Selective α_{1A}-Adrenoceptor Stimulation Induces Mueller's Smooth Muscle Contraction in an Isolated Canine Upper Eyelid Preparation.

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Short running title: Mueller's smooth muscle contraction

ABSTRACT

Purpose: It has been demonstrated that in patients with aponeurotic blepharoptosis, α_1-adrenoceptor stimulation causes the contraction of the upper eyelid tarsal smooth muscle (Mueller's muscle) and opening of the eye. However, α_1-adrenoceptor subtypes mediating the contraction of Mueller's muscle are still unclear. This study was designed to identify the α_1-adrenoceptor subtypes in Mueller's muscle. *Materials and Methods:* A newly developed canine upper eyelid preparation was retrogradely perfused with a drug-containing Krebs-Henseleit solution through the angular vein in a temperature-controlled organ chamber. The contraction of the preparation was measured with a force-displacement transducer. *Results:* Phenylephrine, an α_1-adrenoceptor agonist, increased the upper eyelid contractile force in a dose-dependent manner ($K_{0.5}$ = 110 nmol). Interestingly, the contraction in response to phenylephrine was persistent and hardly recovered to a base line level for more than 100 min after washout of the drug. WB4101 (100 nM), an α_{1A}- and α_{1D}-adrenoceptor antagonist, but not BMY7378 (100 nM), a selective α_{1D}-adrenoceptor antagonist, competitively inhibited the phenylephrine-induced contraction. ABT-866, a selective α_{1A}-adrenoceptor agonist, increased the upper eyelid contractile force as effectively as phenylephrine in a dose-dependent manner ($K_{0.5}$ = 430 nmol), and the contraction continued again for more than 100 min. *Conclusion:* These results suggest that selective α_{1A}-adrenoceptor agonists such as ABT-866 induce the sustained Mueller's muscle contraction and may be useful in pharmacological treatment of blepharoptosis.

KEYWORDS

Mueller's smooth muscle; α_1-adrenoceptors; canine; blepharoptosis; eyelid

INTRODUCTION

Blepharoptosis refers to drooping of the upper eyelid that usually results from a congenital or acquired abnormality of the muscles that elevate the eyelid. Acquired blepharoptosis is a common problem that affects individuals of all ages and ethnicities. It can be classified into the following four categories: aponeurotic, myogenic, neurogenic and mechanical ptosis.[1] In adults, the most common cause of acquired blepharoptosis is aponeurotic blepharoptosis (i.e.spontaneous disinsertion or dehiscence of the levator aponeurosis from the tarsal plate).[2, 3] Clinical features include a high lid crease due to weakened attachments of aponeurosis to the tarsus and skin.

It is well known that the eyelid movement is mediated by the orbicularis oculi and the levater palebrae superioris muscles.[4] The levator palpebrae superioris muscle is one of the extraocular muscles. The fibers are made up with fast-twich and slow-twich muscles. The fast-twitch fibers are the same fibers that occur in the global layer of the superior rectus muscle, and the slow-twitch fibers are the same fibers that occur in the anti gravity skeletal muscles.[5, 6, 7]

Recently, our studies have suggested that stretching of Mueller's muscle stimulates the surrounding mechanoreceptors in connective tissue and leads to the involuntary contraction of the levator muscle.[5, 8] When the eyelids are opened, fast-twich muscle fibers exert voluntary phasic contraction, while an adequate visual field is maintained by involuntary tonic contraction of the slow-twich muscle. The Mueller's muscle is innervated by sympathetic postganglionic fibers, and noradrenalin causes the contraction of Mueller's muscle through α_1-adrenoceptors.[9] In patients with aponeurotic blepharoptosis, instillation of phenylephrine, a non-selective α_1-adrenoceptor agonist, restored involuntary tonic contraction of the levator muscle.[10]

Thus, α_1-adrenoceptors in the Mueller's muscle can be a therapeutic target in treatment of aponeurotic blepharoptosis. α_1-Adrenoceptors are pharmacologically classified into

α_{1A}-, α_{1B}-, α_{1D}- and α_{1L}-subtypes.[11] The genes encoding the first three subtypes have been identified whereas the α_{1L}-subtype was recently identified as one phenotype derived from the α_{1A}-adrenoceptor gene.[12] There coexist multiple α_1-adrenoceptor subtypes in eyes,[13,14] and phenylephrine nonselectively stimulates these receptors.[11] Thus, phenylephrine may cause untoward effects on different parts of eyes such as mydriasis and vasoconstriction. Therefore, it is important to identify the subtype of α_1-adrenoceptor mediating the contraction of Mueller's muscle. Based on pharmacological analyses, we here show that α_{1A}-adrenoceptors are responsible for Mueller's muscle contraction.

