Biochemical Pharmacology, Vol. 37, No. 21, pp. 4055–4061, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
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ALPHA ADRENOCEPTOR SITES IN VASCULAR SMOOTH MUSCLE

DIFFERENTIATION BY SELECTIVE ANTAGONIST BINDING*

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(*Received* 5 *November* 1987; *accepted* 16 *March* 1988)

Abstract—The properties of alpha adrenoceptors in rat-tail artery membranes were studied using tritiated ligands that are selective for the α_1 and α_2 subtypes. High-affinity saturable binding was obtained for the α_1 antagonist prazosin yielding a B_{max} of 144 ± 31.6 fmol/mg protein (mean ± SEM, N = 3) and a K_d of 0.17 ± 0.04 nM, and also for the α_2 antagonist rauwolscine which yielded a B_{max} of 141.3 ± 19.3 fmol/mg protein and a K_d of 1.57 ± 0.32 nM. The [^{3}H]prazosin-labelled sites displayed a pharmacological profile characteristic of an α_1 adrenoceptor, whereas the [^{3}H]rauwolscine-labelled sites exhibited the expected α_2 adrenoceptor profile. Agonist affinity for [^{3}H]rauwolscine sites was reduced by Gpp(NH)p and Na^+, and the effects appeared synergistic for adrenaline, but non-interactive for UK-14304. Agonist interaction with [^{3}H]prazosin sites in the rat-tail artery was also regulated by Gpp(NH)p and Na^+, although clearly in a qualitatively and quantitatively different manner from the [^{3}H]rauwolscine sites. These results suggest that distinct binding sites for [^{3}H]prazosin and [^{3}H]rauwolscine could be differentiated with antagonist ligands. These distinct antagonist recognition sites demonstrate the pharmacological profile expected for α_1 and α_2 adrenoceptors, and the quantitatively differing abilities of Na^+ and Gpp(NH)p to regulate agonist interactions with these sites are suggestive, but do not necessarily prove, that different G proteins may be involved in this regulation.

It is now generally recognized that α adrenoceptors can be divided into the α_1 and α_2 subtypes. Over the last decade or so, experimental evidence has accumulated suggesting the role for both α_1 and α_2 postsynaptic adrenoceptors mediating vasoconstriction [1–4]. However, evidence for the existence of both α_1 and α_2 postsynaptic adrenoceptors in vascular smooth muscle was obtained primarily from *in vivo* and *in vitro* functional studies. Few reports have been published describing radioligand binding to α adrenoceptor subtypes in vascular smooth muscle, although relatively selective receptor probes have been available in recent years. [^{3}H]Prazosin and [^{3}H]rauwolscine have now been widely used to study α_1 and α_2 adrenoceptors, respectively, in a variety of tissues [5]. In previous studies, we have also used these ligands to characterise α_1 and α_2 adrenoceptor binding sites in the rat cerebral cortex, human platelet and rat renal cortex [6, 7].

In the present study, the pharmacological properties of the [^{3}H]prazosin and [^{3}H]rauwolscine binding sites in a membrane preparation of the rat-tail artery, a tissue in which both receptor subtypes appear to exhibit a functional role in vasoconstriction [8, 9], were examined. Furthermore, the modulatory effects of monovalent and guanine nucleotide on agonist interaction with α_1 and α_2 adrenoreceptor sites in the rat-tail artery were also investigated. The results indicate the presence of two distinct populations of α adrenoceptor binding sites in the rat-tail artery exhibiting differential pharmacological properties. Moreover, agonist interaction with either site was modulated by guanine nucleotide and monovalent cation in a qualitatively and quantitatively different manner and, since it is now recognized that there is an expanding number of distinct G proteins involved in cell signalling [10], this could be interpreted as reflecting different membrane regulatory components for the α_1 and α_2 sites, thus suggesting that receptor–effector coupling may involve different membrane regulatory components.

