Br. J. Pharmac. (1984), **83**, 103–111

Calcium-dependence and antagonism of responses to α_1- and α_2-adrenoceptor agonists in vascular tissues from hypertensive and normotensive rats

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1 Inhibition by D600 (methoxyverapamil) of responses to an α_2-adrenoceptor selective agonist, B-HT 920, (6-allyl-2-amino-5, 6, 7, 8-tetrahydro-4H-thiazolo [4, 5-d] azepin dihydrochloride), an α_1-adrenoceptor selective agonist, phenylephrine (PE), and a nonselective agonist, noradrenaline (NA), was studied in isolated preparations of the aortae and carotid arteries obtained from young (5–7 weeks) and old (15–17 weeks) hypertensive (SHR) and normotensive (WKY) rats.

2 Maximum responses of WKY tissues to B-HT 920 were the most sensitive, PE-induced responses the least sensitive and maximum responses to NA were intermediate in their sensitivity to inhibition by D600.

3 Sub-maximal responses to NA and PE were not different in their sensitivity to inhibition by D600, but were less sensitive than the responses to B-HT 20. Sub-maximal responses to PE were significantly more sensitive to D600 inhibition than were the maximal responses to this agonist.

4 NA-induced responses of tissues from older SHR were less sensitive to inhibition by D600 when compared to responses in WKY rats.

5 Responses to B-HT 920, in tissues suspended in calcium-free solutions, showed the largest decline compared to NA- and PE-induced responses.

6 We conclude that responses to B-HT 920 largely utilize extracellular calcium. PE- and NA-induced responses mobilize extracellular calcium to varying degrees depending upon the concentration of the agonist employed to elicit the response.

Introduction

There is growing awareness that postsynaptic α-adrenoceptors may comprise at least two sub-types which, on the basis of *in vivo* observations, can be classified into α_1- and α_2-adrenoceptors (Starke, 1981; Timmermans & Van Zweiten, 1981; McGrath, 1982). An interesting development in this area was the report that pressor responses of a pithed rat to B-HT 920, a selective α_2-adrenoceptor agonist, were more sensitive to inhibition by calcium antagonists than were responses to an α_1-adrenoceptor agonist (Van Meel *et al.*, 1981a). These findings, indicative of a greater dependence on extracellular calcium of responses mediated via α_2-adrenoceptors, have since been confirmed by *in vivo* (Van Meel *et al.*, 1981b) and *in vitro* (Nghiem *et al.*, 1982a; Godfraind *et al.*, 1982) observations in the rat.

Our earlier work (Nghiem *et al.*, 1982a) showed that the responses to clonidine, an α_2-adrenoceptor agonist, were less sensitive to inhibition by D600 in aortic strips from hypertensive rats than those from normotensive rats. In view of the evidence that calcium transport and sequestration are altered in vascular tissues of hypertensive rats (Jones, 1974; Webb & Bhalla, 1976; Pedersen *et al.*, 1978; Daniel & Kwan, 1981), we have extended our previous work by comparing the dependency on extracellular calcium of the responses of isolated vascular tissues from hypertensive and normotensive rats to selective α-adrenoceptor agonists. The criteria chosen were sensitivity to inhibition by D600 and the persistence of responses to adrenoceptor agonists by vascular strips suspended in calcium-free solution. Our results clearly demonstrate differing degrees of calcium-dependency, which vary not only with the agonist but also with the concentrations of the agonist employed to elicit the response.

Methods

Animals

The spontaneously hypertensive rats (SHR) used in this study are derived from rats originally obtained from the National Institutes of Health, Washington, D.C. and which have been maintained at our facilities by brother-sister mating for approximately 12 years. Their normotensive counterparts, Kyoto Wistar (WKY) rats, were obtained from commercial sources (Charles River). All rats were fed on common laboratory food (Purina Lab Chow) and allowed free access to tap water.

