(−)-α-Bisabolol inhibits preferentially electromechanical coupling on rat isolated arteries

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ABSTRACT

Previous findings enable us to hypothesize that (−)-α-bisabolol acts as inhibitor of voltage-dependent Ca^{2+} channels in smooth muscle. The current study was aimed at consolidating such hypothesis through the recording of isometric tension, measurement of intracellular Ca^{2+} as well as discovery of channel target using in silico analysis. In rat aortic rings, (−)-α-bisabolol (1–1000 μM) relaxed KCl- and phenylephrine-elicited contractions, but the IC_{50} differed significantly (22.8 [17.6–27.7] and 200.7 [120.4–334.6] μM, respectively). The relaxation of phenylephrine contractions remained unaffected by L-NAME, indomethacin, 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one, tetraethylammonium, glibenclamide or KT-5720. Under Ca^{2+}-free conditions, (−)-α-bisabolol did not alter the contractions evoked by phenylephrine or caffeine whereas it reduced those evoked by $CaCl_2$ in KCl-, but not in PHE-stimulated preparations. Furthermore, it did not significantly alter the contractions evoked by phorbol 12,13-dibutyrate or induced by the extracellular Ca^{2+} restoration in cyclopiazonic acid-treated preparations. In mesenteric rings loaded with Fluo-4 AM, (−)-α-bisabolol blunted the tension and the cytosolic levels of Ca^{2+} in response to K^+ but not to norepinephrine. Silico docking analysis of the $Ca_v\beta2a$ subunit of voltage-dependent Ca^{2+} channel indicated putative docking sites for (−)-α-bisabolol. These findings reinforce the ability of (−)-α-bisabolol to inhibit preferentially contractile responses evoked by Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

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1. Introduction

(−)-α-Bisabolol, also known as levomenol, is a sesquiterpenic monocyclic alcohol abundantly found as the major constituent of the essential oil of chamomile (*Matricaria chamomilla* L., Asteraceae). It has been attributed to this compound the healing attributes of chamomile, the feature that argues for the use of (−)-α-bisabolol in pharmaceutical formulations in virtue of its anti-inflammatory and antiallergic properties [10]. In fact, it has been reported that (−)-α-bisabolol has protective effects against gastric damage caused by acetylsalicylic acid [12, 26]. The antitumor activity of (−)-α-bisabolol, which is mediated by its proapoptotic effects, has also recently received special attention in the literature [2–4].

In the cardiovascular system, intravenous injections of increasing doses of (−)-α-bisabolol induced dose-dependent hypotension and bradycardia in awake rats [13]. However, the mechanism of action of these cardiovascular effects has not been addressed. The hypotensive response to (−)-α-bisabolol could be mediated, at least partially, through an active vascular relaxation. We recently found that (−)-α-bisabolol induced endothelium-independent relaxation with a pharmacological potency that was higher in mesenteric than in aortic rings [5]. In only aortic rings, (−)-α-bisabolol relaxed KCl-induced contractions with pharmacological potency significantly higher than that observed in vessels contracted with phenylephrine. This data indicates that (−)-α-bisabolol counteracts contractile responses that preferentially recruit voltage-gated Ca^{2+} channels, similar to what was reported for tracheal smooth muscle preparations [5].

Putative Ca^{2+} channel blocking activity as the underlying mechanism for vasodilator action induced by (−)-α-bisabolol was previously demonstrated by Vuorela et al. [28]. Such activity has been also reported for bisabolol oxide and (+)-T-cadinol, two analogues of (−)-α-bisabolol, in experiments with isolated papillary muscle from guinea-pig hearts [16] and binding assays with dihydropyridine on the voltage-operated Ca^{2+} channels [30]. However, direct demonstration that (−)-α-bisabolol is able to interfere with

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cytoplasmic Ca^{2+} dynamics was not demonstrated in smooth muscle cells hitherto.

Therefore, the present study was designed to test the hypothesis that pharmacological effects of $(-)$-α-bisabolol on vascular smooth muscle result from its ability to interfere with the intracellular levels of Ca^{2+}, especially on those events mediated electromechanically. Docking experiments and simultaneous measurement of mechanical force and $[Ca^{2+}]_i$ in Fluo-4 loaded mesenteric artery rings stimulated with either KCl or norepinephrine were performed to support the underlying mechanism involved in the vasorelaxant effects of $(-)$-α-bisabolol.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300 g) were obtained from our local colonies (vivarium of the Department of Physiology and Pharmacology, Federal University of Ceará) and maintained under constant temperature (22 ± 2 °C) with a 12 h light/12 h dark cycle and free access to food and water. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). All procedures described herein were reviewed by and had prior approval from the local animal ethics committee (Protocol n° 48/09 — CEPA).

2.2. Isolated ring-like aortic preparations

Rats were euthanized under sodium pentobarbital anesthesia and a segment of thoracic aorta was removed and immersed in perfusion medium at room temperature. After removing adhering fat and connective tissue, the aorta was cut transversally into cylindrical ring-like segments (1 × 5 mm) receiving careful transluminal insertion of steel wire triangular pieces (0.3 mm diameter) that allowed tissue suspension into 5-mL organ bath containing Krebs–Henseleit solution (continuously aerated at 37 °C with 5% CO_2 in O_2). Endothelium-containing preparations were stretched with a passive tension of 1 g and tension was recorded using isometric force transducer (ML870B60/C-V, AD Instruments, Australia) connected to a data acquisition system (PowerLab™ 8/30, AD Instruments). After an equilibration period of 60 min, control contractions were induced by adding a submaximal concentration (60 mM) of KCl to the bath. When two successive contractions showed similar amplitude, preparations were considered in equilibrium. At the beginning of the experiment, each aortic or mesenteric ring was precontracted with phenylephrine (0.1 μM) and thereafter challenged by acetylcholine (1 μM) to evaluate the integrity of endothelium. Preparations were considered to possess an intact endothelium when the vasorelaxant response to acetylcholine was 80% or greater. The concentration-effect curves were obtained by exposing the aortic preparation to cumulatively increasing concentrations of $(-)$-a-bisabolol (1–1000 μmol/L), which was added to the bath and maintained at a given concentration for 10 min.