MATERIALS AND METHODS

Ethical Approval

All experiments were carried out in accordance with the Guidelines for Animal Experimentation of Shinshu University.

Isolated Upper Eyelid Preparation

Twenty-two beagle dogs (weighing 10-15 kg each) were treated with sodium heparin (500 USP units/kg i.v.), anesthetized with sodium pentobarbital (30 mg/kg i.v.) and then, sacrificed by exsanguination of venous blood from the right femoral vein. After that, the upper eyelid with surrounding tissues were quickly removed and placed in cold Krebs-Henseleit solution of the following composition (mM): NaCl, 120; KCl, 5.9; $CaCl_2$, 2.5; NaOH, 4.6; $MgCl_2$, 1.2; HEPES, 11.5 and glucose, 5.55 (pH 7.4 at room temperature). Then, an upper eyelid preparation was created by removing surrounding tissues.[15] Then, the angular vein was cannulated, and leaking vessels were ligated. The preparation was fixed to the base of a custom-made chamber (Fig. 1A). The preparation was perfused retrogradely under a constant flow condition with oxygenated (with 100 % oxygen) Krebs-Henseleit solution at 36 ± 1 $^{\circ}$C. To avoid surface cooling, the preparation was immersed in the effluent from the tissue, which was maintained at a constant temperature (equal to the perfusion temperature) with a heat exchanger. Perfusion pressure was measured with a pressure transducer (AP-630G, Nihon Kohden Co, Tokyo, Japan) and maintained within a range between 40 and 60 mmHg by adjusting the flow. The four distinct sites of the edge of the eyelid were connected to a force-displacement transducer (TB-611T, Nihon Kohden Co., Tokyo, Japan,) through silk threads. The preparation was stretched to exhibit the resting tension of 1 g. Isometric tension was recorded on a thermo-writing retigraph (WT-685G, Nihon Kohden Co., Tokyo, Japan).

Our experimental protocol typically ended within 3 hours during which preparations were not deteriorated. After each experiment, the tissue was fixed with a 30 % solution of formalin in phosphate-buffered saline at room temperature for more than 24 hours. The tissue was cut along a sagittal plane and embedded in paraffin.

Immunohistochemistry

After deparaffinization, the sections (5 μm) were treated with 3 % H_2O_2 in methanol for 30 min to inhibit the endogenous peroxidase activity. After washout with phosphate-buffered saline (PBS), sections were incubated in PBS with 1 % normal goat serum for 1h at room temperature. The primary antibody solution containing, mouse monoclonal anti-human alpha-smooth muscle actin (anti-SMA, 1:100, DAKO, Glostrup, Denmark) and 1 % normal goat serum, was applied to the sections and incubated for 60 min at 37 °C. Then, the secondary antibody, peroxidase-conjugated anti-mouse IgG (1:50, DAKO, Glostrup, Denmark) was added to the tissue and incubated for 60 min at 37 °C. Every step was followed by three washes with PBS for 5 min. Finally, the sections were treated with 3,3'-diaminobenzidine-tetrachloride-H_2O_2 (DAB- H_2O_2) and then counterstained with hematoxylin. Three sections were stained with an anti-smooth muscle actin antibody to retrospectively confirm the existence of the Mueller's muscle in each preparation.

Experimental Protocol

All experiments were started after a 30 min stabilization period. We carried out the following three series of experiments. In these experiments, the given amount of agonists of α_1-adrenoceptors was applied to the perfusate as a single shot by a syringe, whereas the antagonists were applied to the tissue after dissolved into the perfusate at a given concentration. In the preliminary experiments, we found that phenylephrine induced

the maximum contraction at 30 μmol with resting tension of 1 g. Therefore, in the first series, we examined the effect of phenylephrine (0.001-30 μmol) on the upper eyelid contraction. In the second series, we measured the upper eyelid contractile force in response to phenylephrine in the presence of either WB4101 (100 nM, n = 5), an α_{1A}- and α_{1D}-adrenoceptor antagonist or BMY7378 (100 nM, n = 5), a selective α_{1D}-adrenoceptor antagonist.[16, 17] In the third series, we measured the effect of ABT-866, a selective α_{1A}-adrenoceptor agonist on the upper eyelid contractile force in 7 preparations.[18]

In addition, in order to confirm that ABT-866 increased the contractile force through Mueller's muscle but not through the levator skeletal muscle contained in the preparations, the effects of ABT-866 on the upper eyelid contractile force was examined in the presence of vecronium, a competitive antagonist of cholinergic receptors at the motor end plate which inhibits the skeletal muscle contraction in the canine upper eyelid preparations.