The systolic blood pressure was measured from tails of unanaesthetized rats by use of a pulse transducer and an electrosphygmomanometer (Narco Biosystems, Inc.). Three blood pressure determinations were made on each rat and measurements were made routinely every week. The systolic blood pressure of SHR at 5 and 17 weeks of age was 106.2 ± 3.1 mmHg ($n = 15$) and 185.0 ± 6.1 mmHg ($n = 16$), respectively, while that of age-matched WKY rats was 107 ± 4.6 mmHg ($n = 8$) and 129.5 ± 5.0 mmHg ($n = 9$).

Tissue preparations

Spirally cut strips of the thoracic aorta and the carotid artery were prepared according to procedures described earlier (Nghiem *et al.*, 1982b). Tissues were equilibrated in 10 ml jacketed organ baths under tension (350–400 mg for carotid arteries; 4.0g for aortae) for 1–3 h in warm (37.5°C) physiological solutions through which a mixture of 95% O_2 + 5% CO_2 was constantly bubbled. Isometric contractions were recorded via a force displacement transducer (Grass FTO3) on a Grass polygraph recorder (Model 7B).

Vascular responses

(A) Cumulative dose-response curves to phenylephrine (PE), B-HT 920 and noradrenaline (NA) were determined in tissues obtained from 5–7 and 15–17 week old rats. The response to each concentration of the agonist was recorded until a steady level was reached (5–10 min). Each tissue preparation was exposed to only one agonist and the sensitivity of the tissues to the three agonists has been reported in terms of ED_{50} values (Table 1).

(B) The concentration-effect relationship of D600 was studied under conditions where either maximum or submaximum control responses to agonists were elicited by tissues obtained from both age-groups of rats. The concentrations employed to elicit maximum responses were 1×10^{-5} M for all three agonists in experiments with aortic strips. In experiments with carotid strips, 1×10^{-6} M and 1×10^{-5} M NA was used for strips from 5–7 and 15–17 week old rats, respectively, while maximum responses to PE and B-HT 920 were uniformly obtained with a concentration of 1×10^{-5} M of the agonists. Experiments with submaximal responses were performed with aortic strips only and the concentrations used (ED_{50}) are listed in Table 1.

In a typical experiment, a control response to an agonist was recorded followed by a recovery period of approximately 1 h. Tissues were exposed to a concentration of D600 (30 min) before recording a second response to the agonist. Each tissue preparation was exposed to only one concentration of D600 and the effect of D600 is given in terms of percentage inhibition of the control responses (Figures 1–5).

(C) The persistence of agonist-induced responses in (nominally) calcium-free physiological solution (CFS) was studied in the following manner. A control response was recorded to an agonist in tissues suspended in normal physiological solution (NPS). Tissues were allowed to recover for approximately 1 h and a second response to the agonist was recorded following replacement of NPS by CFS for 30 min. The responses of tissues suspended in CFS are reported with reference (%) to the magnitude of the control responses in NPS (Figures 6 and 7).

Drugs and solutions

The composition (mM) of the physiological solutions (NPS) used in the experiments was as follows: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.18, $NaHCO_3$ 12.5, KH_2PO_4 1.18 and glucose 5.5 in twice-distilled water. The calcium-free solutions (CFS) refers to the above physiological solution from which calcium was omitted.

D(−)-Noradrenaline (Sigma), D(−)-phenylephrine (Sigma) and B-HT 920 (6-allyl-2-amino-5, 6, 7, 8-tetrahydro-4H-thiazolo [4, 5-d] azepin dihydro-chloride; Dr K. Thomae Gmbh) were dissolved in saline (0.9%NaCl, w/v) containing sodium metabisulphite (0.05%, w/v). D600 (methoxyverapamil, Knoll AG) was dissolved in twice distilled water. All drug concentrations in the text refer to the final bath concentration.

Statistical analysis

The results are reported as arithmetic means (s.e.mean) except for the ED_{50} values (Table 1) which are given as geometric means (Fleming *et al.*, 1972). The homogeneity of obervations obtained in experiments with the three agonists was tested by employing one-way analysis of variance procedures. Whenever this initial procedure indicated differences

within the experimental observations, a comparison between the three groups was made according to the Newman-Keul Multiple range test (Snedecor & Cochran, 1976). Comparison in all cases was made at the 5% level of significance.