MATERIALS AND METHODS

[^{3}H]Prazosin (sp. act. 80–85 Ci/mmol) and [^{3}H]rauwolscine (sp. act. 85–90 Ci/mmol) were purchased from New England Nuclear. Yohimbine HCl, corynanthine HCl, epinephrine bitartrate, phenylephrine HCl, prazosin HCl and propranolol HCl were obtained from Sigma. Rauwolscine HCl and phentolamine HCl were from Carl Roth and Burroughs Wellcome respectively. UK-14304 tartrate was a gift from Pfizer, and 5′-guanylyl imidodiphosphate (Gpp(NH)p) was purchased from Boehringer Mannheim.

The tail-artery was dissected from mostly young female (10–12 weeks) Sprague–Dawley rats, pooled, and chopped into small pieces with scissors. Tissues from sixteen to twenty rats were usually pooled for each assay. The tissue was subsequently homogenized in 20 vol. of ice-cold 5 mM Tris, 5 mM EDTA, pH 7.4, using a Polytron homogenizer (3 × 10 sec bursts) and centrifuged at 3000 *g* for 10 min at 4°. The supernatant fraction was filtered through six layers of cheesecloth before being centrifuged at 43,000 *g* for 10 min at 4°. The resultant pellet

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Table 1. Equilibrium binding parameters for [^{3}H]prazosin and [^{3}H]rauwolscine in rat-tail artery membranes

	B_{max} (fmol/mg protein)	K_d (nM)
[^{3}H]Prazosin	144 ± 31.6	0.17 ± 0.04
[^{3}H]Rauwolscine	141.3 ± 19.3	1.57 ± 0.32

B_{max} and K_d values were obtained from Scatchard analysis of equilibrium binding data. Assays were carried out in triplicate at room temperature (22°) as described in Materials and Methods. Results are means ± SEM of three to four separate experiments.

was resuspended in an appropriate volume of assay buffer (50 mM Tris–HCl, 0.5 mM EDTA, 0.1% ascorbate, pH 7.4) and used for binding assays. In those protocols in which the effect of Na^+ was examined, a stock solution of NaCl (2 M) was diluted in the assay buffer to a final assay concentration of 200 mM. Previous studies [11] have demonstrated that in a number of tissues the maximum effect of Na^+ on agonist affinity occurs between 100 and 200 mM and, thus, in the present study 200 mM was chosen.

Binding assays were carried out at room temperature (23°) in a final volume of 250 μl. Incubation was initiated upon the addition of 40–100 μg membrane protein and continued toward equilibrium. Reaction was stopped by adding 3 ml of ice-cold assay buffer, and the contents were rapidly filtered under vacuum on GF/B filters. Radioactivity retained on filters was subsequently measured in 10 ml scintillant (NEN Formula 963) using a Beckman Scintillation Counter at an efficiency of 40%. Specific [^{3}H]prazosin or [^{3}H]rauwolscine binding was defined as that binding displaceable by 10 μM phentolamine, and represents 60–70% of total binding at 1–2 nM [^{3}H]prazosin or 4–5 nM [^{3}H]rauwolscine. Protein was determined by the method of Lowry *et al.* [12]. Specific binding was analyzed by both the Scatchard and Hill plots as previously utilized by one of the investigators [6, 11].

RESULTS

[^{3}H]Prazosin and [^{3}H]rauwolscine binding to tail-artery membranes. Both [^{3}H]prazosin and [^{3}H]rauwolscine bound rapidly to rat-tail artery membranes at 23°. Equilibrium binding for either ligand was attained in less than 20 min. The binding was saturable and reversible and, on the basis of both Scatchard and Hill plot analyses, appeared homogeneous. Scatchard analysis of equilibrium binding revealed a B_{max} of 144 ± 31.6 fmol/mg protein for [^{3}H]prazosin, with an apparent K_d of 0.17 ± 0.04 nM. A similar binding capacity for [^{3}H]rauwolscine, with a K_d of 1.57 ± 0.32 nM, was obtained (Table 1; Fig. 1). K_d values determined from equilibrium binding were in close agreement with those derived from kinetics studies (0.112 and 1.81 nM, respectively, for [^{3}H]prazosin and [^{3}H]rauwolscine), as well as from drug displacement studies (Table 2).

