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Interaction between α_{1B}- and other α_1- and α_2-adrenoceptors in producing contractions of mouse spleen

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Abstract

We have investigated the interaction of α_1- and α_2-adrenoceptor subtypes in producing isometric contractions to NA in mouse whole spleen. The α_1-adrenoceptor antagonist prazosin (10^{-8}M) or the α_2-adrenoceptor antagonist yohimbine (10^{-6}M) alone produced only small shifts in NA potency in wild type (WT) mice, but the combination produced a large shift in NA potency. In spleen from $\alpha_{1A/D}$-KO mice, the effects of prazosin and the combination of prazosin and yohimbine were similar to their effects in WT mice. Hence, in $\alpha_{1A/D}$-KO mice, in which the only α_1-adrenoceptor present is the α_{1B}-adrenoceptor, prazosin still antagonized contractions to NA. The α_{1A}-adrenoceptor antagonist RS100329 ($3x10^{-9}$M) produced significant shifts in the effects of higher concentrations of NA (EC_{50} and EC_{75} levels) and the α_{1D}-adrenoceptor antagonist BMY7378 ($3x10^{-8}$M) produced significant shifts in the effects of lower concentrations of NA (EC_{25} and EC_{50} levels). The effects of BMY7378 and RS00329 demonstrate α_{1D}-adrenoceptor and α_{1A}-adrenoceptor components, and suggest that the α_{1B}-adrenoceptor interacts with an α_{1D}-adrenoceptor, and to a lesser extent an α_{1A}-adrenoceptor, at low, and an α_{1A}-adrenoceptor at high, NA concentrations. This study demonstrates the complex interaction between α_1- and α_2-adrenoceptor subtypes in producing contractions of mouse spleen and may have general implications for α-adrenoceptor mediated control of smooth muscle.

Key words α_{1A}-adrenoceptors; α_{1B}-adrenoceptors; α_{1D}-adrenoceptors; splenic contraction; mouse spleen

1. INTRODUCTION

The spleen is a relatively understudied organ but yet is of interest both in terms of cardiovascular function and in terms of adrenergic control. The spleen acts as a reservoir particularly for platelets and erythrocytes, and contraction of the human spleen increases the circulating platelet volume and platelet count and increases erythrocytes in apnoea.[1,2] The human spleen contracts to sympathetic stimulation, and sympathetic activation for the fight or flight response may result in contraction of the spleen during exercise, in diving and hypoxia.[3-6] Contraction of the spleen to mobilise platelets may potentially be exploited as a therapeutic target to treat hypersplenism in portal hypertension.

However, the sympathetic control of the spleen is also of interest in terms of adrenergic responses. Smooth muscle contractions commonly involve predominantly α_{1A}- or α_{1D}-adrenoceptors, yet the spleen appears to be unusual in this respect.[7] In pharmacological studies using relatively non-selective antagonists, predominantly α_{1B}-adrenoceptor-mediated contractions have been reported in rat and mouse spleen.[8-12] Indeed, adrenergic contractions of the spleen from rat, guinea pig or mouse have often been used as a functional α_{1B}-adrenoceptor screen for test drugs.[13-15] In addition, contractions of rat spleen, and presumably mouse spleen, to noradrenaline (NA) resistant to prazosin involve α_2-adrenoceptors.[16] Recently, we have shown that contractions of rat spleen to NA involve predominantly α_2-adrenoceptors and a receptor that is probably an α_{1B}-adrenoceptor, with a lesser role for α_{1A}-adrenoceptors.[17]

Currently, there is no widely trusted selective antagonist for the α_{1B}-adrenoceptor subtype.[18] AH11110A was found not to be α_{1B}-adrenoceptor selective when comparing rat spleen and vas deferens in functional studies.[13] Chloroethylclonidine (CEC) is an effective non-competitive antagonist of contractions to NA in rat spleen, but also binds non-competitively to α_{2A}-adrenoceptors.[8,9,19,20] Stam et al. reported that the putative α_{1B}-adrenoceptor selective antagonist cyclazosin was potent and

produced relatively parallel shifts in the potency of phenylephrine (Phe) in rat spleen, but produced only small shifts that were not concentration-dependent against NA in mouse spleen.[21,22] Hence, in the absence of a reliable selective α_{1B}-adrenoceptor antagonist, it cannot be definitively concluded that the major α_1-adrenoceptor in spleen is an α_{1B}-adrenoceptor. However, this problem can be studied by gene knock-out (KO) technology in the mouse.

The objectives of this study were to investigate the interaction of α_1- and α_2-adrenoceptor subtypes in producing isometric contractions in mouse spleen and how NA selectively interacts with subtypes. We have employed WT and $\alpha_{1A/D}$-adrenoceptor KO ($\alpha_{1A/D}$-KO) mice to study α_{1B}-adrenoceptor function, and selective antagonists to study α_{1A}-and α_{1D}-adrenoceptor function in the mouse spleen.

Some of these results have been published in abstract form.[23]

2. METHODS

2.1. General

$\alpha_{1A/D}$-KO mice were generated by cross-breeding single knockout mice of the α_{1A}-adrenoceptor (α_{1A}-KO) and α_{1D}-adrenoceptor (α_{1D}-KO) KO genotypes, as described previously.[9] Mice were of C57BL6/J background and were bred and genotyped at the University of Glasgow. All other studies were carried out using male C57BL6/J wild type (WT) mice obtained from *Envigo*, UK.

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[24] All studies were approved by the Department of Health/Health Products Regulatory Agency (HPRA) in Ireland (licenses B100/762, AE 19127/I185) and by the RCSI Research Ethics Committee (REC1284), and comply with EU Directive 2010/63/EU. The animals were housed in a controlled environment with a 12-hour light, 12-hour dark cycle and were fed a standard diet.

2.2. Mouse Spleen

This study employed 42 male WT, 8 male KO and 18 female WT, adult mice, 2-3 months old. Mice were euthanized by overdose of CO_2 and cervical dislocation. The abdomen was opened and the whole spleen was carefully removed, separated from extra-splenic loose connective tissue, and transferred to a petri dish where threads were carefully inserted top and bottom. Spleens were attached to a fixed rod and to myograph transducers under 0.5 g tension in organ baths[9,10] with Krebs-Henseleit solution of the following composition (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 11.1, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.0, EDTA 0.03, ascorbic acid 0.28. In addition, the NA transporter (NET) inhibitor cocaine (3 μM) was present.