Results

(A) *Vascular response to α-adrenoceptor agonists*

The aortic and the carotid arterial strips responded in graded fashion to increasing concentrations of the three agonists. The maximum responses to NA and PE were approximately equal in magnitude in tissues from rats of both age groups (5–7 weeks, 15–17 weeks) of both strains and larger than that induced by B-HT 920. Maximum responses to B-HT 920, relative to those of NA or PE, varied with age, approximately 74% and 86% in carotid and aortic strips from young rats and 48% and 55% in tissues from old rats, respectively.

The sensitivity of the arterial strips, in terms of ED_{50} values, is given in Table 1. Tissues from both strains of rats show lower ($P<0.05$) sensitivity to B-HT 920 than to NA or PE, except in aortic strips from older SHR, where NA is approximately equipotent to B-HT 920. Responses to NA and PE were generally equipotent except for aortic strips from SHR which were less sensitive to NA, and carotid strips from young WKY rats, where NA was more sensitive than PE.

Concentration-effect curves of D600

The inhibition by D600 of the maximum responses to the three agonists is illustrated in Figures 1–4. High concentrations of either NA or PE elicited a characteristic response from both arterial preparations which could be differentiated into an initial rapid contraction (within 30–45 s) followed by a slow, sustained contraction which reaches a steady level in 10–20 min. Maximum responses to B-HT 920 had a noticeably slower onset characterized, generally, by an absence of the initial rapid component. The peak response to all three agonists was represented by the slow component and the inhibition depicted in Figures 1–5 thus reflects the reduction in the magnitude of the slow component of the response relative to control.

Inhibition of NA-induced responses of tissues from older WKY rats (Figures 1 and 2) shows three distinct concentration-dependent phases. An initial phase of inhibition is seen with low ($<10^{-6}$ M) concentration of D600 followed by a phase where large increments (<100 fold) in concentration of D600 resulted in a minor increase in inhibition. A third phase (of inhibition) is seen at high concentrations ($>10^{-4}$ M) and is characterized by an abrupt large increase in inhibition. This triphasic inhibition of NA-induced responses of WKY tissues is a good example of the dual sensitivity to D600 (and other calcium channel antagonists) described by Golenhofen (1981) and which forms the basis for his hypothesis of two calcium activation systems in

Table 1 Sensitivity[a] of isolated aortae and carotid arteries of normotensive (WKY) and hypertensive (SHR) rats to α-adrenoceptor agonists

Aorta	*Noradrenaline* (M)	*Phenylephrine* (M)	*B-HT 920* (M)
WKY (5–7 weeks)	$5.2\pm1.3\times10^{-9}$ (6)	$4.7\pm1.2\times10^{-9}$ (8)	$3.9\pm1.3\times10^{-7}$* (6)
WKY (15–17 weeks)	$2.2\pm1.2\times10^{-8}$ (7)	$5.4\pm1.3\times10^{-8}$ (6)	$1.9\pm0.9\times10^{-7}$* (12)
SHR (5–7 weeks)	$1.7\pm0.8\times10^{-8}$ (7)	$4.4\pm1.4\times10^{-9}$* (6)	$2.2\pm1.5\times10^{-7}$* (6)
SHR (15–17 weeks)	$1.4\pm1.1\times10^{-7}$ (10)	$3.7\pm1.4\times10^{-8}$* (7)	$3.2\pm1.3\times10^{-7}$ (12)
Carotid			
WKY (5–7 weeks)	$3.9\pm0.7\times10^{-9}$ (14)	$1.1\pm0.5\times10^{-8}$* (11)	$5.6\pm0.7\times10^{-7}$* (13)
WKY (15–17 weeks)	$1.5\pm0.9\times10^{-8}$ (14)	$8.1\pm0.4\times10^{-9}$ (10)	$1.0\pm1.3\times10^{-6}$* (8)
SHR (5–7 weeks)	$7.9\pm1.1\times10^{-9}$ (8)	$2.9\pm0.6\times10^{-8}$ (7)	$5.9\pm1.2\times10^{-7}$* (6)
SHR 15–17 weeks)	$9.4\pm0.8\times10^{-8}$ (13)	$4.5\pm0.9\times10^{-8}$ (11)	$1.0\pm1.2\times10^{-6}$* (14)

[a]Sensitivity is reported as the geometric mean of ED_{50} values. Numbers in parentheses denote the number of observations.