Pharmacological characteristics of [^{3}H]prazosin and [^{3}H]rauwolscine binding sites on rat-tail artery membranes. [^{3}H]Prazosin and [^{3}H]rauwolscine binding to rat-tail artery membranes could be competitively displayed by various adrenergic antagonists. The pharmacological profiles generated resemble those expected of interaction at α_1 and α_2 adrenoceptor sites. Thus, α_2 selective antagonists rauwolscine and yohimbine displaced [^{3}H]rauwolscine binding more potently than the relatively nonselective antagonist phentolamine, and this was fol-

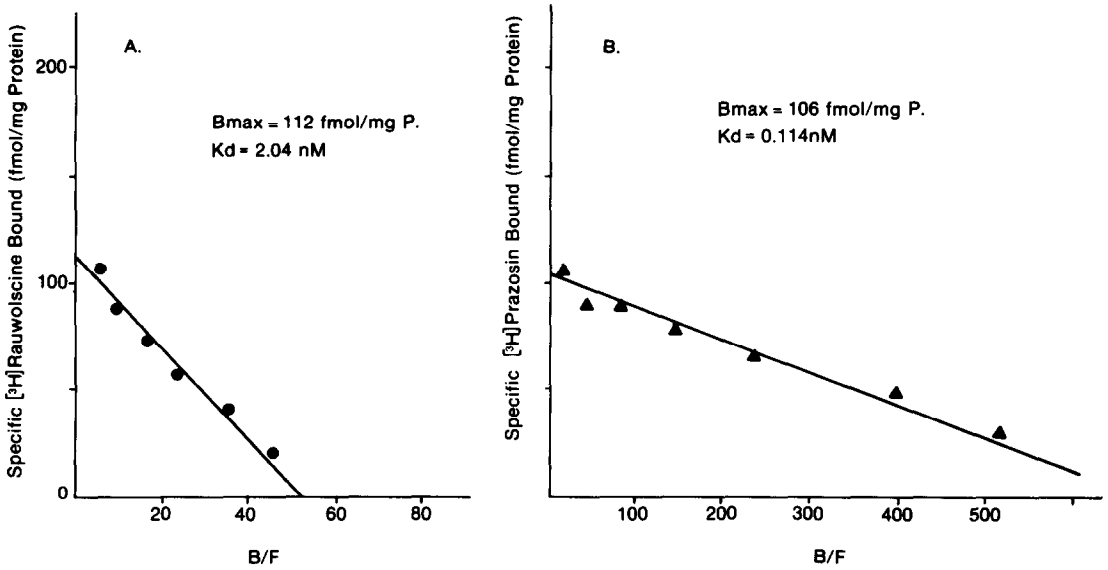

Fig. 1. Scatchard plots of equilibrium binding data. (A) [^{3}H]Rauwolscine. (B) [^{3}H]Prazosin. Rat-tail membranes were incubated for 45 min at room temperature with 0–5 nM [^{3}H]prazosin or 0–45 nM [^{3}H]rauwolscine as described in Materials and Methods. Data are from one experiment representative of three to four separate experiments performed in triplicate.

Table 2. Inhibitory constants of antagonists at [^{3}H]prazosin and [^{3}H]rauwolscine-labelled sites in rat-tail artery membranes

	K_i (nM)	
	[^{3}H]Prazosin	[^{3}H]Rauwolscine
Rauwolscine	3,001 ± 488 (1.03)	1.64 ± 0.52 (0.96)
Yohimbine	1,182 ± 236 (0.97)	5.57 ± 1 (0.97)
Phentolamine	12.3 ± 2.5 (0.96)	45.9 ± 2.7 (0.94)
Corynanthine	168.3 ± 26.5 (0.93)	611.6 ± 35 (1.01)
Prazosin	0.18 ± 0.04 (0.99)	148 ± 1.7 (1.08)
Propranolol		19,877.8 ± 1,569 (0.98)

K_i values were calculated from IC_{50} values obtained graphically using the formula:

$$K_i = IC_{50}/(1 + S/K_d)$$

where S is the radioligand concentration and K_d is the equilibrium dissociation constant. The numbers in parentheses represent slope factors of mean displacement curves for individual antagonists. Data are means ± SEM of three to five separate experiments performed in duplicate.