Data Analysis

All numerical data are shown as the mean ± SE. Student's t-test for paired or unpaired data was used for comparisons between 2 groups. An analysis of variance with Bonferroni's test was used for the statistical analysis of multiple comparisons of data. $P < 0.05$ was considered statistically significant. The relationship between the dose of agonists and the contractile force of the preparation was fit with the following Hill equation:

$$y = F_{max}/(1 + (K_{0.5}/[A])^h), \qquad \text{Eq. 1}$$

where y is the contractile force; F_{max}, the maximum contractile force; $K_{0.5}$, the half

maximum effective dose of an agonist; [A], the dose of an agonist; and h, the Hill coefficient. This fit was done with a commercially available software (Delta Graph 4.5; Polaroid Computing Japan, Tokyo, Japan).

Drugs

Phenylephrine hydrochloride and prazosin hydrochloride were purchased from Wako Pure Chemical, Osaka, Japan. BMY7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride) and WB4101 (2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane) hydrochloride were from Research Biochemicals, Inc., Natick, MA, USA. ABT-866 (*N*-[3(1*H*-imidazol-4-ylmethyl)phenyl]ethanesulfonamide, maleate) was kindly provided by Abbott laboratories (Abbott Park, IL, USA). All drugs used were dissolved in distilled water. The stock solutions were kept at -20 $^{\circ}$C until use. Agonists were infused into the angular vein through a rubber tube by a microsyringe. Drug solutions (0.01 to 0.05 ml) were injected within 2 sec.

RESULTS

Effects of phenylephrine on the upper eyelid contraction

We could identify the Mueller's muscle in each preparation used in this study with immunohistochemistry (Fig. 1B). Phenylephrine dose-dependently increased the contractile force of an isolated perfused canine upper eyelid preparation (Fig. 2A). Interestingly, the contractile force persisted for 92 ± 6.4 min and then recovered to a base line level (n =7). The pretreatment with WB4101, an α_{1A}- and α_{1D}-adrenoceptor antagonist, inhibited the phenylephrine-induced contraction (Fig. 2B).[16] Figure 2C shows the averaged data. In the absence of the antagonist, phenylephrine (0.001-3 μmol) significantly increased the contractile force in a dose-dependent manner ($P < 0.001$). The fit of the data with the Hill equation (Eq. 1) yielded F_{max}, $K_{0.5,}$ and h of 0.70 g, 0.11 μmol and 1.50, respectively. WB4101 (100 nM) shifted the dose-response curve rightward without substantially suppressing the maximum contractile force ($P < 0.001$). In the presence of WB4101, $F_{max,}$ $K_{0.5,}$ and h were 0.64 g, 2.9 μmol and 0.86, respectively.

Receptors Mediating the Contraction Induced by Phenylephrine

WB4101 (100 nM), an α_{1A}- and α_{1D}-adrenoceptor antagonist, but not BMY7378 (100 nM), a selective α_{1D}-adrenoceptor antagonist, inhibited the contractile responses to phenylephrine (Fig. 3A).[16, 17] Figure 3B shows the averaged time-dependent change in the phenylephrine-induced contraction in the presence of BMY7378 (100 nM) or WB4101 (100 nM) (n = 5 of each). WB 4101 but not BMY7378 significantly inhibited the contraction in a time-dependent manner. Prazosin, a nonselective α_1-adrenoceptor antagonist, also inhibited the contractile response (data not shown).

Effect of a Selective α_{1A}-Adrenoceptor Agonist (ABT-866) on the Upper Eyelid Contractile Force

ABT-866, a selective α_{1A}-adrenoceptor agonist, increased the contractile force of an isolated perfused canine upper eyelid preparation in a dose-dependent manner (Fig. 4A).[18] The contractile force induced by ABT-866 was also persistent and hardly recovered to a base line level for more than 100 min after the drug was washed out. Figure 4B shows the averaged relationship between the dose of ABT-866 and the contractile force (n = **7**). ABT-866 (0.001-3 μmol) significantly increased the upper eyelid contractile force in a dose-dependent manner ($P < 0.001$). The fit of the data with the Hill equation (Eq. 1) yielded F_{max}, $K_{0.5}$ and h of 0.67 g, 0.19 μmol and 0.72, respectively. ABT-866 also induced the sustained contraction of the upper eyelid preparations in a similar dose-dependent manner in the presence of vecuronium (500 μg i.v.), a competitive antagonist of cholinergic receptors at the motor end plate of skeletal muscles (data not shown).