* Significantly different ($P<0.05$) from values for noradrenaline.

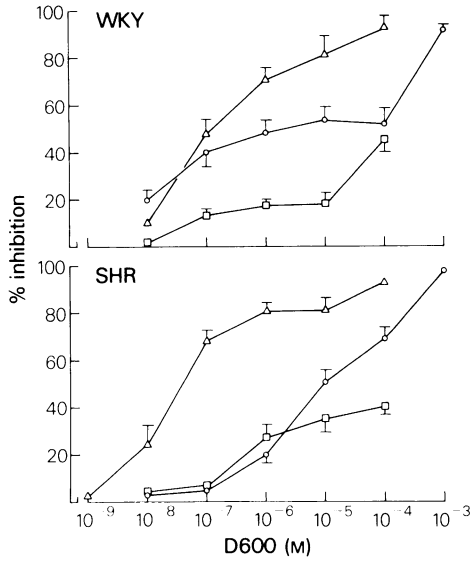

Figure 1 Inhibition by D600 of the maximal responses of the isolated aorta from 15–17 week old rats to noradrenaline (○), phenylephrine (□) and B-HT 920 (△). Each point represents an average of at least six observations and vertical lines denote s.e.mean.

smooth muscle. In contrast, the inhibition by D600 of NA-induced responses of SHR tissues (Figures 1 and 2: lower panels) comprised a single and essentially linear component of inhibition. Inhibition at low ($< 10^{-6}$ M) concentrations of D600 was significantly smaller than that seen with WKY tissues while that at higher ($> 10^{-5}$ M) concentrations was approximately equal in both strains.

Responses to B-HT 920 in tissues from both strains of rats were clearly more sensitive to inhibition by D600 than were those to NA; a large proportion of the inhibition occurred over concentrations ($< 10^{-6}$ M) defining the sensitive phase of inhibition of NA-induced responses in WKY rats. No large differences were seen in the inhibition of responses to B-HT 920 in tissues from SHR and WKY rats. Responses of tissues from WKY rats to PE were consistently less sensitive to inhibition than those to NA in contrast to PE-induced responses in SHR, where lesser sensitivity was observed only at high ($> 10^{-5}$ M) concentrations of D600.

Maximum responses elicited in tissues from young (5–7 weeks) rats, showed a similar order of sensitivity to D600 (B-HT 920 > NA > PE) as in older rats (Figures 3 and 4). No large differences were seen in the inhibition of tissue responses of SHR and WKY rats. Responses to NA of aortae from young WKY rats were less sensitive to low ($< 10^{-6}$ M) concentrations of D600 than those seen in older rats, in contrast to SHR tissues, where greater sensitivity to inhibition was seen with younger rats.

An increased sensitivity to inhibition was observed when aortic responses to concentrations of agonists

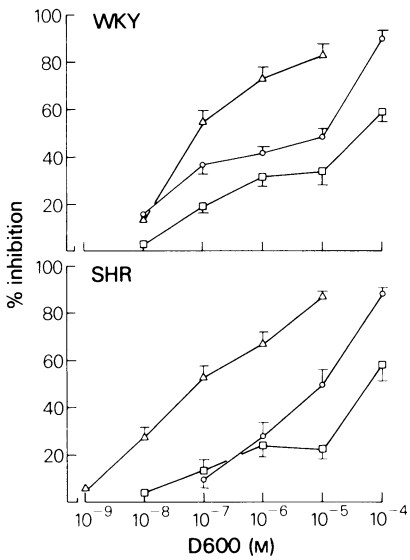

Figure 2 Inhibition by D600 of the maximal responses of the isolated carotid artery from 15–17 week old rats to noradrenaline (○), phenylephrine (□) and B-HT 920 (△). Each point represents an average of at least six observations and vertical lines denote s.e.mean.