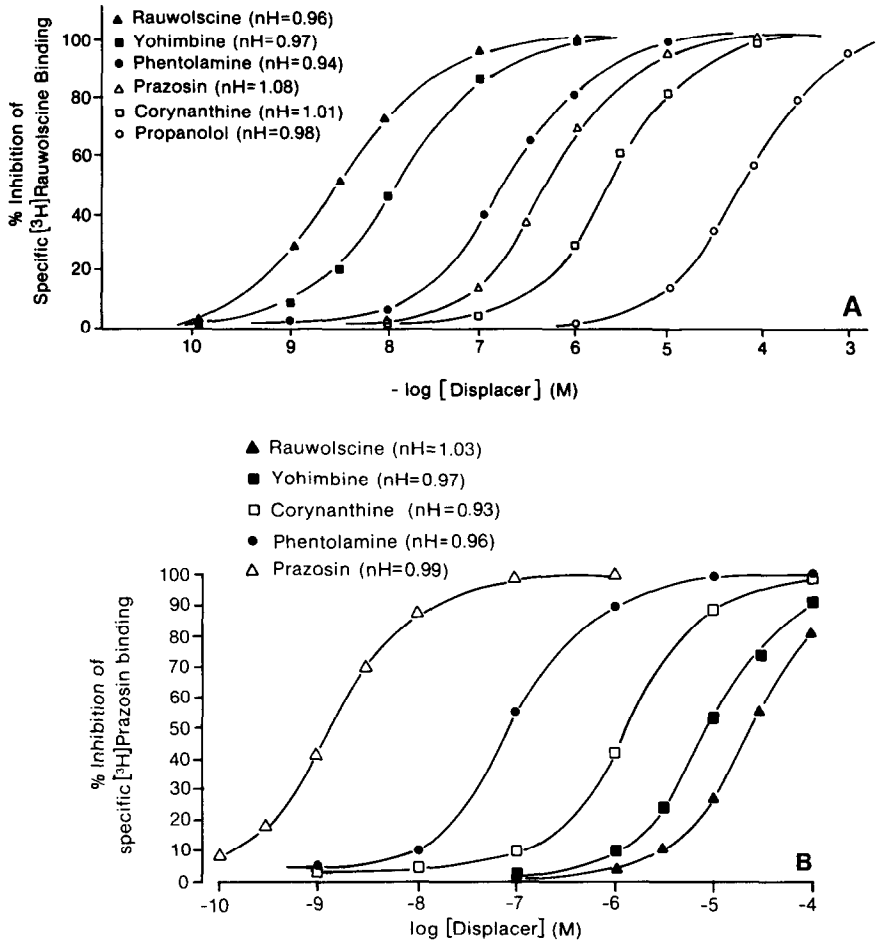

Fig. 2. Pharmacological profiles of [^{3}H]rauwolscine and [^{3}H]prazosin binding to rat-tail artery. Rat-tail artery membranes were incubated with approximately 5 nM [^{3}H]rauwolscine or 1 nM [^{3}H]prazosin and increasing concentrations of antagonists. (A) Displacement of [^{3}H]rauwolscine binding. (B) Displacement of [^{3}H]prazosin binding. Each data point represents the mean of three to five separate experiments performed in duplicate. The numbers in parentheses are mean slope factors of individual curves.