DISCUSSION

In this study, we found that phenylephrine increases the contractile force of isolated canine upper eyelid preparations in a dose-dependent manner. Because the preparation was denervated, it is likely that the contractile force was generated by the Mueller's muscle but not the levator skeletal muscles contained in the preparations. Consistent with this, vecuronium did not suppress the ABT-866-induced contraction of the upper eyelid preparations (data not shown).

We found that WB4101, an α_{1A}- and α_{1D}-adrenoceptor antagonist, but not BMY7378, a selective α_{1D}-adrenoceptor antagonist, inhibited the phenylephrine-induced contraction of the Mueller's muscle.[16, 17] In addition, ABT-866, a selective α_{1A}-adrenoceptor agonist with antagonism against α_{1B}- and α_{1D}-adrenoceptors, induced the Mueller's muscle contraction as effectively as phenylephrine (Fig. 4).[18, 19] Compared with phenylephrine, ABT-866 has 80, 11 and 0 % of intrinsic activity for α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, respectively.[19] These results clearly indicate that α_{1A}-adrenoceptors are responsible for the contraction of the Mueller's muscle.

It was recently reported that α_{1D}-adrenoceptors are expressed in human Mueller's muscle.[20] However, eyelid elevation response to phenylephrine was not correlated with the amount of the receptors, consistent with this study. The authors found that the effect of phenylephrine was inversely related to the amount of α_{2C}-adrenoceptor staining. Although the mechanistic interpretation of this observation is difficult, it is tempting to examine the effect of α_2-adrenoceptor agonists or antagonists on the effect of phenylephrine on Mueller's muscle.

Interestingly, the contraction induced by phenylephrine and ABT-866 persisted for more than 100 min after washout of the drugs (Figs. 2 and 4). We cannot rule out the possibility that this phenomenon was due to a slow washout of drugs in our preparations.

However, it is also reported that phenylephrine injected into the femoral vein induces the sustained contraction of Mueller's muscle in anesthetized rats. [21] The inhibition of the sustained contraction by WB4101 (Fig. 3) indicates that the sustained activation of α_{1A}-adrenoceptors is responsible for the persistent contraction of the Mueller's muscle. In contrast, the sympathetic nerve-induced contraction of the Mueller's muscle does not sustain as long as 100 min. This is probably because noradrenalin released from sympathetic nerve terminals but not phenylephrine or ABT-866 is easily metabolized by monoamine oxidase. Thus, ABT-866 seems to be useful to induce the sustained contraction of Mueller's muscle and thus, to generate a continuous mechanosensory signal to invoke the involuntary contraction of the levator slow-twitch muscle fibers. [5, 8]

Our previous clinical studies have shown that activation of α_1-adreneroceptors of the Mueller's muscle with phenylephrine has a therapeutic benefit in the treatment of aponeurotic blepharoptosis.[3, 10] However, the nonselective activation of α_1-adrenoceptors by phenylephrine may cause untoward effects at different parts of eyes.[13, 14] For instance, iris dilator muscle contracts in response to stimulation of α_{1A}-, α_{1B}- or α_{1L}-adrenoceptors.[22-24] Vascular smooth muscles express all of α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors.[25] Thus, phenylephrine can cause mydriasis and vasoconstriction through these receptors. In this aspect, a selective α_{1A}-adrenoceptor agonist may cause lesser untoward effects on different parts of eyes than phenylephrine. Moreover, a recent study demonstrated the presence of α_{1A}-adrenoreceptors in the Mueller's muscle of humans.[26] Our finding that ABT-866 effectively induced the contraction of the Mueller's muscle suggests that topically applied ABT-866 may improve aponeurotic blepharoptosis with minimum adverse effects because ABT-866 also has antagonistic effects on α_{1B}- and α_{1D}-adrenoceptors.[18, 19]

Study limitation

A previous study showed that phenylephrine injected into the femoral vein induced the contraction of Mueller's muscle in anesthetized rats.[21] However, it is uncertain in the whole-animal model whether phenylephrine induced the Mueller's muscle contraction directly or indirectly through unknown mechanisms. In order to show that the former is the case, we needed isolated and denervated eyelid preparations, for which dogs are more suitable than small animals such as rats. In addition, dogs have been used as a model of a number of human diseases, including genetic diseases. Indeed, it has been shown that α_{1A}-adrenoreceptors also exist in the Mueller's muscle of humans,[26] consistent with this study. Thus, we have used the dog model in this study. However, it is necessary to examine in future study whether the pharmacology of the canine Muller's muscle can be reasonably extrapolated to that of humans.