The spleens were gassed with 5% CO_2 in O_2 and allowed to equilibrate for 30 minutes at 37°C. The spleens were then contracted with NA (10 μM). The bathing fluid was then changed every 15 minutes for the next hour. Following this, the first (curve 1: control) cumulative concentration response curve to NA was carried out in 0.5 log unit increments in concentrations from 1 nM to 1000 μM, or until a clear maximum was reached. This first, control, curve was not used in calculations. The bathing fluid was changed every 15 minutes for the next hour. This was followed by 1 h exposure to antagonist or vehicle, again changing bathing fluid every 15 min, at 15, 30 and 45 min, and replacing antagonist or vehicle. At 60 min, bathing fluid was not changed and a second (curve 2: test 1) concentration response curve to NA was carried out. A third concentration-response curve (curve 3: test 2) was used for all spleens from KO animals, to maximize results, given the limited number of KO mice available. Hence, for consistency, in all studies, prazosin/yohimbine or BMY7378/RS100329 interactions were obtained in curve 3 (test 2) with the relevant vehicle. However, potencies of NA (pEC_{50}) for vehicle experiments in test 1 and test 2 were almost identical in WT (7.23±0.08, n=6 & 7.17±0.09, n=6) and KO (7.25±0.06, n=4 & 7.20±0.12, n=3), as were maximum responses in WT (0.11±0.02g, n=6 & 0.11±0.02g, n=6) and KO (0.14±0.02g, n=4 & 0.12±0.02g, n=3). Hence, for simplicity, vehicle (test 1) was used throughout.

Splenic contractions to NA were measured after 1 h exposure to, and in the continuing presence of, test drug or vehicle. Antagonist potency was assessed in terms of the ability of antagonists to produce a significant shift in NA potency from response in the presence of vehicle at the EC_{25}, EC_{50} or EC_{75} levels. Antagonist potency was calculated only where there was a significant shift in agonist potency. Antagonist potency was expressed as an apparent dissociation constant pK_B from the equation K_B =[B]/(DR-1), where [B] is the concentration of antagonist and DR is the agonist dose-ratio produced by the antagonist, calculated from NA potency in the presence of antagonist in each individual experiment as compared to the mean NA potency in vehicle experiments.

Experiments were carried out in three groups with different vehicle experiments. Group 1 was the initial study of the effects of prazosin and yohimbine in spleen from male WT and KO mice (Figures 2 & 3). Group 2 was the follow-up study of the effects of the subtype selective antagonists BMY7378 and RS100329 in spleen from male WT mice (Figure 5). Group 3 was a separate study on female WT mice (Figure 4).

At the end of each experiment, the spleens were removed, placed on paper tissue to partially dry, and weighed. This is the wet weight of spleen. Wet weight of spleen was also related to weight of animal as % of body weight**.** These data are shown in the Results section.

2.3. Chemicals

BMY7378 (8-[2-(4-(2-methoxyphenyl) piperazin-1-yl)ethyl]-8-azaspiro[4,5]decane-7,9-dione) (Tocris Bioscience, Bristol, UK); noradrenaline bitartrate (Sigma Aldrich, Wicklow, Ireland); RS100329 (5-methyl-3-[3-[4-[2-(2,2,2,-trifluoroethoxy)phenyl]-1-piperazinyl]propyl]-2,4-(1H)-pyrimidinedione)(Tocris Bioscience); prazosin hydrochloride (Sigma Aldrich), yohimbine hydrochloride (Sigma Aldrich). Drug stocks were dissolved in distilled water.

2.4. Statistical analysis

Except for studies employing KO mice, group sizes were planned as n=6. The results are expressed as mean ± SE of mean. NA potency at producing splenic contractions was expressed as a pEC_x (–log EC_x, where x is 25%, 50% or 75% of maximum response). pEC_x values were obtained by non-linear regression using GraphPad Prism 5 for MacIntosh.

Differences within WT or KO groups were compared by one-way analysis of Variance (ANOVA) employing GraphPad Prism for Macintosh and, if there were no significant differences in variances, by Bartlett's test, this was followed by the Bonferroni test (comparison between groups) and Dunnett's test (comparison with vehicle), as appropriate. Differences between WT and KO, and the interaction between pairs of antagonists were assessed by two-way ANOVA followed by a Bonferroni test, as appropriate. *P* value of less than 0.05 was considered to be significant. Graphical analysis was carried out using GraphPad Prism for Macintosh.

3. RESULTS

3.1. General

Mouse weights were as follows: male WT 25±1 g (n=31), male KO 26±1 g (n=8), female WT 18±1 g (n=13)(body weight significantly lower for female WT mice, *P* <0.001), and spleen wet weights were as follows: male WT 68±3 mg (n=31), male KO 74±4 mg (n=7), female WT 55±3 mg (n=13) (spleen weight significantly lower for female WT mice, *P* <0.05). Wet weight of spleen was also expressed as a % of body weight, but there were no significant differences, at 0.27±0.01% (n=31), 0.28±0.01% (n=7) and 0.29±0.01% (n=9), of body weight for male WT, male KO and female WT, respectively.

Concentration response curves were carried out to NA in the presence of vehicle, antagonist or antagonist combination. Figure 1a shows typical vehicle responses obtained to NA in spleens from male WT, female WT and male α_{1A}-/α_{1D}-

adrenoceptor KO mice, which were very similar (Figure 1a). Figure 1b shows typical responses obtained to NA in tissues from male WT mice, in the presence of prazosin (10^{-8}M), yohimbine (10^{-6}M) or vehicle, and the shifts in the NA concentration response curve produced by these antagonists as compared to vehicle (Figure 1b).

Antagonists employed did not significantly affect the maximum response to NA as compared to the response in the presence of the respective vehicle (comparisons within groups) (one-way ANOVA, NS), and there were no significant differences between male WT and KO animals in effects of antagonists on maximum response (see Table 1). However, contractions were significantly larger in spleens from female WT mice than from male WT or KO mice (one-way ANOVA, P <0.001) (Table 1).

3.2. Male WT mice: prazosin and yohimbine

Both prazosin (10^{-8}M) and yohimbine (10^{-6}M) significantly inhibited components of the contraction to NA (Figure 2). Yohimbine produced an approximate parallel shift in NA potency, but prazosin produced a larger shift in the response to higher concentrations of NA, making the NA concentration response curve biphasic (Figure 2 & Table 2).