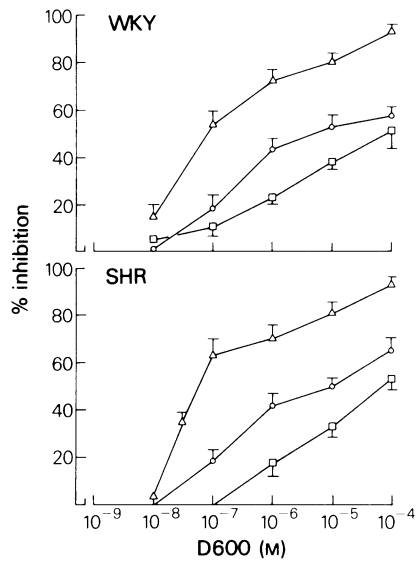

Figure 3 Inhibition by D600 of the maximum responses of the isolated aorta from 5–7 week old rats to noradrenaline (○), phenylephrine (□) and B-HT 920 (△). Each point represents an average of at least six observations and vertical lines denote s.e.mean.

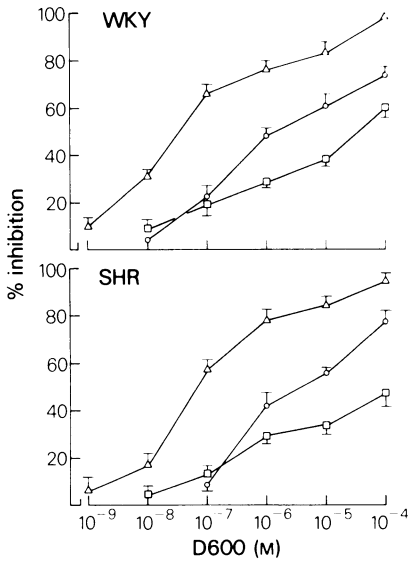

Figure 4 Inhibition by D600 of the maximum responses of the isolated carotid artery from 5–7 week old rats to noradrenaline (○), phenylephrine (□) and B-HT 920 (△). Each point represents an average of at least six observations and vertical lines denote s.e.mean.

causing 50% of the maximum responses (see Methods) were studied (Figure 5). B-HT 920-induced responses of the aortae from both age groups and of both strains were most sensitive to inhibition. No consistent difference was observed in the inhibition of responses to NA and PE. Inhibition of NA- and PE-induced submaximal responses was graded and generally typical of a single component of inhibitory action by D600.

(C) *Responses in calcium-free solution (CFS)*

A diminished response relative to that observed in NPS was observed with 30 min exposure of the aortic (Figure 6) and carotid strips (Figure 7) to CFS. The magnitude of the decline varied with the concentration of the agonists and the age of the animals. The decline in response to B-HT 920 was significantly greater than that to NA or PE in every experimental group. Significant differences between the persistence of PE- and NA-induced responses (PE > NA) were seen when agonist concentrations eliciting maximum responses in tissues from older animals were compared (Figures 6a and 7a). No significant difference was observed between NA- and PE-induced maximum response in aortae from young rats in contrast to the carotid arteries, where the decline in