2.3. Simultaneous measurement of force and intracellular Ca^{2+} in rings of mesenteric artery

Vessel segments from the second branch of the superior mesenteric artery were dissected and maintained in oxygenated Krebs–Henseleit solution at room temperature. After removing adhering fat under a microscope, cylindrical ring-like segments were obtained and carefully mounted in a confocal myograph chamber (DMT120CW Confocal Wire Myograph, Aarhus, Denmark) following the transluminal insertion of two tungsten wires (40 μm diameter). Then, under constant temperature (37 °C), a resting tension of 11.8 kPa was applied to each isolated artery segment. The preparation was incubated by 50 min with the fluorescent Ca^{2+} indicator Fluo-4 AM (5 μM) supplemented with pluronic

acid (0.1% w/v) (Life Technologies, USA). After washing, the Ca^{2+} fluorescence was registered with an inverted confocal microscope (Olympus, IX81) using a 20× magnification and excitation/emission wavelengths of 488/505–515 nm. Sample rate was 1 frame/7 s and the intracellular Ca^{2+} variations were expressed in relative variation of the initial fluorescence (F/F_0). Simultaneously, the variations of the tension were recorded with an isometric force transducer connected to a data acquisition system (PowerLab™ 8/30, AD Instruments).

2.4. In silico docking analysis

We employed AutoDock 4.2 [14] in docking experiments using three-dimensional molecular structure of the ligand $(-)$-α-bisabolol (constructed by the molecular editor Avogadro 1.1.0; [6]) in pharmacophoric alignments with macromolecules based on two crystallographic models of a β-subunit isoform of voltage-gated L-type Ca^{2+} channel, the conserved core $Ca_v\beta_{2a}$ alone or in complex with the α-interaction domain (AID), namely AID-$Ca_v\beta_{2a}$ [27]. The macromolecules and ligand were prepared using the graphical interface AutoDockTools 1.5.6, which allowed the addition of polar hydrogens (Kollman) and partial charges (Gasteiger). For ligand, the number of active torsions was 5, and both macromolecules were considered rigid. Autogrid (part of the AutoDock package) allowed the construction of affinity grid fields, previously to the docking procedure. For all docking experiments, we used the genetic algorithm available in the software. In a first step of docking experiments, we constructed a 0.59 Å-spaced grid field that covered the entire macromolecule of either $Ca_v\beta_{2a}$ or AID-$Ca_v\beta_{2a}$. The numbers of energy evaluations and docking runs were 2,500,000 and 50, respectively, and other parameters were maintained as default. According to the results of the first step, a second series of simulations was performed by constructing two grid fields (with points spaced by 0.38 Å), one centered on the Hook domain (residues 121–169) and the other centered near to the conserved hydrophobic cleft named α-binding pocket. Such region engages $Ca_v\beta_{2a}$ with AID. The parameters were maintained as previously described. The cluster populations and binding energy as well as proximity of the residues were taken into account in the docking analysis.

2.5. Solutions and drugs

The perfusion medium used in isolated organ chamber was fresh modified Krebs–Henseleit solution (pH 7.4; in mM: 118.0 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.18 $MgSO_4 \cdot 7H_2O$, 2.50 $CaCl_2$, 25.0 $NaHCO_3$, and 11.1 glucose). Nominally Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding ethylene glycol bis(2-aminoethyl ether)-N,N, N′N′-tetraacetic acid (EGTA). All drugs were of analytical grade purity purchased from Sigma Co. (St. Louis, MO, USA). They were dissolved directly in Krebs–Henseleit solution except nifedipine, cyclopiazonic acid, KT-5720 and 1H-[1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) that were dissolved directly in DMSO (dimethyl sulfoxide). Solutions of $(-)$-α-bisabolol (bisabolol; Sigma-Aldrich) were prepared in Krebs–Henseleit solution containing Tween 80 (0.5%). Maximal percentage of Tween 80 in bath chamber was 0.06%. Substances were prepared as stock solutions and were brought to volume with Krebs–Henseleit solution in order to achieve a desired concentration in bath chamber.

2.6. Statistical analysis

Data are reported as mean ± SEM and n indicates the number of experiments. The IC_{50} values (i.e., the $(-)$-α-bisabolol concentration in μM that relaxes a contraction by 50%) were calculated by interpolation from semi-logarithmic plots and are expressed as geometric mean [95% confidence interval]. The significance ($p < 0.05$) of results was assessed using paired or unpaired Student's t-test, Mann–Whitney U-

test, and one- or two-way analysis of variance (ANOVA) followed by the Holm–Sidak post hoc test when appropriate.

3. Results

3.1. Vasodilator effects of (−)-α-bisabolol in aortic rings contracted with high KCl or phenylephrine

Aortic rings produced sustained contractions corresponding to 1.52 ± 0.15 (n = 6) and 1.30 ± 0.11 g (n = 7) in response to 1 μM phenylephrine and 60 mM KCl (Fig. 1A). The cumulative addition of (−)-α-bisabolol (1–1000 μM) fully relaxed the phenylephrine- and KCl-induced contractions with IC_{50} values of 200.7 [120.4– 334.6] and 22.8 [17.6–27.7] μM, respectively. However, the potency of (−)-α-bisabolol in inducing relaxant effect was significantly ($p < 0.05$, Mann–Whitney U-test) higher in preparations contracted by KCl. The cumulative addition of vehicle (Tween 80) does not exert significant effect on preparations precontracted with KCl 60 mM (Fig. 1A).

In a separate set of tissues contracted with phenylephrine (1 μM), pretreatment with L-NAME (100 μM), ODQ (10 μM) or indomethacin (INDO, 10 μM) (Fig. 1B) as well as with TEA (1 mM), glibenclamide (10 μM) or KT-5720 (10 μM) (Fig. 1C) did not change the vasodilator effect of (−)-α-bisabolol as none of these pretreatments altered the IC_{50} value of (−)-α-bisabolol-induced vasorelaxation (Table 1; $p > 0.05$, Mann–Whitney U-test).