We did not examine the effect of α_{1A} agonism in eyelids with intact innervation. However, as mentioned above, it has been shown that phenylephrine injected into the femoral vein induces the sustained contraction of the Mueller's muscle in anesthetized rat.[21] It is also well known that topical application of phenylephrine induces the contraction of the Mueller's muscle in human eyelids.[4] These observations are consistent with the present observation with denervated isolated eyelids (Fig. 2). In addition, ABT-866 mimicked the effect of phenylephrine (Fig. 4). Thus, the finding in this study may reflect the effect of α_{1A} agonism in vivo. However, it is needed to verify this hypothesis in whole-animal models.

It is also important to identify the molecules of α_{1A}-adrenoceptors in the canine Mueller's muscles. However, we failed to isolate intact mRNA samples from the canine upper eyelid preparation especially from the region of the Muller's muscle. It may have been due to 1) the large amount of RNase in the tissue, and/or 2) too sticky and adhesive eyelid tissue to be homogenized. We also tried immunohistochemistry with two distinct commercially available antibodies against α_{1A} adrenoreceptors. However, both of the antibodies did

not show enough sensitivity to obtain a specific signal in our preparations. Future studies are awaited to examine the distribution of each subtype of adrenoceptors in the beagle Mueller's muscle.

Conclusion

Selective α_{1A}-adrenoceptor stimulation can induce the sustained Mueller's muscle contraction, and thus, selective α_{1A}-adrenoceptor agonists such as ABT-866 may be useful in pharmacological treatment of blepharoptosis.

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FIGURE'S LEGENDS

FIGURE 1 A: A photograph of the canine upper eyelid with Mueller's muscle preparation (L) fixed in a custom-made chamber (Ch). Angular vein (V) at the nasal side of the lid was cannulated (C). The four distinct sites of the eyelid edge were connected to a force-displacement transducer (T) through silk threads. B: *Upper panel*, Distribution of the Muller's muscle was visualized by immunohistochemical staining using anti-SMA antibody and DAB substrate (brown). *Lower panel*, High magnification image of the box shown in the upper panel. Arrowheads: the Mueller's muscle; arrow: palpebral conjunctiva.

FIGURE 2 A and B: Representative examples of upper eyelid contractile force in response to phenylephrine (PE) injection (0.03-30 μmol) in the absence (A) and presence (B) of 100 nM of WB 4101, an α_{1A}- and α_{1D}-adrenoceptor antagonist. C: The averaged relationship between the dose of phenylephrine (0.001-30 μmol) and the contractile force in the absence (open circles) and presence (closed circles) of 100 nM of WB 4101. The X-axis is the logarithm of phenylephrine dose. Symbols and bars represent the mean and SE (n = 7 for each point), whereas lines are the fit of the data with the Hill equation (Eq. 1, see Text).

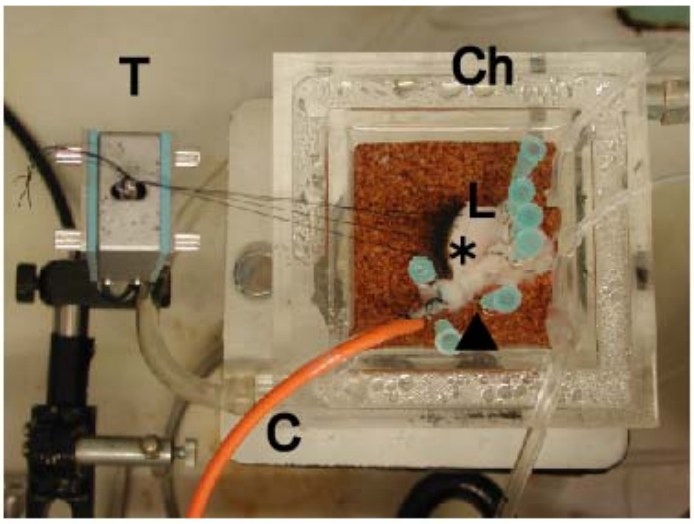
FIGURE 3 A: A representative example of the effects of WB 4101 (100 nM), an α_{1A}- and α_{1D}-adrenoceptor adrenoceptor antagonist and BMY 7378 (100 nM), a selective α_{1A}-adrenoceptor antagonist, on the upper eyelid contractile response to phenylephrine (3 μmol). B: Averaged time-dependent changes in the contractile force in the presence

of WB 4101 (100 nM) and BMY 7378 (100 nM). Symbols and bars represent the mean and SE (n = **5** for each point). *$p<0.05$ vs. values at the corresponding time in the presence of BMY 7378.