The combination of prazosin and yohimbine produced a further shift in NA potency, significantly more than the shift produced by prazosin or yohimbine alone (Figure 2 & Table 2). When the effects of prazosin, yohimbine and the combination of the two were analysed by two-way ANOVA at the NA EC_{50} level, there was no significant interaction overall and prazosin ($F_{1,20}$ = 101.0, P<0.001) and yohimbine ($F_{1,20}$ = 169.0, P <0.001) produced significant effects (see Table 2). Similarly, at the NA EC_{25} level, prazosin ($F_{1,20}$ = 87.2, P<0.001) and yohimbine ($F_{1,20}$ = 202.7, P<0.001) produced significant effects (see Table 2). However, there was a significant interaction overall at the EC_{75} levels ($F_{1,20}$ = 2.99, P <0.05) (two-way ANOVA) so that prazosin affected the response to yohimbine, or vice versa (see Table 3). Hence,

this may suggest that effects of prazosin and yohimbine were additive by actions at different receptors, presumably α_1- and α_2-adrenoceptors, at the NA EC_{25} and EC_{50} levels, but that at the EC_{75} level, one receptor is dominant, presumably the α_1-adrenoceptor (see Figure 2).

3.3. Male $\alpha_{1A/D}$-KO mice: prazosin and yohimbine

The effects of prazosin (10^{-8}M) and of the combination of prazosin and yohimbine in male KO mice were similar to their effects in male WT mice (Fig. 3, compare Fig. 2). In particular, prazosin significantly shifted the NA concentration-response curve (CRC) in a similar way in male WT and in male $\alpha_{1A/D}$-KO mice, both in terms of degree of shift in potency and in change of shape of the CRC (Table 2, compare figures 2 & 3). In addition, the combination of prazosin and yohimbine also produced a large shift in the response to low concentrations of NA in KO as in WT (compare figures 2 & 3). Two-way ANOVA showed no significant differences between male WT and KO mice in the potency of NA at the EC_{50} in the presence of vehicle or antagonists ($F_{1,23}$ = 0.448, NS)(see Table 2).

Shifts in NA potency produced by prazosin and the combination of prazosin and yohimbine were also compared between groups of mice at the EC_{25}, EC_{50} and EC_{75} levels. For the combination of prazosin and yohimbine, there were significant differences between groups only at the EC_{75} level: the shifts at the EC_{75} level were 1.86±0.11 (n=6) and 2.73±0.22 (n=3) (-log M) for male WT and male KO, respectively. The shift in NA potency produced by the combination of prazosin and yohimbine at the EC_{75} level was significantly greater in male KO than in male WT (*P* <0.01).

3.4. Female WT mice: prazosin and yohimbine

Prazosin (10^{-8}M), yohimbine (10^{-6}M) and the combination of prazosin and yohimbine all inhibited components of the contraction to NA in spleens from female WT in a similar way to the effects in spleen from male WT mice (Table 2, compare figures 2

& 4). Prazosin produced a larger shift in the response to high concentrations of NA (Figure 4 and Table 2). The combination of prazosin and yohimbine produced a large shift in NA potency in spleen from female, as was the case for spleen from male, WT mice (compare Figs. 2 & 4). When the effects of prazosin and yohimbine at the NA EC_{50} level were analysed by two-way ANOVA, there was no significant Interaction overall ($F_{1,21}$ = 3.92, NS), so that prazosin did not affect the response to yohimbine, and vice versa. Prazosin ($F_{1,21}$ = 214.0 *P* <0.001) and yohimbine ($F_{1,21}$ = 494.0, *P* <0.001) produced significant effects (see Table 2). Hence, this suggests that effects of prazosin and yohimbine were additive by actions at different receptors, presumably α_1- and α_2-adrenoceptors.

3.5. Prazosin and yohimbine potencies

Prazosin shifted the potency of NA at the EC_{50} level with pK_B values (-log M) of 8.95±0.12, 8.72±0.16 and 8.83±0.05 in tissues from male WT, male KO and female WT mice, respectively. Prazosin shifted the potency of NA at the EC_{75} level with pK_B values (-log M) of 9.32±0.16, 9.09±0.17 and 9.00±0.12 in tissues from male WT, male KO and female WT mice, respectively. Yohimbine shifted the potency of NA at the EC_{50} level with pK_B values (-log M) of 7.24±0.13 and 7.33± 0.08 in tissues from male and female WT mice, respectively (no significant difference).

3.6. Male WT mice: RS100329 and BMY7378

BMY7378 produced a significant shift in the response to lower concentrations of NA at the EC_{25} and the EC_{50} but not the EC_{75} level (one-way ANOVA; Table 3 & Figure 5). RS100329 (10^{-9}M) did not significantly affect responses to NA (Table 3), but RS100329 ($3x10^{-9}$M) produced small but significant shifts particularly in the effects of higher concentrations of NA, making the curve biphasic, with a significant shift in the response to NA at the EC_{50} and the EC_{75} level (one-way ANOVA; Table 3 & Figure 5). However, BMY7378 ($3x10^{-8}$M) and RS100329 ($3x10^{-9}$M) significantly shifted the

potency of NA with pK_B values (-log M) of 7.90±0.13 (EC_{50} level) and 9.28±0.22 (EC_{75} level), respectively, in tissues from male WT mice (see Table 3 & Figure 5). In male WT mice, the combination of BMY7378 ($3x10^{-8}$M) and RS100329 ($3x10^{-9}$M) behaved like prazosin, causing a biphasic shift in the NA CRC (Figure 5).

When the effects of BMY7378 and RS100329 and the combination were analysed by two-way ANOVA (excluding RS100329 (10^{-9}M), there was no significant Interaction overall, so that BMY7378 did not affect the response to RS100329, and vice versa. Hence, this suggests that effects of BMY7378 and RS100329 were additive by actions at different receptors presumably α_{1D}- and α_{1A}-adrenoceptors (Fig. 6). At the NA EC_{25} level, BMY7378 ($3x10^{-8}$M) produced a shift (log M) in NA potency of 0.33±0.09 (n=6); RS100329 ($3x10^{-9}$M) produced a similar shift but this did not reach significance (Figure 5). At the NA EC_{50} level, BMY7378 ($3x10^{-8}$M) and RS100329 ($3x10^{-9}$M) produced shifts in NA potency of 0.32±0.10 and 0.39±0.09, respectively (n=6). At the NA EC_{75} level, RS100329 ($3x10^{-9}$M) produced a shift in NA potency of 0.76±0.22 (n=6); BMY7378 ($3x10^{-8}$M) had no significant effect.

The combination of BMY7378 ($3x10^{-8}$M) and RS100329 ($3x10^{-9}$M) produced shifts of NA potency at EC_{25}, EC_{50} and EC_{75} levels (log M) of 0.98±0.09, 0.92±0.12 and 1.18±0.15, respectively (n=6). By comparison, prazosin (10^{-8}M) produced shifts of NA potency at EC_{25}, EC_{50} and EC_{75} levels (log M) of 0.82±0.20, 0.94±0.16 and 1.28±0.22, respectively (n=6) (no significant differences from effects of combination of BMY7378 & RS100329). Hence, the combination of BMY7378 and RS100329 behaved like prazosin.