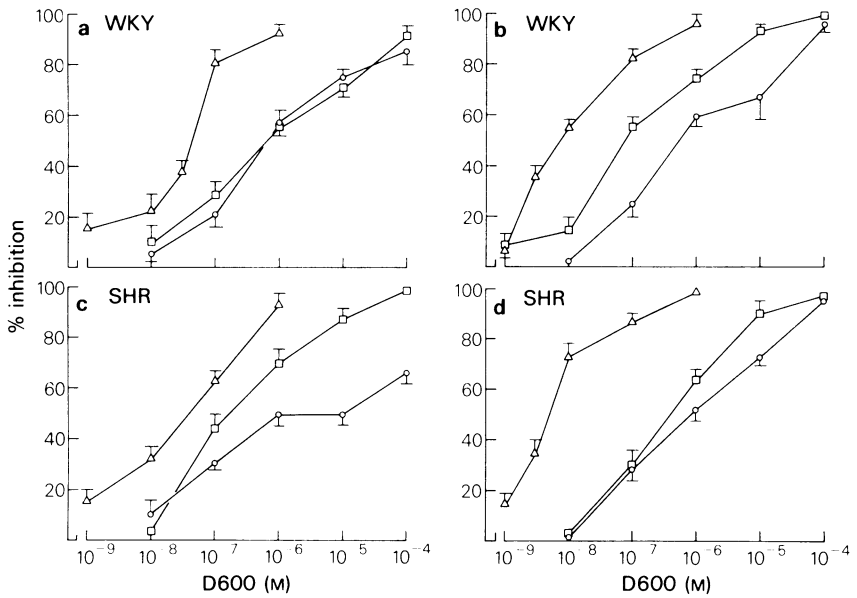

Figure 5 Inhibition by D600 of the sub-maximal responses of the isolated aorta to noradrenaline (○), phenylephrine (□) and B-HT 920 (△). The panels on the left (a, c) depict responses of aortae from 15–17 week old rats and the responses on the right (b, d) are from 5–7 week old rats. The concentration of agonists used to elicit submaximum responses are listed in Table 1 (ED_{50} values). Each point represents an average of at least six observations and vertical lines denote s.e.mean.

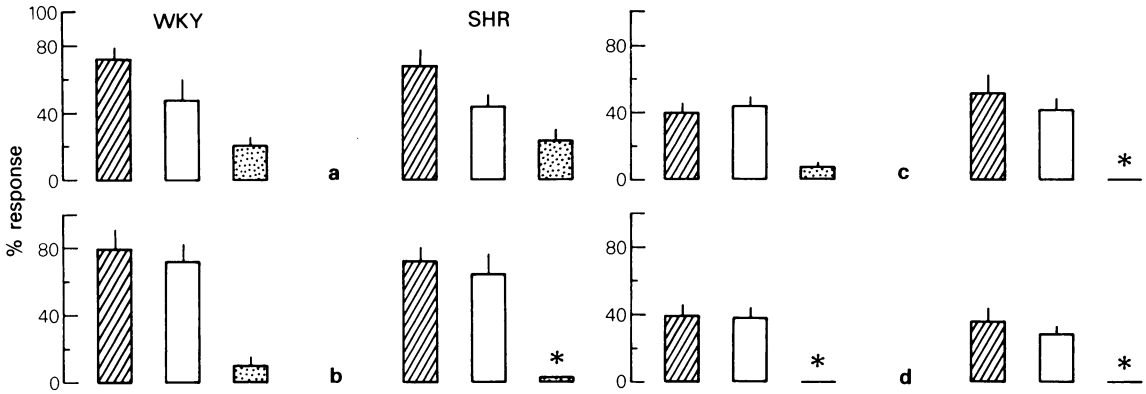

Figure 6 Responses to adrenoceptor agonists by aortic strips suspended in calcium-free solution (30 min). The hatched columns represent responses to phenylephrine, the open colums, responses to noradrenaline and the stippled columns, responses to B-HT 920. Panels (a) and (b) are responses of aortic strips from 15–17 weeks and 5–7 weeks old rats, respectively, to concentrations of agonist eliciting maximum response in normal physiological solution. Panels (c) and (d) are responses of the aotric strips from 15–17 weeks and 5–7 weeks old rats, respectively, to concentrations (ED_{50}) listed in Table 1. Responses are % of control in normal physiological solution. Each column represents an average of at least six observations; vertical lines denote s.e.mean. Asterisks denote responses which were 5% or less in magnitude relative to control responses.

NA-induced response was significantly greater than that of PE (Figures 6b and 7b). No significant difference was seen between the persistence of NA- and PE-induced submaximal responses of the aortae from both age groups (Figure 6c and d).

Discussion

A major object of our experiments was to examine the dependence on extracellular calcium of responses of vascular strips to α-adrenoceptor agonists. Evaluation of the role of calcium was based on the sensitivity of vascular responses to inhibition by D600, a calcium entry blocker, and by the persistence of the responses in calcium-free solution. As judged by the above criteria, the responses to B-HT 920 uniformly showed a marked dependence on extracellular calcium. However the responses to NA and PE varied in their relative dependence on extracellular calcium depending upon experimental conditions, such as the age of rats and the concentration of agonists employed to elicit the response.