3.2. Inhibitory effects of (−)-α-bisabolol on the contractions induced by Ca^{2+} or Ba^{2+} in preparations maintained in the presence of KCl or phenylephrine

Considering that the phenylephrine-induced contraction was reduced almost a half ($59.4 \pm 6.1\%$) by 100 μM (−)-α-bisabolol, this concentration was used in all subsequent experiments. Under Ca^{2+}-free conditions (in the presence of 60 mM KCl and 10 μM EGTA), aortic rings contracted promptly in a concentration-dependent manner ($p < 0.001$, ANOVA) when increasing concentrations (0.1–20 mM) of either Ca^{2+} (Fig. 2A) or Ba^{2+} (Fig. 2B) were added in the extracellular medium (maximal effect of 163.2 ± 4.1 [n = 6] and $130.8 \pm 3.4\%$ [n = 5], respectively; values are % of the reference contraction induced by 60 mM KCl in Ca^{2+}-containing medium). The maximum contractile effect induced by Ca^{2+} or Ba^{2+} was significantly reduced ($p < 0.05$, two-way ANOVA followed by Holm–Sidak test) in the presence of (−)-α-bisabolol (100 μM) to 45.2 ± 5.7 (Fig. 2A, n = 6) and $44.4 \pm 10.3\%$ (Fig. 2B, n = 5), respectively. Interestingly, when phenylephrine (60 μM) was the agonist in Ca^{2+}-free medium (containing 10 μM EGTA and 1 μM nifedipine), contractions due to Ca^{2+} (0.1–20 mM) addition remained significantly ($p > 0.05$, two-way ANOVA) unaffected by 100 μM (−)-α-bisabolol (Fig. 2C, n = 3).

3.3. (−)-α-Bisabolol does not interfere with the contractions mediated intracellularly by phenylephrine or caffeine in aortic rings under Ca^{2+}-free conditions

In aortic rings with intact endothelium under Ca^{2+}-free conditions (in the presence of 1 mM EGTA), phenylephrine (1 μM) induced a transient (phasic) contraction that corresponded to $28.5 \pm 3.4\%$ (n = 8) of the K^+-induced response under nominally normal Ca^{2+} (Fig. 3A). The previous addition of (−)-α-bisabolol (100 μM, n = 6) did not change ($p > 0.05$, paired Student's t-test) this phasic contraction ($30.4 \pm 4.6\%$). In other set of experiments with aortic preparations (with intact endothelium) maintained in Ca^{2+}-free medium at 25 °C, (−)-α-bisabolol (100 μM) was also ineffective ($p > 0.05$, paired Student's t-test) when caffeine (30 mM) was employed as the contractile agent. In fact, the transient contraction induced by
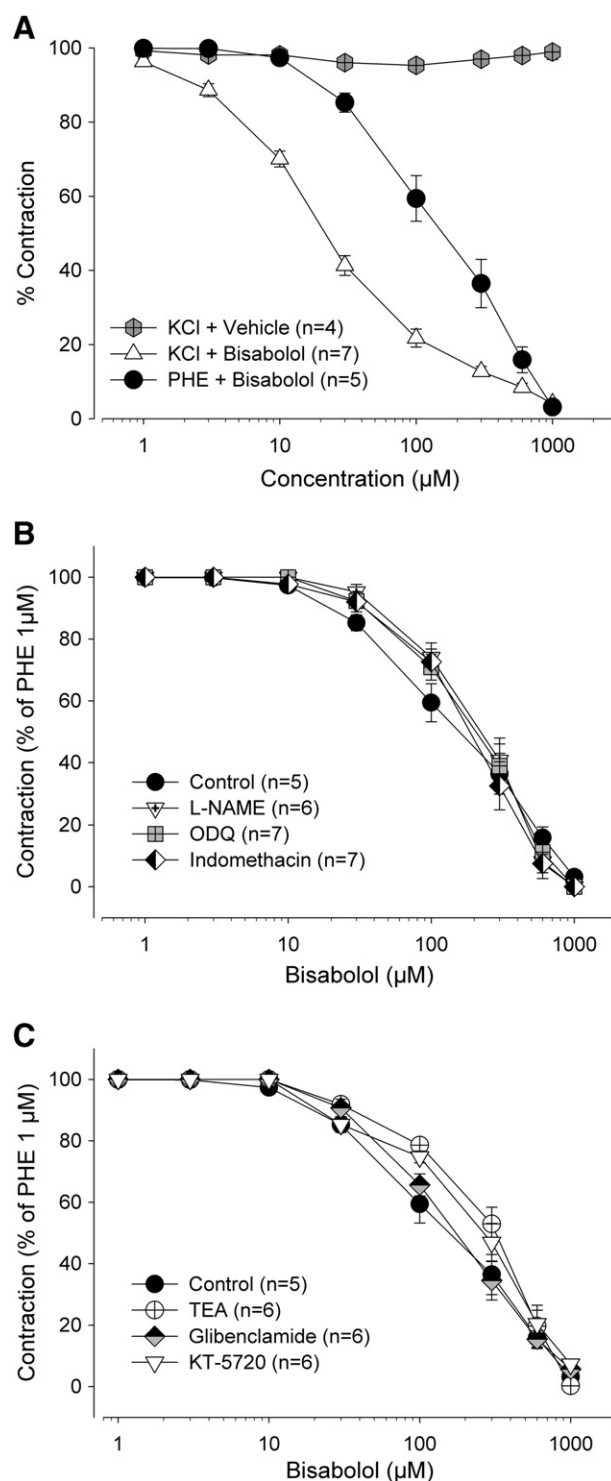

Fig. 1. Effects of (−)-α-bisabolol on phenylephrine- or KCl-induced sustained contractions in rat aortic rings. Concentration–response curves were constructed by the addition of bisabolol (1–1000 μM) or his vehicle (Tween 80; 1–1000 μM) on the steady state of induced contractions (panel A). Bisabolol was cumulatively added after phenylephrine (1 μM)-induced contractions in the absence (control) or in the presence of L-NAME (100 μM), indomethacin (10 μM), or ODQ (10 μM) (panel B) or in the presence of TEA (1 mM), glibenclamide (10 μM) or KT-5720 (10 μM) (panel C). The results are mean ± S.E.M. and the number of experiments is indicated as n.

caffeine corresponded to 15.9 ± 2.0 and $23.4 \pm 3.7\%$ of the reference contraction induced by 60 mM KCl in the absence (n = 8) and in the presence (n = 6) of (−)-α-bisabolol, respectively (Fig. 3B).