4. DISCUSSION

We have investigated the α_1-adrenoceptors involved in contractions of mouse spleen, and the interactions between them. First of all, the results in the mouse spleen will be compared with findings from rat spleen. In rat spleen, contractions to adrenergic agonists have been reported to be mediated predominantly by an α_{1B}-adrenoceptor, although α_2-adrenoceptors are also involved in this response.[8-10,16]

We have previously shown that contractions of rat spleen to adrenergic agonists involve α_2- and probably α_{1B}-adrenoceptors, with a lesser role for α_{1A}-adrenoceptors.[17] However, potency of NA in the present study of mouse spleen was a log unit or more higher than its potency in rat spleen.[9,10,25] This may suggest that the mouse spleen has an additional α_1-adrenoceptor, the α_{1D}-adrenoceptor, at which NA has high potency, as will be confirmed by the use of BMY7378.[7] Indeed, mRNA for all 3 subtypes of α_1-adrenoceptor is expressed in mouse spleen.[26,27]

In studies of subtypes of α_1-adrenoceptor, a major problem is selectivity of antagonists, particularly for α_{1B}-adrenoceptors, but this problem can be overcome by use of receptor KO technology.[7] We compared male and female WT and male $\alpha_{1A/D}$-KO mice in terms of prazosin and yohimbine potency. Vehicle responses to NA were very similar in terms of potency and curve shape between male and female WT and male KO mice. This demonstrates that deletion of α_{1A}- and α_{1D}-adrenoceptors did not affect the overall responsiveness of the spleen to NA. In the KO mice, in which the only α_1-adrenoceptor present is the α_{1B}-adrenoceptor, prazosin behaved as in WT, causing responses to NA to become biphasic, producing a small shift in the effects of low concentrations of NA, but a large shift in the response to high concentrations of NA. Since prazosin at a concentration of 10^{-8}M should not affect α_2-adrenoceptors (prazosin above 10^{-7}M may block α_2-adrenoceptors:[28]), the only possibility in the KO mouse is that α_{1B}-adrenoceptors have two distinct actions (or phases of action) at low and high concentrations of NA. It is clear that, when yohimbine is combined with prazosin, the major change is a large shift in the effects of low to medium concentrations of NA, and this is again most clearly seen in the KO studies. This may suggest that at low concentrations of NA, the α_2-adrenoceptor is dominant but with an α_{1B}-adrenoceptor mediated component or that the α_{1B}-adrenoceptor facilitates the α_2-adrenoceptor. α_1-Adrenoceptors are absent in heart from double α_{1A}/α_{1B}-KO mice, with no evidence for the presence of α_{1D}-adrenoceptors, so that deleted adrenoceptors are not necessarily replaced by other adrenoceptors in KO mice,[31] and the α_{1B}-adrenoceptor is expressed in WT spleen.[26]

It is important to study possible sex differences in responses and as a result the prazosin and yohimbine experiments were also carried out in female mice.[30] There were no sex differences in the response to NA in vehicle experiments or in the effects of prazosin or yohimbine, alone or in combination. However, the maximum response to NA, but not the potency, was significantly greater in spleen from female mice. The differences in maximum response between male and female may be methodological rather that real: female experiments were carried out in a separate study. However, α_{1A}-adrenoceptor expression is greater in renal blood vessels from female than from male rats,[31] so that an increased expression of α_1-adrenoceptors in female mice cannot be ruled out in our studies. Although we did not study subtype selective antagonists in spleen from female mice, the unusual biphasic effects of prazosin found in spleen from both male and female mice indicates that the two components of the contraction to NA were similar in both sexes.

The pK_B for yohimbine of 7.24 (male) and 7.33 (female) in WT mice compares with its potency (pK_B) of 7.23-7.37 at inhibiting contractions to NA at postjunctional α_2-adrenoceptors in the human saphenous vein and 7.50 at rat prejunctional α_2-adrenoceptors.[32-34] Hence, the potency of yohimbine is consistent with α_2-adrenoceptor actions. The high potency of prazosin (pK_B of 9.32 in male and 9.00 in female) against high concentrations of NA (EC_{75} level) is inconsistent with actions at a homogeneous population of α_{1A}-adrenoceptor, at which prazosin has low potency with a pK_B value of 8.40 (average of reported values).[7] Indeed, the pK_B of prazosin at α_{1B}-adrenoceptor was 9.09 in $\alpha_{1A/D}$-KO mice. Hence, prazosin probably acts against high concentrations of NA largely by blocking α_{1B}-adrenoceptors (since α_{1D}-adrenoceptors are activated by low concentrations of NA). However, α_{1A}-adrenoceptors are presumably also involved in contractions to high concentrations of NA, but these contractions are modulated by α_{1B}-adrenoceptors. Low apparent potency of prazosin (8.72) against low concentrations of NA in male KO mice may be simply due to the additional involvement of an α_2-adrenoceptor in this response, as NA has high potency at α_{1B}-adrenoceptors. It should be noted that the greater shift in the NA EC_{75} produced by the combination of prazosin and yohimbine in spleen from male KO mice could be explained by the absence of α_{1A}-adrenoceptors at

which prazosin has low potency. However, such a difference between male WT and KO mice was not seen for prazosin alone, probably due to the presence of α_2-adrenoceptors.