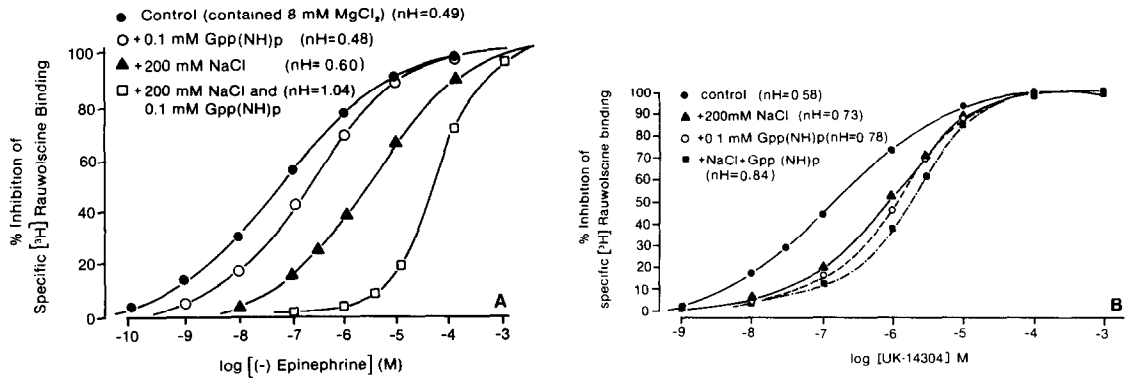

Fig. 3. Modulation by Na^+ and Gpp(NH)p of agonist interaction with [^{3}H]rauwolscine-labelled sites in the rat-tail artery. Tail-artery membranes were incubated with approximately 5 nM [^{3}H]rauwolscine and increasing concentrations of agonist in the absence and presence of 200 mM NaCl, 0.1 mM Gpp(NH)p or both. (A) Interaction of (−)-epinephrine with [^{3}H]rauwolscine binding. (B) Interaction of UK-14304 with [^{3}H]rauwolscine binding. Each data point represents the mean of three to six separate experiments performed in duplicate. Numbers in parentheses are mean slope factors of individual displacement curves.

lowed by the α_1 selective agents prazosin and corynanthine. The beta antagonist propranolol was a very weak competitor for [^{3}H]rauwolscine binding in this preparation (Table 2; Fig. 2A).

In contrast, a profile displaying an exactly reverse order of drug potency was exhibited at [^{3}H]prazosin binding sites. Thus, α_1 selective antagonists were much more potent competitors than α_2 antagonists for [^{3}H]prazosin binding (Table 2; Fig. 2B), suggesting interaction at an α_1 adrenoceptor.

It should be noted that all the displacement curves generated steep slopes, indicating that drug interaction with either ligand occurred at a homogeneous population of binding site or state (Table 2; Fig. 2).

Modulation of agonist interaction with [^{3}H]rauwolscine binding sites by guanine nucleotide and monovalent cation. There is now evidence that agonists, but not antagonists, interact with α_2 adrenoceptor sites in high- and low-affinity states interconvertible under the influence of guanine nucleotides. Moreover, monovalent cations such as Na^+ also selectively reduce against affinity [13–15].

Figure 3A shows the effects of Na^+ and Gpp(NH)p, a non-hydrolysable GTP analogue, on epinephrine interaction with [^{3}H]rauwolscine-labelled sites in rat-tail artery. In the absence of guanine nucleotide or monovalent cation, the agonist displacement curve displayed a shallow slope, suggesting interaction with the binding site in more than one affinity state. It is clear that 0.1 mM Gpp(NH)p alone reduced the affinity of epinephrine weakly, compared to 200 mM NaCl. However, the effects of Na^+ and Gpp(NH)p appeared to be synergistic. Na^+ promoted or facilitated the modulatory effect of Gpp(NH)p. In the presence of 200 mM NaCl, 0.1 mM Gpp(NH)p produced a larger shift of the displacement curve to the right. Moreover, there was a steepening of the curve toward unity, suggesting that epinephrine interacted with a homogeneous population of affinity state under the influence of the guanine nucleotide.

In contrast to epinephrine, interaction of UK-14304 with [^{3}H]rauwolscine-labelled sites in rat-tail artery was modulated by Na^+ and Gpp(NH)p in a somewhat different manner (Fig. 3B). A 0.1 mM concentration of Gpp(NH)p alone produced a 10-