Table 1
IC$_{50}$ values for the relaxing effects induced by (−)-α-bisabolol in aortic rings (pretreated or not with L-NAME, indomethacin, ODQ, TEA, glibenclamide or KT-5720) that were pre-contracted with 1 μM phenylephrine or 60 mM KCl. Values are geometric mean [95% confidence interval].

Treatment	IC$_{50}$ (μM)
Phenylephrine	200.7 [120.4–334.6]
+ L-NAME (100 μM)	213.5 [138.6–329.1]
+ Indomethacin (10 μM)	190.1 [130.4–277.1]
+ ODQ (10 μM)	216.9 [141.7–332.1]
+ TEA (1 mM)	318.3 [210.3–481.8]
+ Glibenclamide (10 μM)	186.1 [114.5–302.4]
+ KT-5720 (10 μM)	261.2 [179.3–380.5]
KCl	22.1 [17.6–27.7][a]

[a] p < 0.05 by Mann–Whitney U-test vs. phenylephrine alone.

3.4. (−)-α-Bisabolol does not interfere with the contractions induced by internal Ca^{2+} store depletion or by phorbol 12,13-dibutyrate in aortic rings under Ca^{2+}-free conditions

In order to assess the effects of (−)-α-bisabolol on contractions induced by Ca^{2+} influx after internal Ca^{2+} store depletion, aortic rings were exposed to successive stimuli (3–4 times) induced by phenylephrine (10 μM) in Ca^{2+}-free medium containing nifedipine (1 μM) and cyclopiazonic acid (10 μM) until testifying no measurable contraction in response to phenylephrine. After phenylephrine removal from the extracellular medium, the extracellular Ca^{2+} was restored to 2 mM still in the presence of nifedipine and cyclopiazonic acid. This procedure allowed aortic rings to produce sustained contractions corresponding to 54.7 ± 13.0% (n = 5) of the reference contraction induced by KCl (60 mM). Pretreatment with (−)-α-bisabolol (100 μM) did not change significantly (p > 0.05, paired Student's t-test) this contraction induced by the Ca^{2+} restoration (45.1 ± 8.5%, n = 5). In a separate set of experiments, the addition of the protein kinase C (PKC) activator phorbol 12,13-dibutyrate (1 μM) to aortic rings with intact endothelium and maintained in Ca^{2+}-free medium (with 1 mM EGTA) produces a sustained contraction corresponding to 171.1 ± 18.5% (n = 6) of the reference contraction. In the same aortic preparation, the addiction of (−)-α-bisabolol (100 μM, n = 6) did not significantly changed (p > 0.05, paired Student's t-test) the sustained contraction (172.0 ± 19.6% of the reference contraction).

3.5. Inhibitory effects of (−)-α-bisabolol on K$^+$-induced contractions occur simultaneously to a decrease in cytoplasmic Ca^{2+} levels in mesenteric rings

In Fluo-4 AM-loaded mesenteric vessels stimulated by KCl (mean amplitude of 3.50 ± 0.64 mN; n = 4), (−)-α-bisabolol (100 μM, n = 4) significantly reduced the fluorescence ratio (F/F0) related to the cytoplasmic Ca^{2+} to 50.7 ± 5.5% of the fluorescence observed in control preparations (Fig. 4A, B, C), whereas it significantly reduced the contractile effect of K$^+$ to 0.68 ± 0.10 mN (Fig. 4D) (p < 0.05, paired Student's t-test). In contrast, such reduction induced by (−)-α-bisabolol was not seen when mesenteric vessels were stimulated by norepinephrine (60 μM, n = 4). As one can see in Fig. 4 (panels E to H), neither the contractile force (Fig. 4H) nor the cytoplasmic fluorescence (Fig. 4G) was significantly changed by the pretreatment with 100 μM (−)-α-bisabolol (p > 0.05, paired Student's t-test, n = 4).

3.6. In silico analysis of the molecular interaction between (−)-α-bisabolol and Ca^{2+} channels

The first step of docking experiments indicated two preferential regions in the surface of Ca$_v$β$_{2a}$ subunit (Fig. 5A–C) for (−)-α-bisabolol interactions. The first one is located near the α$_2$-helix in the HOOK
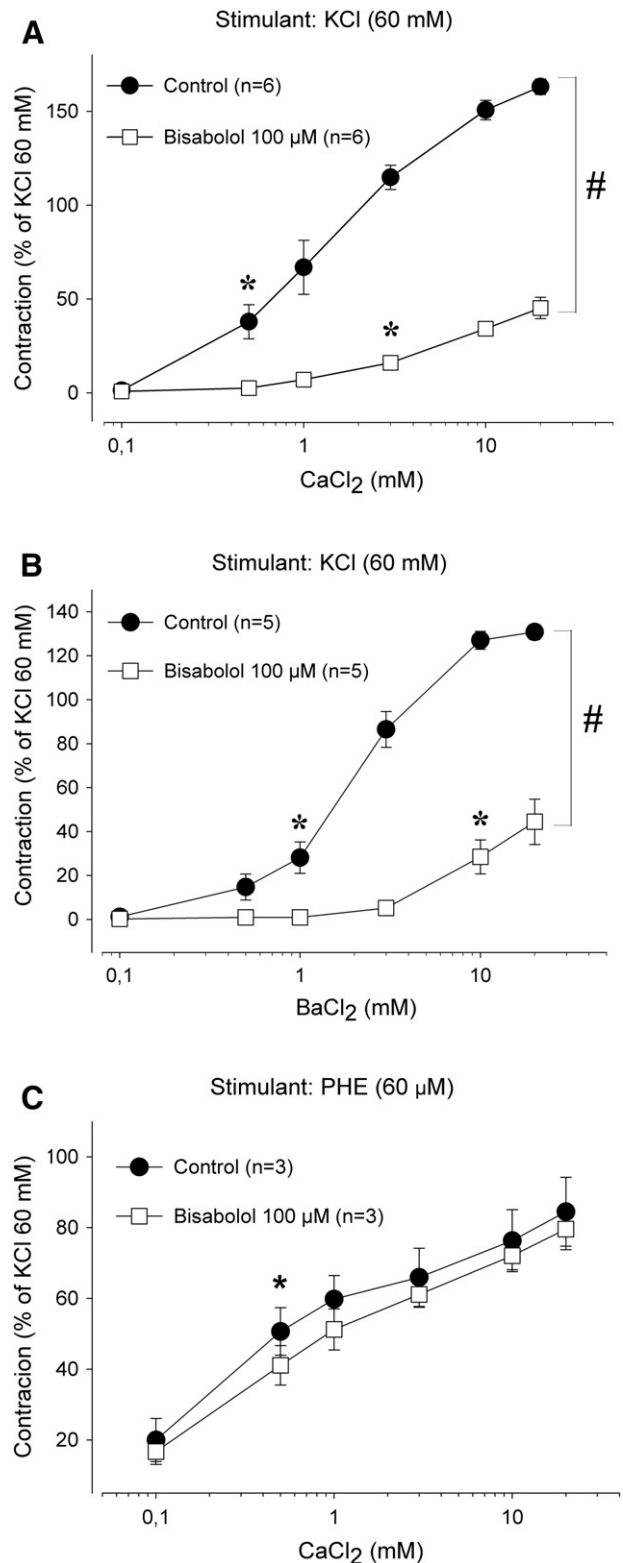