In the absence of a reliable selective α_{1B}-adrenoceptor antagonist, α_{1B}-adrenoceptors can be identifed by the use of three antagonists, prazosin (non-selective), RS100329 (α_{1A}-adrenoceptor selective) and BMY7378 (α_{1D}-adrenoceptor selective). The α_{1B}-adrenoceptor can be identified in terms of high potency of prazosin and low potency of RS100329 and BMY7378. RS100329 is a potent α_{1A}-adrenoceptor antagonist, with pK_B of 9.84 in rat vas deferens (average of reported values).[7] We have previously reported that RS100329 ($3x10^{-9}$M) abolished α_{1A}-adrenoceptor mediated contractions to NA (1μM) in rat vas deferens.[35] Hence, any small effects of RS100329 ($3x10^{-9}$M) seen against low concentrations of NA (0.03–0.1μM) in mouse spleen in the present study may indeed involve α_{1A}-adrenoceptors but it is possible that they involve other α_1-adrenoceptor subtypes. RS100329 has low potency at α_1-adrenoceptors in a number of tissues, with a pK_B value of around 8.15 (average of reported values), indicating actions at α_{1B}- or α_{1D}-adrenoceptors.[7] RS100329 ($3x10^{-9}$M) significantly shifted responses to higher concentrations of NA, with a pK_B in the present study of greater than 9.0. BMY7378 is a potent α_{1D}-adrenoceptor antagonist, with pK_B of 8.60 in rat aorta (average of reported values).[7] BMY7378 has low potency at α_1-adrenoceptors in a number of tissues, with a pK_B value of 6.55 (average of reported values).[7] BMY7378 ($3x10^{-8}$ M) significantly shifted responses to lower concentrations of NA, with a pK_B in the present study of around 8.0. Hence, potencies of RS100329 and BMY7378 in this study are consistent with their selectivities in previous studies. In a previous study of mouse spleen, BMY7378 had a pA_2 of 6.76 against contractions to NA, but the concentrations of BMY7378 chosen were $3x10^{-7}$M, 10^{-6}M and $3x10^{-6}$M, a concentration range in which BMY7378 may act at all subtypes of α_1-adrenoceptor, and lower concentrations of BMY were not studied.[12] In another study of mouse spleen, the α_1-adrenoceptor antagonist cyclazosin (0.1μM) produced a shift in potency of NA, particularly of high concentrations, presumably by actions at α_{1B}-adrenoceptors or a combination of α_{1D}- and α_{1A}-adrenoceptor, or all three.[21]

From this it can be seen that the α_1-adrenoceptor mediated contractions in mouse spleen have two components, a predominantly α_{1D}-adrenoceptor mediated response at low, and an α_{1A}-adrenoceptor mediated response at high concentrations of NA. Furthermore, studies in $\alpha_{1A/D}$-KO mice indicate that both of these components also involve α_{1B}-adrenoceptors. Hence, low concentrations of NA produce contractions involving (in addition to α_2-adrenoceptors) α_{1D}-adrenoceptors and α_{1B}-adrenoceptors, and high concentrations of NA produce contractions involving α_{1A}-adrenoceptors and α_{1B}-adrenoceptors (see Figure 6). This suggests that the α_{1B}-adrenoceptor may have a different mode of action from the other α_1-adrenoceptors, perhaps calcium sensitization involving Rho kinase as opposed to increased calcium levels.[36] Indeed, we have demonstrated that the Rho kinase inhibitor fasudil inhibits tonic but not phasic contractions in rat portal vein, and that these tonic contractions involve α_{1B}-adrenoceptors.[37] However, given the fact that the α_{1B}-adrenoceptor component occurs over a very wide range of concentrations of NA in the present study, NA may act at this receptor by biased agonism, acting by increasing calcium availability at low concentrations, and by calcium sensitization at higher concentrations (or vice versa). This can be seen as a form of receptor pleiotropy where subtypes of receptor interact in multiple combinations, linked to several different second messenger systems, to produce subtle differences in response. The receptors involved in contractions to NA in mouse spleen are summarized in Figure 6. Hence, it is suggested that the α_{1B}-adrenoceptor modulates contractions mediated by the other adrenoceptor subtypes: the α_{1A}-, α_{1D}- and α_2-adrenoceptors, in mouse spleen.

5. CONCLUSIONS

It is concluded that both α_2- and α_1-adrenoceptors mediate contractions of mouse spleen and that the major, but not exclusive, α_1-adrenoceptor involved is the α_{1B}-adrenoceptor, with smaller α_{1D}- and α_{1A}-adrenoceptor mediated components. Receptor subtypes interact to produce contractions.

CONFLICT OF INTEREST

The authors declare that they do not have a conflict of interest

AUTHOR CONTRIBUTIONS

All authors contributed to the acquisition, analysis or interpretation of data and in drafting the manuscript. JRD had primary role in drafting revised manuscript. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data are available on reasonable request from the authors.

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Table 1. Effects of vehicle or antagonists on maximum isometric contraction to noradrenaline (NA) in spleen from (a) male Wild Type (WT) and $\alpha_{1A/D}$-KO (KO) mice (Group 1), (b) male WT mice (Group 2) and (c) female WT mice, expressed as tension (T) in g. Abbreviations: BMY/RS: combination of BMY7378 and RS100329; Praz /Yoh: combination of prazosin and yohimbine. Values are mean ± SE, from n experiments, indicated in parenthesis. There were no significant differences between vehicle and antagonist (one-way ANOVA) or between male WT and KO (two-way ANOVA), although responses in female were significantly larger than in male WT in vehicle experiments and combining vehicle and antagonist experiments (0.18±0.01g, n=25 in female, 0.12±0.01g, n=24; *P* <0.001).

	Male WT T(g)	**Male KO T(g)**
(a) Group 1		
Vehicle	0.11±0.02g (6)	0.14±0.02(4)
Prazosin 10^{-8} M	0.12±0.03 (6)	0.11±0.02 (4)
Yohimbine 10^{-6} M	0.13±0.03 (6)	/
Praz 10^{-8} M/yoh 10^{-6} M	0.13 ± 0.02(6)	0.12±0.02 (3)
(b) Group 2		
Vehicle	0.12±0.02 (6)	
BMY7378 $3x10^{-8}$ M	0.10±0.02 (6)	
RS100329 10^{-9} M	0.10±0.01 (6)	
RS100329 $3x10^{-9}$ M	0.12±0.02 (6)	
BMY $3x10^{-8}$ M/RS $3x10^{-9}$ M	0.14±0.03 (6)	

	Female WT T (g)
(c) Group 3	
Vehicle	0.20±0.02 (6)
Prazosin 10^{-8} M	0.16±0.02 (6)
Yohimbine 10^{-6} M	0.20±0.02 (6)
Praz 10^{-8} M/yoh 10^{-6} M	0.17±0.02 (7)

Table 2. Effects of antagonists on potency (expressed as pEC_{25}, pEC_{50} and pEC_{75}) of noradrenaline (NA) at producing isometric contractions of spleen from (a) male Wild Type (WT) mice, (b) male $\alpha_{1A/D}$-KO (KO) mice and (c) female WT mice. Values are mean ± SE, and number of experiments are indicated in Figure 1. Abbreviations: Praz /Yoh: combination of Prazosin and Yohimbine. Asterisks denote significance of difference between NA potency in the presence of vehicle and the potency in the presence of the antagonist concentration (one-way ANOVA and Dunnett's multiple comparison test: * P<0.05; ** P< 0.01; *** P< 0.001). All antagonist/antagonist combinations produced a significant shift in NA potency. Crosses denote significance of difference between NA potency in the presence of combination of yohimbine and prazosin and the potency in the presence of either antagonist alone (one-way ANOVA and Bonferroni test: + P < 0.05).