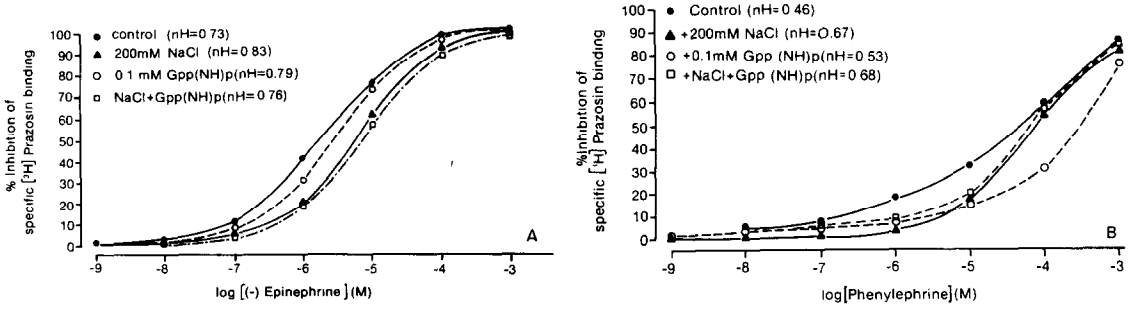

Fig. 4. Modulation of agonist interaction with [^{3}H]prazosin-labelled sites in the rat-tail artery by Na^+ and Gpp(NH)p. Rat-tail artery membranes were incubated with approximately 1 nM [^{3}H]prazosin and increasing concentrations of agonists in the absence and presence of 200 mM NaCl, 0.1 mM Gpp(NH)p or both. (A) Interaction of (−)-epinephrine with [^{3}H]prazosin binding. (B) Interaction of phenylephrine with [^{3}H]prazosin binding. Each data point represents three to four separate experiments performed in duplicate. Numbers in parentheses are mean slope factors of individual displacement curves.

fold shift of the displacement curve to the right. There was also a steepening of the slope from 0.58 to 0.78. At a concentration of 200 mM, NaCl alone produced a 6- to 7-fold rightward shift, though the presence of Na^+ apparently did not further enhance the effect of Gpp(NH)p. These results suggest that Na^+ could modulate the interaction of UK-14304 with α_2 adrenoceptor sites independently of Gpp(NH)p and may involve different sites of mechanism of action.

Modulation of agonist interaction with [^{3}H]prazosin binding by guanine nucleotide and monovalent cation. In comparison with α_2 adrenoceptor sites, guanine nucleotide and monovalent cation modulation of agonist binding to α_1 adrenoceptor sites has not been widely reported. Figure 4A shows the effects of Gpp(NH)p and Na^+ on epinephrine displacement of [^{3}H]prazosin binding to rat-tail artery membranes. In this system, 0.1 mM Gpp(NH)p alone shifted the agonist displacement curve weakly to the right, and although 200 mM NaCl produced a 2-fold rightward shift on its own, it had a minimal effect on the modulatory action of Gpp(NH)p; this is in sharp contrast to that observed at [^{3}H]rauwolscine-labelled sites.

It is of interest to note that the interaction of [^{3}H]prazosin-labelled sites with phenylephrine, a relatively α_1-selective agonist, appeared to exhibit greater sensitivity to Gpp(NH)p in the rat-tail artery (Fig. 4B). In the presence of 0.1 mM Gpp(NH)p, there was a 6-fold shift of the agonist displacement curve to the right, although the slope was only steepened slightly. A steeper curve was generated in the presence of 200 mM NaCl in spite of a lack of reduction in overall agonist affinity. In fact, in the presence of 200 mM NaCl, the modulatory effect of Gpp(NH)p even appeared to be inhibited, at least with respect to the overall affinity.

DISCUSSION

Much of the evidence supporting the co-existence of postsynaptic α_1 and α_2 adrenoceptors in vascular smooth muscle has come from *in vivo* whole animal [16, 17] or isolated blood vessel [3, 8, 9, 18] studies. That postsynaptic α_2 adrenoceptor subtype is also present in the rat-tail artery and may play a role in regulating vasoconstriction is still a controversial issue. For instance, Hicks *et al.* [9] found that α_2-adrenoceptor mediated vasoconstrictive responses to agonists are only apparent in spontaneously hypertensive, as compared to normotensive, animals. In fact, in more recent studies questions have been raised regarding the existence of distinct α_1 and α_2 adrenoceptors in the rat-tail artery [19, 20]. In view of such controversy, it was considered worth investigating the nature of postsynaptic alpha adrenoceptors in this tissue using radioligand binding techniques.

In the present study, [^{3}H]prazosin and [^{3}H]rauwolscine differentially labelled distinct, and apparently homogeneous, populations of receptor sites in the rat-tail artery membrane preparation. At least with respect to antagonist binding, pharmacological profiles characteristic of α_1 and α_2 adrenoceptors, respectively, were evident (Table 2; Fig. 2). It is of interest to note that prazosin appeared to exhibit a relatively high affinity for the [^{3}H]rauwolscine-labelled sites, considering the accepted high α_1 selectivity of this antagonist.