Fig. 2. Effects of (−)-α-bisabolol on contractions induced by cumulative addition of Ca^{2+} or Ba^{2+} in aortic rings maintained in the presence of KCl or phenylephrine under Ca^{2+}-free conditions. The cumulative addition of Ca^{2+} (0.1 to 20 mM; panels A and C) or Ba^{2+} (0.1 to 20 mM; panel B) contracted aortic rings stimulated with 60 mM KCl (panels A and B) or 60 μM phenylephrine (panel C) under Ca^{2+}-free conditions in a concentration-dependent manner (control; p < 0.001, one-way ANOVA). Pretreatment with 100 μM bisabolol significantly blunted the contractile response to exogenous Ca^{2+} in KCl- but not in phenylephrine-stimulated preparations. Results are mean ± S.E.M. and the number of experiments is indicated as n. *p < 0.05, first significant effect by one-way ANOVA followed by Holm–Sidak test; #p < 0.05, two-way ANOVA, (−)-α-bisabolol vs. control.
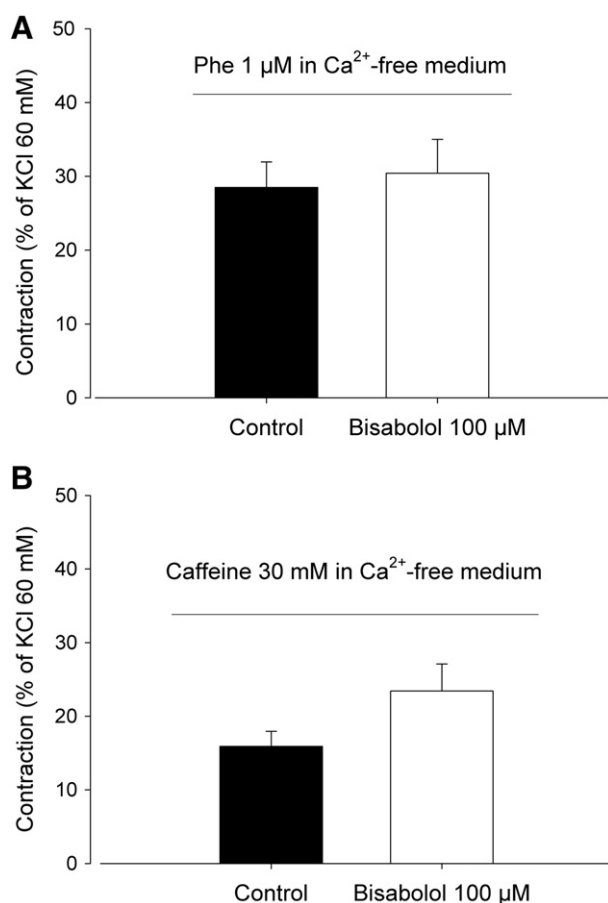
Fig. 3. (−)-α-Bisabolol does not interfere with the phasic contractions induced by phenylephrine or caffeine in rat aortic rings under Ca^{2+}-free conditions. Phasic (unsustained) contractions were induced in aortic rings maintained in Ca^{2+}-free medium after stimulus with phenylephrine (1 μM; panel A) or caffeine (30 mM; panel B) and their magnitude was expressed as % of the reference contraction induced by 60 mM KCl under Ca^{2+}-containing conditions. Pretreatment with bisabolol (100 μM) did not change the magnitude of these contractions (p > 0.05, paired Student's t-test). Results are mean ± S.E.M. (n = 6–8 preparations by group).

domain (Fig. 5A) as it revealed the five biggest population clusters with binding energy lower than −5 kcal/mol. The other, located near the α-binding pocket, presented only two clusters, but with the lowest energy (−6.11 and 6.08 kcal/mol; Fig. 5A). When docking experiments were performed with the complex AID-$Ca_v\beta_{2a}$ (Fig. 5D–F) the clusters were distributed only near the HOOK domain with binding energy of −6.17 kcal/mol (Fig. 5D).