	pEC_{25}	pEC_{50}	pEC_{75}
(a) Male WT			
Vehicle	7.99±0.12	7.23±0.08	6.46±0.12
Prazosin 10^{-8}M	7.18±0.13***	6.28±0.12***	5.18±0.15***
Yohimbine 10^{-6}M	6.65±0.12***	5.99±0.12***	5.32±0.12***
praz 10^{-8}M/yoh 10^{-6}M	5.41±0.05***+	4.96±0.06***+	4.55±0.06***+
(b) male KO			
vehicle	7.82±0.03	7.25±0.06	6.70±0.08
Prazosin 10^{-8}M	7.38±0.14*	6.52±0.16 **	5.45±0.38**
praz 10^{-8}M/yoh 10^{-6}M	5.04±0.16***+	4.53±0.07 ***+	4.05±0.08***+

(c) Female WT

Vehicle	7.79±0.08	7.18±0.08	6.57±0.11
Prazosin 10^{-8}M	7.06±0.05***	6.35±0.05***	5.52±0.11***
Yohimbine 10^{-6}M	6.56±0.12***	5.85±0.08***	5.13±0.14***
praz 10^{-8}M/yoh 10^{-6}M	5.24±0.06***+	4.76±0.05***+	4.29±0.5***+

Table 3. Effects of antagonists on potency of noradrenaline (NA) at producing isometric contractions in spleen from male wild type mice, expressed as pEC_{25}, pEC_{50} or pEC_{75} values. Abbreviations: BMY/RS: combination of BMY7378 and RS100329. Values are mean ± SE from 6 experiments. Asterisks denote potency of NA in presence of antagonist significantly different from NA potency in the vehicle experiments (one-way ANOVA and Dunnett's multiple comparison test: * $P < 0.05$; *** $P < 0.001$).

NA potency (-log M)	**pEC_{25}**	**pEC_{50}**	**pEC_{75}**
Group 2			
Vehicle	7.84±0.12	7.24±0.16	6.56±0.22
BMY7378 $3x10^{-8}$M	7.36±0.08*****	6.71±0.10*	6.11±0.11
RS100329 10^{-9}M	7.57±0.06	6.94±0.14	6.16±0.26
RS100329 $3x10^{-9}$M	7.55±0.17	6.71±0.12*	5.76±0.24*
BMY $3x10^{-8}$M/RS $3x10^{-9}$M	6.94±0.12***	6.21±0.09***	5.38 ± 0.15***

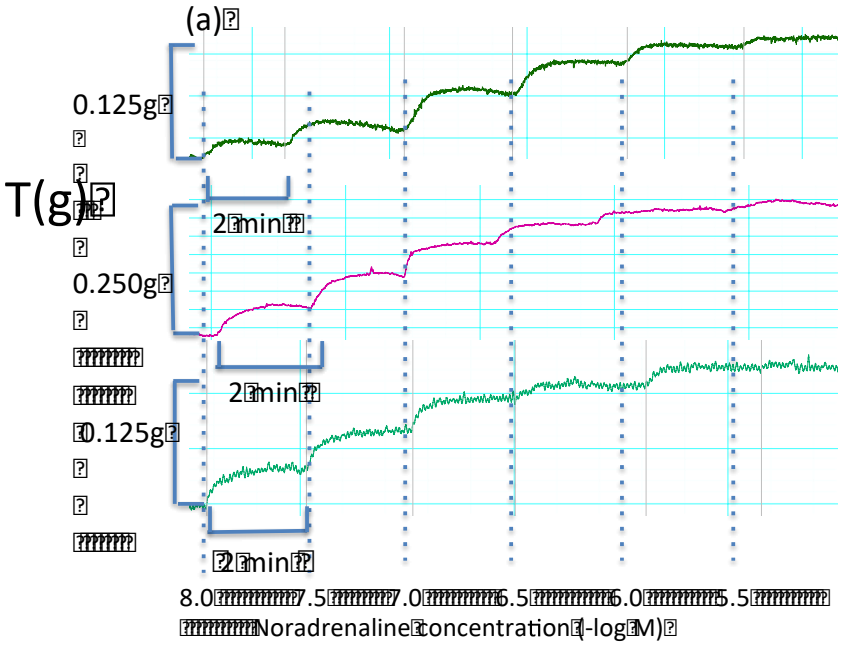

Figure 1(a)

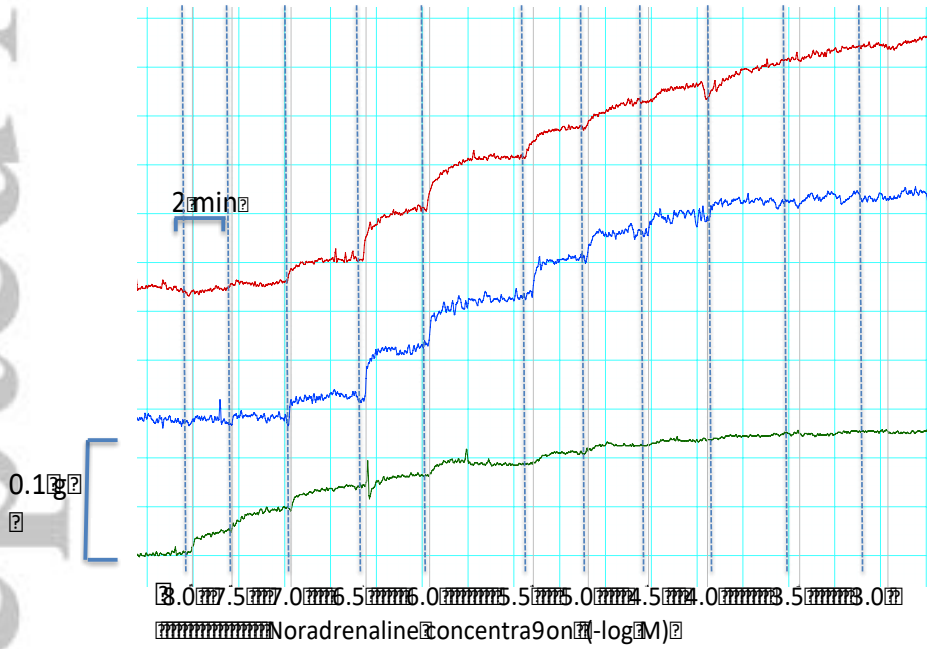

Figure 1(b)

Figure 1. Original recordings of concentration response curves obtained to NA in mouse spleen. (a) Responses to NA obtained in the presence of vehicle in spleens from male WT (top), female WT (middle) and male α_{1A}-/α_{1D}-adrenoceptor KO (bottom) mice. Traces were obtained from different experiments, so that isometric tension scales differ. Time scale of 2 min is indicated for each experiment, and individual time scales have been altered to allow approximate matching of NA administration. Note that responses were similar in spleens from male WT, female WT and male α_{1A}-/α_{1D}-KO mice. (b) Responses to NA obtained in spleens from male WT mice, in the presence of prazosin (10^{-8} M) (top), yohimbine (10^{-6} M)

(middle) and vehicle (bottom). Three experiments carried out in parallel have been superimposed employing the same isometric tension scale (0.1 g is indicated). Time scale of 2 min is also indicated, and increasing cumulative concentrations of NA were added as indicated. Note that responses to low concentrations of NA were shifted markedly by both prazosin and yohimbine, but that the response to NA required higher concentrations to reach a maximum in the presence of prazosin than yohimbine.