Various lines of evidence (reviewed by Timmermans & Van Zweiten, 1981; McGrath, 1982) point to the presence of postsynaptic α_1- and α_2-adrenoceptors in smooth muscle. The adrenoceptor agonists used in this study reportedly show varying specificity for α_1- and α_2-adrenoceptors on the basis of *in vivo* observations notably of pressor responses of pithed rats to selective agonists (Bentley *et al.*, 1977; Docherty & McGrath, 1980; Van Meel *et al.*, 1980). PE and B-HT 920 are relatively specific activators of α_1- and α_2-adrenoceptors, respectively, while NA, the naturally occurring neurotransmitter, is a non-specific agonist.

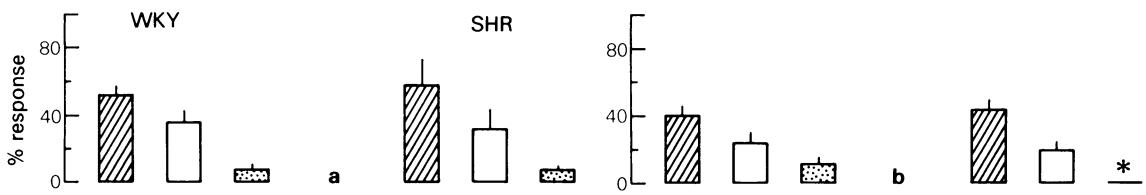

Figure 7 Responses to adrenoceptor agonists by the carotid arterial strip suspended in calcium-free solution (30 min). The hatched columns represent responses to phenylephrine, the open columns responses to noradrenaline and the stippled columns responses to B-HT 920. Panels (a) and (b) are responses of carotid strips from 15–17 weeks and 5–7 weeks old rats respectively, to concentrations of agonists eliciting maximum response in normal physiological solution. Responses are % of control in normal physiological solution. Each column represents an average of at least six observations; vertical lines denote s.e.mean. Asterisks denote responses which were 5% or less in magnitude relative to control responses.

However, confirmation of this classification under *in vitro* conditions has proved elusive. The high affinity of clonidine, as judged by pharmacological and radioligand binding studies led Ruffolo *et al.* (1980) and Weiss *et al.* (1983) to conclude that the rat aorta and tail artery contained α_2-adrenoceptors. Other studies with rat aortic strips concluded that it contained α_2-adrenoceptors although more than one subclass of α_1-adrenoceptors may be present (Digges & Summers, 1983; Randriantsoa *et al.*, 1981; Beckenringh *et al.*, 1983). Thus, in view of the difficulty of demonstrating the presence of the two subtypes of α-adrenoceptors *in vitro*, it may be premature to attribute with certainty the responses of isolated aortic and carotid strips in this study to the activation of either α_1 or α_2-adrenoceptors.

Nonetheless, distinction between the three agonists can be made on the basis of their mobilization of calcium. Maximum responses of tissue preparations from WKY rats of both age groups showed a similar order of sensitivity (B-HT 920 > NA > PE) to inhibition by D600 (Figures 1–4). The maximum response to B-HT 920 showed a significantly greater decline than those induced by either NA or PE when elicited in calcium-free solutions (Figures 6 and 7). These results demonstrate a strong dependence on extracellular calcium by responses to B-HT 920, in contrast to the maximum response to PE where dependence on extracellular calcium is minimum. NA-induced responses occupy an intermediate position with respect to their dependence on extracellular calcium. Similar conclusions have been reached by others on the basis of differential effects of calcium antagonists on the pressor responses to α_1- and α_2-adrenoceptor agonists and on the contractile responses of isolated vascular tissues of the rat (Van Meel *et al.*, 1981b; Godfraind *et al.*, 1982 Van Meel *et al.*, 1983).