Second step of analysis centering at the HOOK domain of the $Ca_v\beta_{2a}$ revealed the occurrence of a hydrogen bond between docked (−)-α-bisabolol and LEU84 residue within a distance of 1.976 Å in its best docked conformation (−6.34 kcal/mol). The closest residues with atoms closer than 4 Å from docked (−)-α-bisabolol were VAL28, ARG29, TYR30, SER31, PRO81, LEU84, GLU85, MET87, ARG88, THR101, and PRO103 (Fig. 5B). When the analysis was centered at the α-binding pocket region, the lowest binding energy was −6.88 kcal/mol to the best docked conformation and two hydrogen bonds (distance of 2.204 Å and 2.182 Å) were seen between (−)-α-bisabolol and ASN258, a residue of in the $Ca_v\beta_{2a}$ α8-helix. The residues with atoms closer than 4 Å from docked (−)-α-bisabolol were VAL215, LYS216, ILE217, SER218, SER219, MET129, LEU255, ASP256, GLU257, ASN258, and ALA263 (Fig. 5C). All these residues are part of the $Ca_v\beta_{2a}$ α-binding pocket (residues highlighted in blue in Fig. 5D). The results of the second step with AID-$Ca_v\beta_{2a}$ subunit revealed a binding energy of

−6.49 kcal/mol in the best docked conformation of (−)-α-bisabolol near the HOOK domain (Fig. 5E), which established one hydrogen bond with ARG128 residue in the AID-$Ca_v\beta_{2a}$ α2-helix (distance of 2.179 Å) (Fig. 5L) and VAL68, ARG69, TYR70, PRO121, LEU124, GLU125, MET127, THR217, and PRO219 and were the residues with atoms closer than 4 Å from docked (−)-α-bisabolol (Fig. 5E).

4. Discussion

Previously we showed that in rat tracheal smooth muscle (−)-α-bisabolol induced its antispasmodic effects preferentially through inhibition of cellular mechanisms recruiting plasmalemmal voltage-dependent Ca^{2+} influx [5]. The present findings using vascular smooth muscle rat preparations expand this previous work showing that such a mechanism is not peculiar to tracheal smooth muscle. This mode of action for the vasorelaxant effect of (−)-α-bisabolol was presently supported by data obtained from simultaneous measurement of force and intracellular Ca^{2+} in rings of mesenteric artery as well as from in silico docking analysis of the $Ca_v\beta_{2a}$ subunit of voltage-dependent Ca^{2+} channel.

Putative participation of the vascular endothelium in modulating (−)-α-bisabolol-induced relaxation has been previously investigated [5]. Our previous results show that the smooth muscle-relaxing activity of (−)-α-bisabolol in aortic preparations is independent of the integrity of endothelial layer as it remained unaltered by vascular endothelium removal [5]. The present study expands this finding showing that the vasorelaxant effects of (−)-α-bisabolol are resistant to pretreatment with L-NAME and indomethacin indicating that neither oxide nitric nor prostaglandin release is involved. Furthermore, vasorelaxation of (−)-α-bisabolol could not be attributed to a putative ability of this substance in producing K^+-channel opening as there was no effect of pharmacological blockade of potassium channels with either glibenclamide or TEA. Finally, neither soluble guanylate cyclase (sGC) nor adenylyl cyclase activation is involved in the mediation of vasorelaxant effects of (−)-α-bisabolol as they were unaffected following pre-incubation of aortic preparations with the sGC inhibitor ODQ and the cAMP-dependent protein kinase A inhibitor KT-5720, respectively. These data are consistent with a recent study showing vasodilatory effect of bioactive components from Matricaria recutita L., including the (−)-α-bisabolol, in porcine vessels [21].