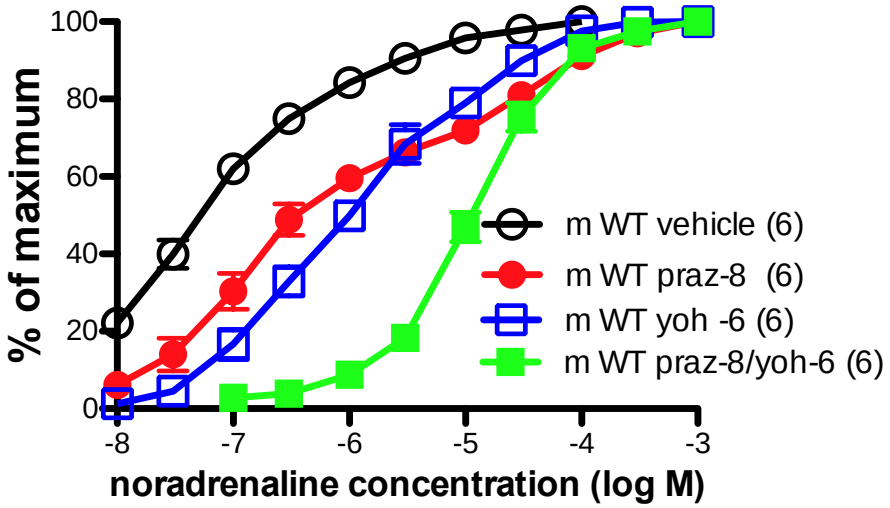

Figure 2. Effects of vehicle or antagonists on the isometric contractions produced by NA in spleens from male WT (m WT) mice. Responses are expressed as % of the maximum response obtained to NA in each individual experiment. Responses were obtained in the presence of vehicle (veh), prazosin 10^{-8}M (praz-8), yohimbine (10^{-6}M) (yoh-6) and the combination of prazosin (10^{-8}M)/yohimbine (10^{-6}M) (praz-8/yoh-6). Values are mean ± SE from 6 experiments. For statistical analysis, see Table 2 and Results.

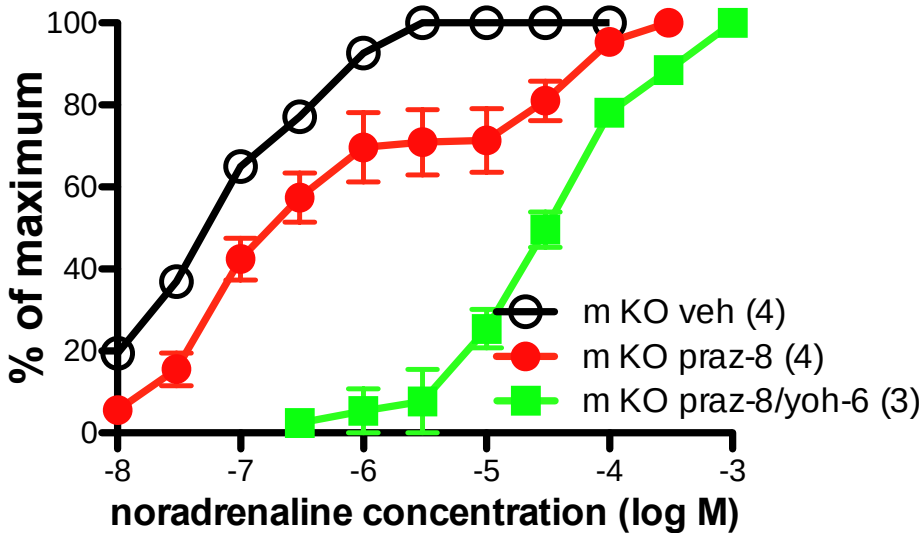

Figure 3. Effects of vehicle or antagonists on the isometric contractions produced by NA in spleens from male α_{1A}-/α_{1D}-KO (m KO) mice. Responses are expressed as % of the maximum response obtained to NA in each individual experiment. Responses were obtained in the presence of vehicle (veh), prazosin 10^{-8}M (praz-8) and the combination of prazosin (10^{-8} M)/yohimbine (10^{-6} M) (praz-8/yoh-6). Values are mean ± SE from 3-4 experiments. For statistical analysis, see Tables 2 & 3 and Results.