In fact, a similar finding has also been observed for [^{3}H]rauwolscine sites in a number of rat tissues [6, 21–24]. This apparent α_1-like nature of the [^{3}H]rauwolscine sites has been considered as suggesting a possible heterogeneity for α_2 adrenoceptors among different tissues and/or species [24, 25].

Functional studies also suggest that the α_2 site in rat tissues may be "α_1-like". Thus, Agrawal *et al.* [26] also demonstrated, in isolated rat mesenteric artery preparations, the high sensitivity of putative α_2 mediated contraction to inhibition by prazosin.

It is well established that α_2 adrenoceptors in various tissues are coupled negatively to adenylate cyclase and that agonists, compared to antagonists, interact with the receptor sites via a G-protein to form a guanine nucleotide sensitive high-affinity state [13, 14, 27, 28]. Moreover, monovalent cations such as Na^+ can preferentially regulate agonist interaction with α_2 adrenoceptor sites [13–15], although it is yet unclear if guanine nucleotides and monovalent cations both act on the G-protein [29, 30]. Few reports have been available describing the modulation of agonist interaction with α_2 adrenoceptor sites in vascular tissues. Results of this study demonstrate that agonist interaction with [^{3}H]rauwolscine-labelled sites in the rat-tail artery membrane preparation could be modulated by guanine nucleotide and Na^+ in a manner similar to that observed in other rat tissues, such as the cerebral cortex or renal cortex [11, 31]. However, in the rat-tail artery, guanine nucleotide modulation of epinephrine interaction with α_2 adrenoceptor sites appears to be facilitated by Na^+. This is in contrast to that for another α_2 full agonist, UK-14304, which seems to be independent of the presence of Na^+. This difference cannot be clearly explained at the moment. However, it remains possible that, being a non-selective full agonist, epinephrine, compared to UK-14304, may invoke receptor interaction with other membrane regulatory components whose influence on α_2 receptor–G-protein interaction could be modulated by Na^+. In any case, the role of α_2 receptor coupled inhibition of adenylate cyclase in vasoconstriction remains unclear, especially in view of the apparent dependency upon extracellular Ca^{2+} influx of α_2 mediated vascular smooth muscle contraction [32, 33].

In contrast to α_2 adrenoceptors, there is now increasing evidence suggesting that α_1 adrenoceptors are coupled via phospholipase C to membrane polyphosphoinositide breakdown in vascular smooth muscle [34, 35]. One metabolite, inositol 1,4,5-trisphosphate, can act as a second messenger to relate intracellular Ca^{2+} [36] required for contractile activity. It is also believed that some kind of G-protein may be involved in α_1 receptor coupling with phospholipase C in a way similar to that between α_2 receptor and adenylate cyclase [37]. Compared to α_2 adrenoceptors, reports on the regulation of agonist interaction with α_1 adrenoceptor by guanine nucleotides and monovalent cations have been limited. However, evidence for such modulation has been emerging from recent studies on such tissues as rat

liver [38], rat kidney [15], and rat mysenteric artery [39].

In the present study, we also provide evidence for the guanine nucleotide and monovalent cation modulation of agonist interaction with rat-tail artery α_1 adrenoceptor binding sites (Fig. 4). The modulation appears qualitatively and quantitatively different from that with the α_2 adrenoceptor sites. Nevertheless, these findings support the view that a guanine nucleotide binding protein, though not necessarily identical to the inhibitory G-protein, G_i, may be involved in the α_1 adrenoceptor–phospholipase C coupling event [37].

Current work in our laboratory has been directed toward evaluating the role of α_1 and α_2 adrenoceptor mediated breakdown of polyphosphoinositide in the rat-tail artery. The results should provide more information regarding the nature of the alpha adrenoceptors in this tissue and the mechanisms by which receptor activation–contraction is coupled.

Acknowledgements—We gratefully acknowledge financial support from the Canadial Heart Foundation. We would like also to thank Betty Granter for typing the manuscript.

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