High KCl induces membrane depolarization, which, in turn, opens the VOCCs, promotes Ca^{2+} influx, increases $[Ca^{2+}]_i$, and elicits sustained contraction following myosin light chain phosphorylation [25]. On the other hand, phenylephrine contracts vascular smooth muscle as result of the α_1-adrenoceptor causing an initial phase of contraction elicited by IP_3-induced intracellular Ca^{2+} release from sarcoplasmic reticulum, followed by a second sustained phase that develops slowly and depends on dihydropyridine-insensitive Ca^{2+} influx through receptor-operated channels (ROCCs) [29]. The present study showed that in aortic preparations with intact endothelium, (−)-α-bisabolol was able to inhibit KCl-induced contractions with pharmacological potency significantly higher (almost 10-fold increase) than that observed in the same preparations contracted with phenylephrine. This implies that in aortic preparations (−)-α-bisabolol also acts against contractile responses that recruit preferentially voltage-gated Ca^{2+} channels as was previously reported for the tracheal smooth muscle. Such mode of action of (−)-α-bisabolol is further supported by some experiments performed under Ca^{2+}-free conditions. In aortic preparations depolarized with high KCl maintained under such conditions, the contractions induced by $CaCl_2$, which are due to an increase in Ca^{2+} influx through VOCCs, were significantly reduced by (−)-α-bisabolol. Under the same conditions, (−)-α-bisabolol was equally effective against contractile responses to increasing concentrations of Ba^{2+}, an ion that selectively passes through VOCCs but is poorly permeable on ROCCs [15,23]. However, in the presence of nifedipine to remove the
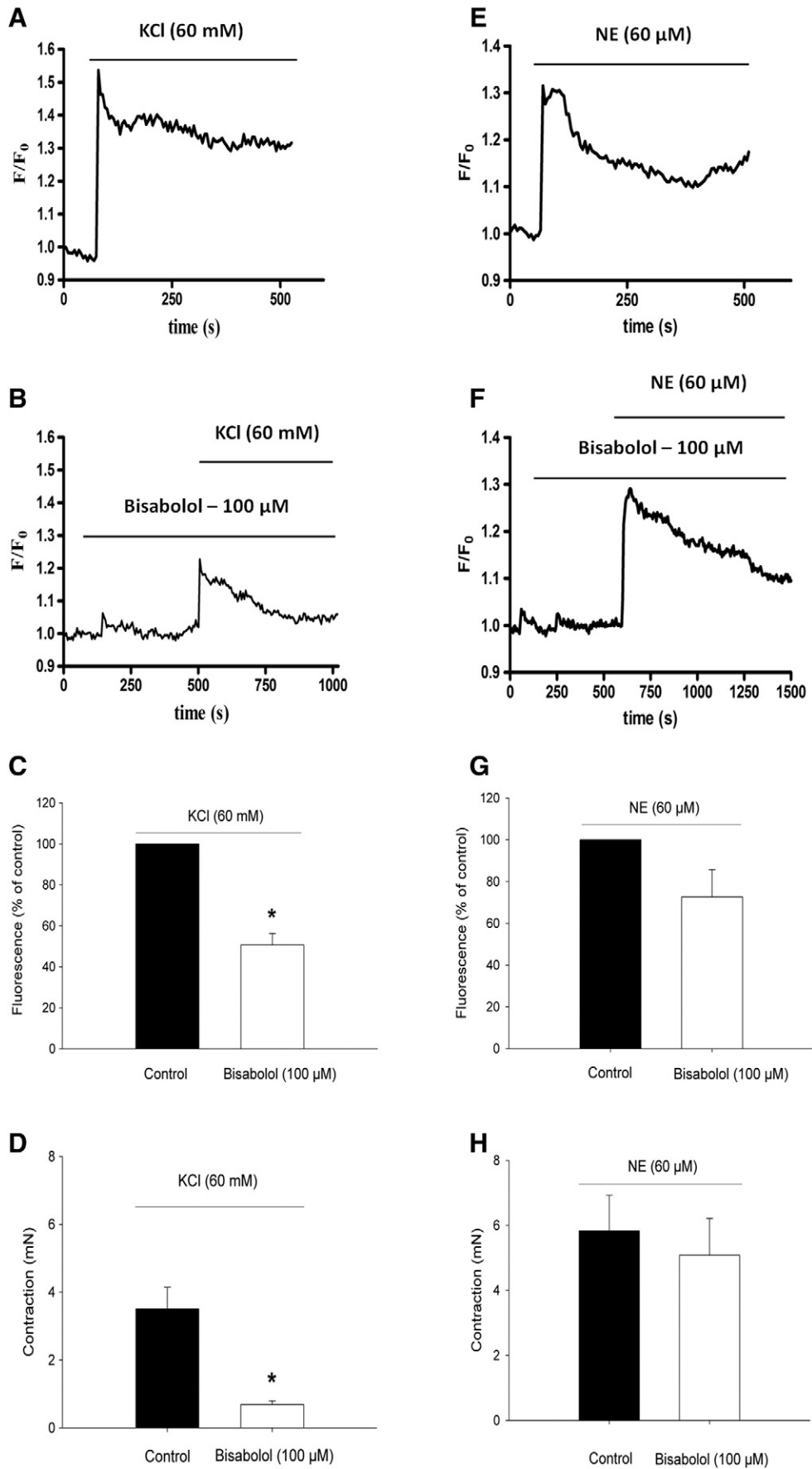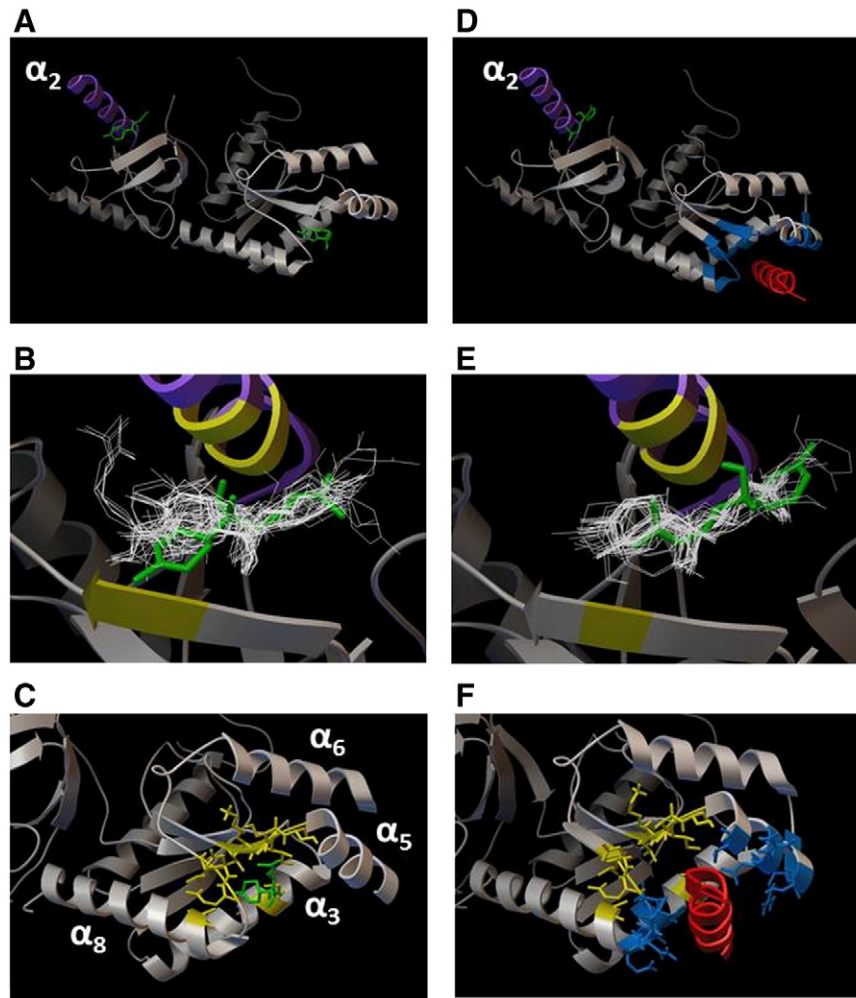
Fig. 5. In silico analysis of the molecular interaction between (−)-α-bisabolol and Ca^{2+} channels. In silico experiments indicated two preferential sites for docking of bisabolol (green molecule) in the surface of $Ca_v\beta_{2a}$ subunit alone (panels A–C) or in complex with AID (red domain in B), namely AID-$Ca_v\beta_{2a}$ (D–F). The first one is located near the α_2-helix (purple helix) in the HOOK domain, while the second one was seen near the sequence known as α-binding pocket (blue residues), but only in the $Ca_v\beta_{2a}$ model. Panels B and E show the 50 lower energy docked conformations at HOOK domain and residues closer than 4 Å are highlighted in yellow. Panels C and F show that bisabolol docked with its lowest energy conformation within a distance smaller than 4 Å apart from α-binding pocket residues, which are highlighted in yellow and compose the sequence of residues in α_3, α_5, α_6, and α_8 helices. Note that when AID is present (F), bisabolol shows no docked conformation in α-binding pocket.

indirect influence of VOCC-mediated Ca^{2+} influx, (−)-α-bisabolol was ineffective against the $CaCl_2$-induced contractions in aortic preparations pretreated with phenylephrine under Ca^{2+}-free conditions. These findings do not only reinforce the hypothesis that (−)-α-bisabolol acts preferentially upon cellular mechanism that involves inhibition of plasmalemmal Ca^{2+} influx by VOCCs (electromechanical coupling) in aortic rings but also argues against the idea of a myogenic action as the underlying mechanism by which (−)-α-bisabolol induces its vaso-dilator effect.