FIGURE 4 A: A representative example of upper eyelid contractile force in response to injection of ABT-866 (0.001-3 μmol), a selective α_{1A}-adrenoceptor agonist. B: The averaged relationship between the dose of ABT-866 and the contractile force. The X-axis is the logarithm of ABT-866 dose. Symbols and bars represent the mean and SE (n = 7 for each point). A line is the fit of the data with the Hill equation (Eq. 1). C: Chemical structure of ABT-866.[1]

Fig.1

A



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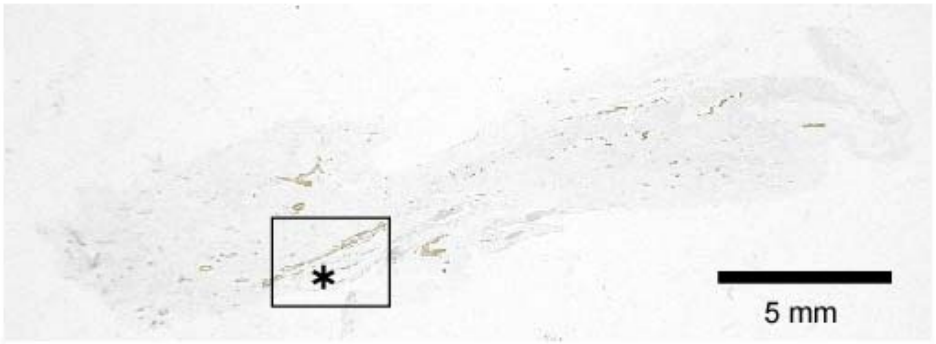


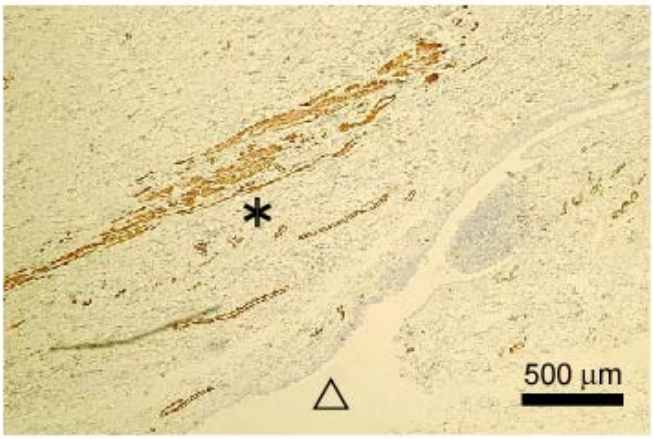
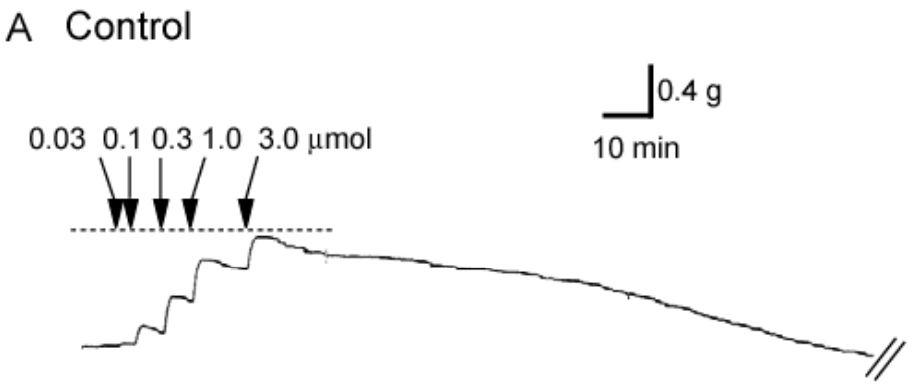
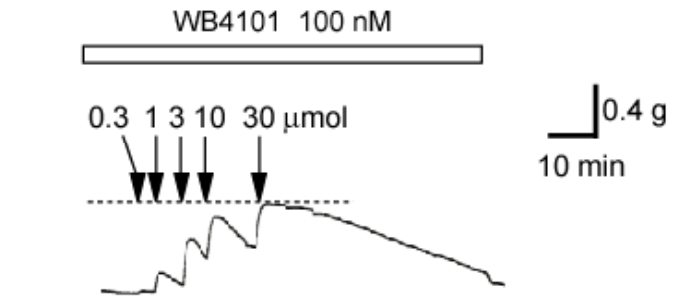