The distinction between responses to the three adrenoceptor agonists, in terms of calcium mobilization, was considerably diminished when submaximal responses are compared (Figure 5). Responses to B-HT 920 continue to be more sensitive to inhibition than responses to NA or PE but no significant differences are seen in the inhibition of the two latter agonists (Figure 5; WKY strips). The altered sensitivity to D600-induced inhibition is suggested by a simple, if imprecise, comparison of the inhibitory potencies of D600 in the two experimental groups (Figures 1, 3 and 5). The concentration of D600 required to cause 50% inhibition increased approximately 2 fold when comparing maximum with submaximal responses to B-HT 920 in aortae from old rats and 10 fold when the comparison was made in young rats. The shifts in potency were approximately the same for NA-induced responses but, in the case of PE-induced responses, large (> 1000 fold) increases in potency were seen with submaximal responses. Furthermore, when responses to approximately equieffective concentrations of the agonists are compared, i.e. ED_{50} concentration of PE (Figure 5) with those of maximum response to B-HT 920 (Figures 1 and 3), it is clear that differences in the sensitivity of these responses to D600 are absent. Similar findings have been reported by Van Breemen *et al.* (1981) who studied the inhibition by diltiazem of calcium influx accompanying contractile responses of rabbit aorta to various concentrations of NA. Although the maximum response to NA is markedly resistant to inhibition these authors found a large (> 10, 000 fold) increase in sensitivity to diltiazem with lower concentrations of NA. If such large shifts in the potency of D600 are indicative of the contribution made by calcium influx through D600-sensitive channels towards an observed response, then it could be concluded that mobilization of extracellular calcium by low concentrations of full agonists such as PE or NA is essentially similar to that employed by agonists such as B-HT 920 which elicit responses of smaller magnitude. However, higher concentrations of full agonists show a shift to D600-insensitive calcium sources or mechanisms of calcium translocation in contrast to the responses of B-HT 920 whose mobilization of calcium remains qualitatively unchanged throughout its range of effective concentrations. The behaviour of B-HT 920 responses seen in these experiments resembles that of K^+-induced responses in their approximate equisensivity to inhibition by D600 (Nghiem *et al.*, 1982b).

In principle, several explanations and combinations thereof may underlie both the differential sensitivity of PE-, NA- and B-HT 920-induced maximum responses to D600 (and other calcium entry blockers) and the enhanced sensitivity to inhibition seen when responses to submaximal concentrations of full agonists are determined. It is possible that maximal responses to PE use a relatively larger proportion of intracellular (and D600-insensitive) sources of cacium relative to submaximal responses. If contractile responses in CFS reflect the degree of mobilization of intracellular calcium, then our observations on PE-induced responses clearly support this explanation. A larger proportion (approximately 80%; Figure 6a and b) of maximal PE-induced responses is retained in CFS in contrast to the smaller (approximately 40%; Figure 6c and d) retention of submaximal responses. Alternatively, the sensitivity of calcium channels to antagonists may vary with the degree of their activation by agonists, such that full activation by high concentrations of the agonist might render them insensitive to D600, whereas partial activation by low concentrations of full agonists might be accompanied by sensitivity to inhibition by calcium antagonists (Cauvin *et al.*, 1983).

It was expected that calcium utilization by tissues from SHR would be different because of an altered distribution and/or utilization of the two subtypes of α-adrenoceptors (Gheyouche *et al.*, 1980; Hicks & Nahorski, 1981). Comparison of responses to the relatively specific agonists B-HT 920 and PE, in tissues from SHR and WKY rats showed no differences in their inhibition by D600. Maximum responses to NA of SHR tissues (Figures 1 and 2) showed decreased inhibition by low concentrations of D600 while submaximal responses in SHR aortae showed reduced maximal inhibition (Figure 5) when compared to WKY tissues. These findings indicate an altered D600-calcium interaction in SHR that is peculiar to NA-induced responses. It is noteworthy, also, that the above changes in D600 effect were seen only in tissues from older rats which would suggest that these changes accompany the development of hypertension and are not intrinsic to this strain of rats.

This work was supported by a grant from the National Institutes of Health (HL 16003). Additional support from the Miles Institute for Pre-clinical Pharmacology is gratefully acknowledged.

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(*Received December 6, 1983.*
Revised March 7, 1984.)