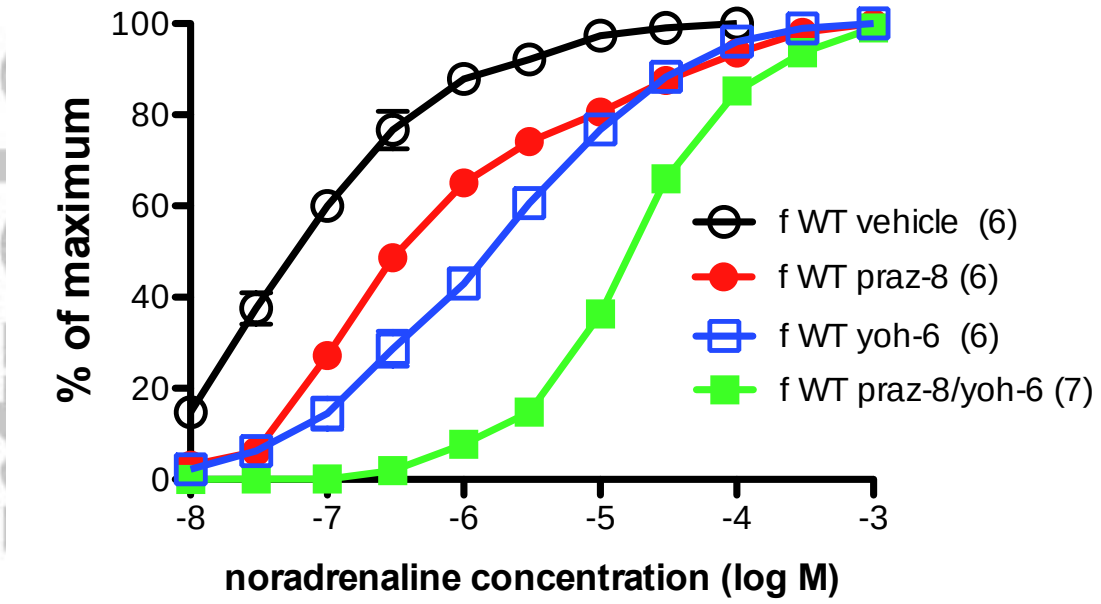

Figure 4. Effects of vehicle or antagonists on the isometric contractions produced by NA in spleens from female WT (f WT) mice. Responses are expressed as % of the maximum response obtained to NA in each individual experiment. Responses were obtained in the presence of vehicle (veh), prazosin 10^{-8} M (praz-8), yohimbine 10^{-6} M (yoh-6) and the combination of prazosin (10^{-8} M)/yohimbine (10^{-6} M) (praz-8/yoh-6). Values are mean ± SE from 6-7 experiments. For statistical analysis, see Table 2 and Results.

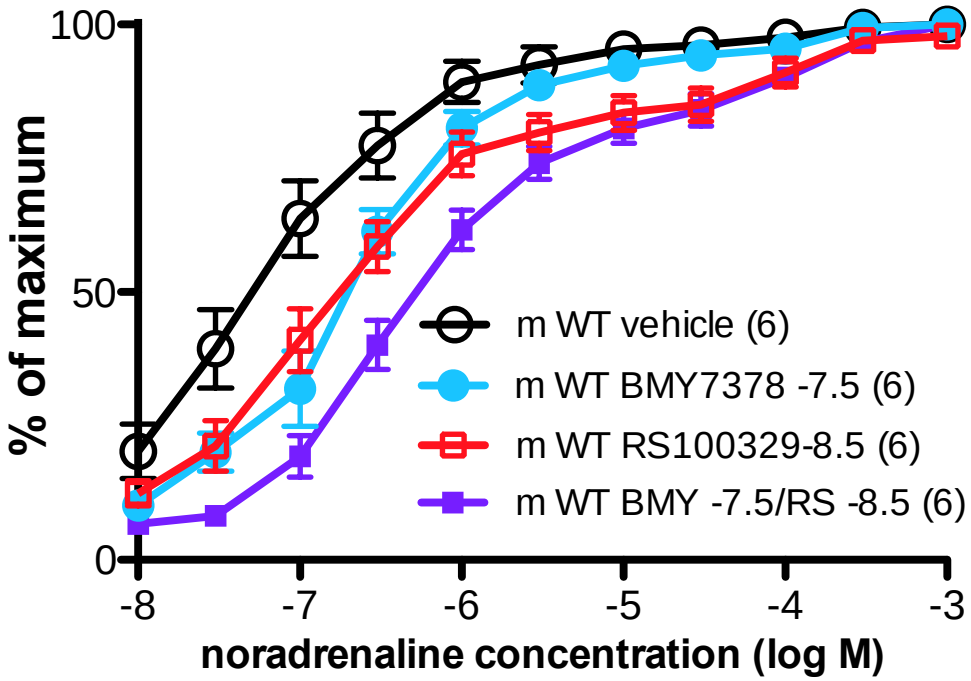

Figure 5. Effects of vehicle or antagonists on the isometric contractions produced by NA in spleens from male WT (m WT) mice. Responses are expressed as % of the maximum response obtained to NA in each individual experiment. Responses were obtained in the presence of vehicle (veh), BMY7378 $3x10^{-8}$M (BMY-7.5), RS100329 $3x10^{-9}$M (RS-8.5), and the combination of BMY7378 $3x10^{-8}$M/RS100329 $3x10^{-9}$M (BMY-7.5/RS-8.5). Values are mean ± SE from 6 experiments. For statistical analysis, see Tables 3 and Results.

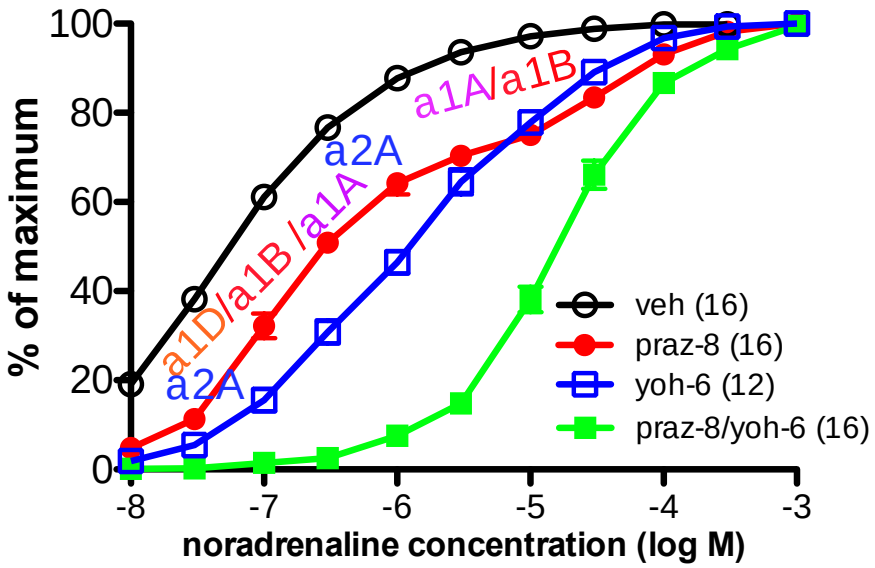

Figure 6. Graphical summary. This graph combines data from male WT, female WT and KO mice to produce composite data. Responses are expressed as % of the maximum response obtained to NA in each individual experiment. Prazosin produces a clear biphasic shift in NA potency, yohimbine produces a more parallel shift, but with a lesser shift against high concentrations of NA. The combination of prazosin and yohimbine produces the clearest parallel shift. Also indicated is an explanation of results presented in this paper. It is suggested that low concentrations of NA produce contractions by action at α_{1D}-/α_{1B}-, and possibly α_{1A}-, adrenoceptors, and high concentrations of NA produce contractions by actions at α_{1A}-/α_{1B}-adrenoceptors, with α_{2A}-adrenoceptors acting at low and medium concentrations of NA. However, the α_{1B}-adrenoceptor may modulate contractions mediated by α_{1A}- or α_{1D}-adrenoceptor, or indeed α_2-adrenoceptor, stimulation.