Fig. 2


A Control
0.4 g
10 min
0.03 0.1 0.3 1.0 3.0 μmol


B WB4101
WB4101 100 nM
0.3 1 3 10 30 μmol
0.4 g
10 min

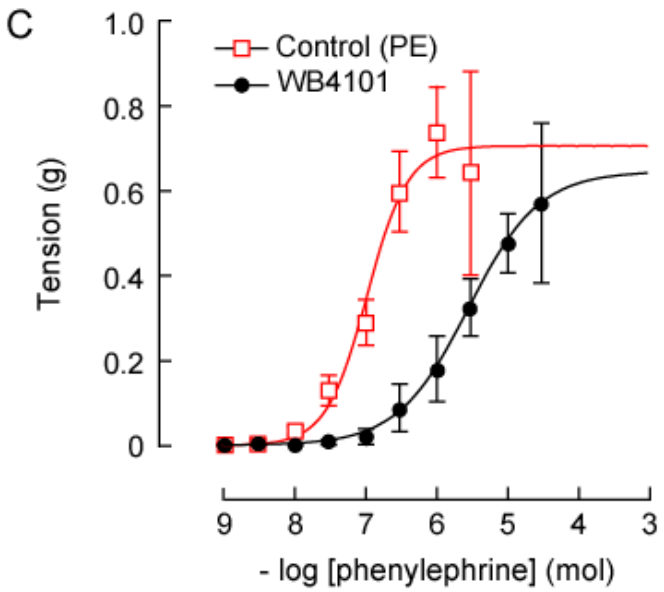
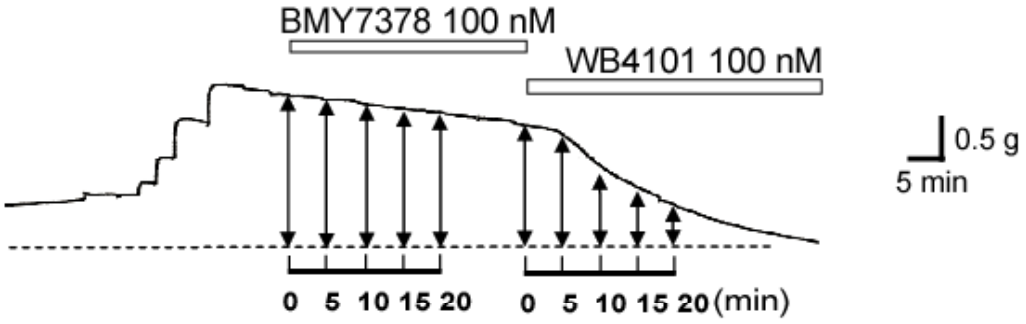

C
Control (PE)
WB4101
Tension (g)
1.0
0.8
0.6
0.4
0.2
0
9 8 7 6 5 4 3
- log [phenylephrine] (mol)

Fig. 3

A


BMY7378 100 nM
WB4101 100 nM
0.5 g
5 min
0 5 10 15 20 0 5 10 15 20 (min)

