. Such a phenomenon probably reflects an interaction between these modulators and the residual endogenous agonist at the receptors in cerebral membrane preparations.

These observations have significant wider implications since they could clearly influence the interpretation of apparent receptor "down regulation" by exogenous agonists or by drugs that release endogenous agonists. Thus, unless membranes are fully cleared of agonist, an apparent reduction in B_{max} for a labelled antagonist without a change in apparent K_d could be interpreted as receptor "down regulation". It would seem likely that this may be avoided by preparing well washed membranes in hypotonic buffer and/or performing assays in the presence of Na^+ and guanine nucleotides.

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