B

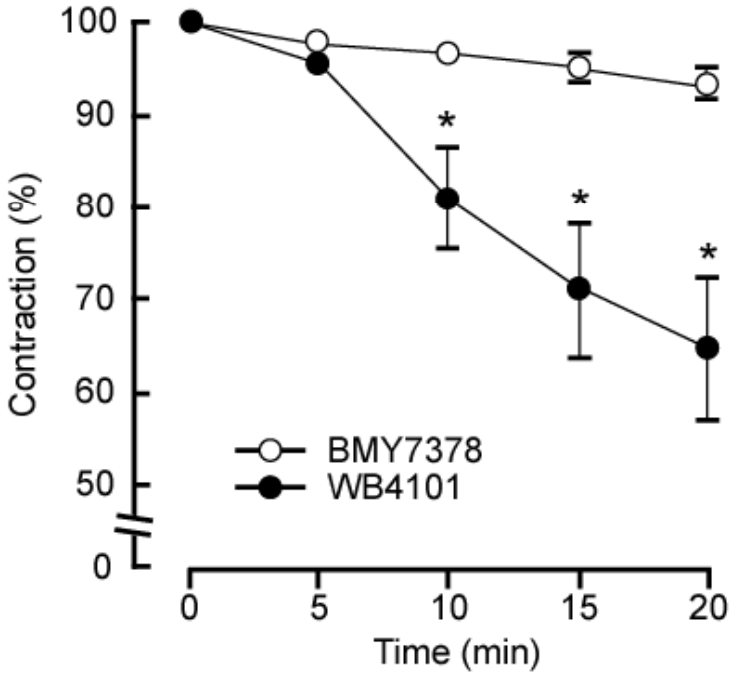
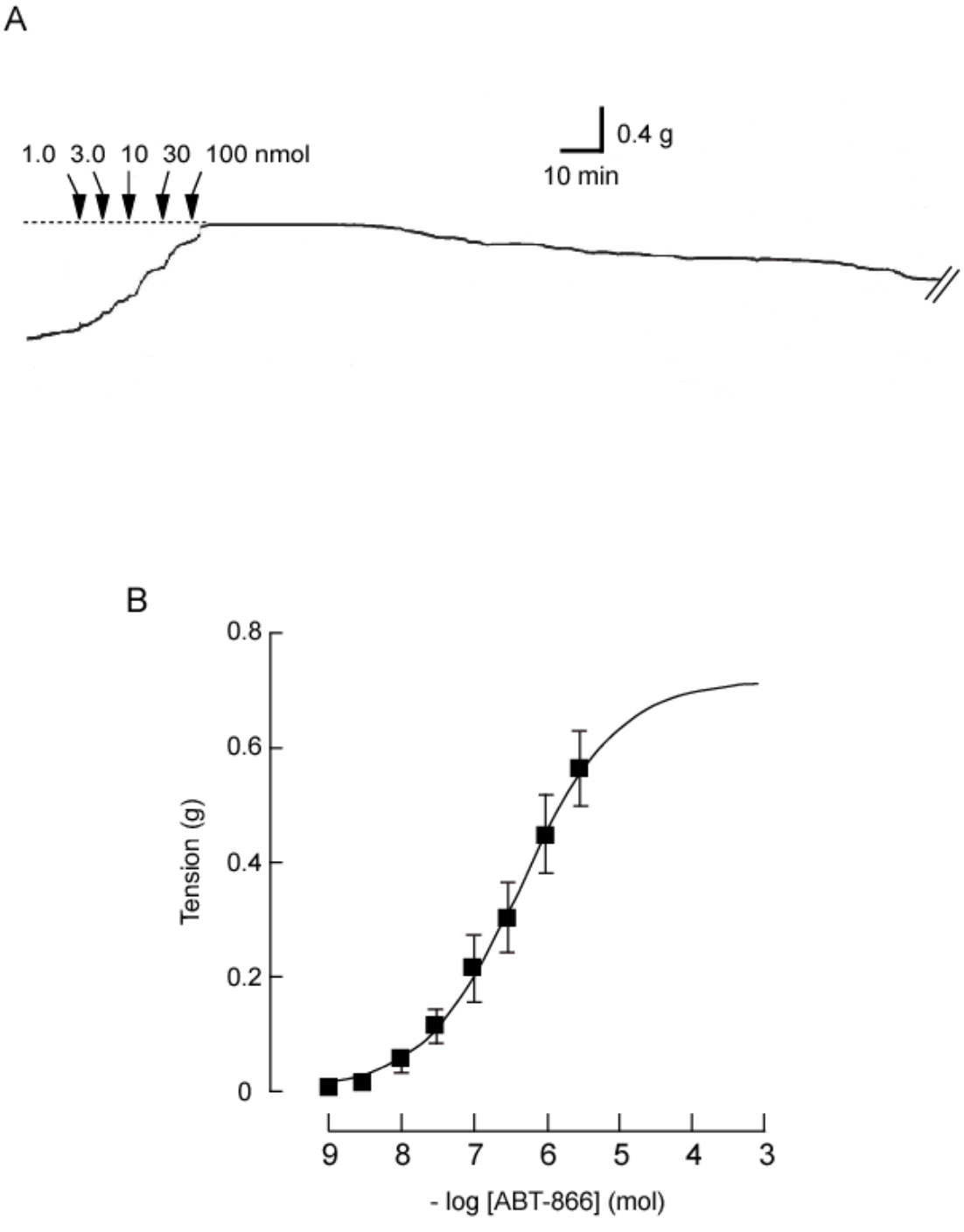

100
90
80
70
60
50
0
Contraction (%)
*
*
*
BMY7378
WB4101
0
5
10
15
20
Time (min)

Fig. 4


A
1.0 3.0 10 30 100 nmol
0.4 g
10 min
B
Tension (g)
0.8
0.6
0.4
0.2
0
9 8 7 6 5 4 3
- log [ABT-866] (mol)