In this direction, putative effects of (−)-α-bisabolol on contractions induced by intracellular events were therefore investigated herein by studying the four following mechanisms under Ca^{2+}-free conditions. Under such conditions, (−)-α-bisabolol did not significantly change (1) the development of transient contraction activated by a phenylephrine-induced stimulus which is expected to result from Ca^{2+} released from sarcoplasmic reticulum stores upon activation of IP_3-sensitive Ca^{2+} channels [7,20], (2) the caffeine-elicited transient contractions which are expected to result from Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors [11,22], (3) the sustained contraction in response to the PKC activator phorbol 12,13-dibutyrate [31,32] which results from cellular events that increase PKC-mediated sensitivity of contractile proteins to intracellular Ca^{2+} [24], and finally (4) the sustained contractions evoked by exogenous Ca^{2+} restoration after its internal store depletion with cyclopiazonic acid, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). This exogenous addition of Ca^{2+} promotes sustained contractions by opening plasmalemmal store-operated Ca^{2+} channels (SOCCs) [17,19].

Fig. 4. Inhibitory effects of (−)-α-bisabolol on force and cytoplasmic fluorescence measured simultaneously in rings of rat mesenteric artery. Rings from the second branch of the superior mesenteric artery were stimulated with KCl or norepinephrine (NE; 60 μM, in presence of verapamil) to produce typical increases in Ca^{2+}-elicited cytoplasmic fluorescence (panels A–B and E–F, respectively), at the same time that contractile responses were recorded (panels D and H, respectively). As one can see, the relative fluorescence (F/F_0) and force were significantly reduced by bisabolol (100 μM) in KCl- (C and D, respectively) but not in norepinephrine-stimulated ones (panels G and H, respectively). All graphs were constructed with mean ± S.E.M. *p < 0.05, paired Student's t-test (n = 4 preparations for each group).

It is then reasonable to conclude that the relaxant effects of $(-)$-α-bisabolol on vascular smooth muscle occur through its inhibitory properties preferentially against contractions mediated by voltage-dependent Ca^{2+} influx. This conclusion is strengthened by the current data obtained during simultaneous measurement of mechanical force and $[Ca^{2+}]_i$ in Fluo-4 loaded mesenteric artery rings stimulated with either KCl or norepinephrine. In fact, our results clearly show that $(-)$-α-bisabolol at 100 μM decreases both the contractile force and the increase in the $[Ca^{2+}]_i$ in response to the KCl stimulus but not in response to norepinephrine. To the best for our knowledge, this is the first study demonstrating that $(-)$-α-bisabolol has the ability to blunt intracellular Ca^{2+} after a contractile stimulus.

Crystal structures of $Ca_v\beta_{2a}$ were employed in the docking experiments of the present study. Found in aortic tissues of rats [9], this β-subunit is an intracellular auxiliary protein that binds to the pore-forming α1 subunit via interaction with ADI, the α-interaction domain. β-Subunit is involved with regulation of channel properties including its voltage-dependent activation [27]. In a first analysis, two docking sites for $(-)$-α-bisabolol were revealed in $Ca_v\beta_{2a}$, one near the HOOK domain, and the other near the α-binding pocket. The docking site for $(-)$-α-bisabolol in the HOOK region was located at the α2-helix and concentrated most docked clusters as in $Ca_v\beta_{2a}$ as in AID-$Ca_v\beta_{2a}$. Although the HOOK region has been described to exert important regulatory roles allowing interactions of $Ca_v\beta$ with other proteins such as calmodulin [18], the present in silico analysis could not definitely establish whether the interaction of $(-)$-α-bisabolol with this subunit of voltage-gated Ca^{2+} channels may result in inhibition of the contractile behavior in live cells.

In contrast, when we adopted the molecular model $Ca_v\beta_{2a}$ in complex with the α-interaction domain (AID-$Ca_v\beta_{2a}$), only one docking site was seen probably in virtue of the spatial impairment imposed by AID in the present in silico approaching of $(-)$-α-bisabolol with the $Ca_v\beta_{2a}$ α-binding pocket. Such finding appears of importance as this region is involved with the engagement between $Ca_v\beta_{2a}$ and AID, a peptide consisting of 18 residues found in all Cav1 L-type pore-forming α1 subunit [1]. As previous studies established that the interaction between α and β subunits of the voltage-dependent Ca^{2+} channels is reversible even in intact cells [1,8], it is reasonable to postulate that $(-)$-α-bisabolol may produce its vasodilator effects through its putative ability to uncouple $Ca_v\beta_{2a}$ from AID. It is well-known that co-expression of α1 and β subunits may improve Ca^{2+} channel currents, although β subunits could not interfere directly with ion permeation through the channel [1]. Notwithstanding, considering that the direct blockade of the Ca^{2+} channel could not be discarded, if an inhibitory action can be confirmed as result of such molecular interaction between $(-)$-α-bisabolol and $Ca_v\beta_{2a}$, then its vasodilator effects may likely be derived from an allosteric influence of $(-)$-α-bisabolol on $Ca_v\beta_{2a}$ rather than from a direct blocking action on Ca^{2+} channel pore in α1 subunit.

In conclusion, our findings indicate that $(-)$-α-bisabolol had concentration-dependent vasorelaxant effects which were independent of the involvement of nitric oxide and prostaglandin release as well as ATP-sensitive K^+ channels, sGC or adenylyl cyclase activation. Rather, the present confocal imaging and in silico analysis of data reinforce the hypothesis that $(-)$-α-bisabolol interfere preferentially with contractile responses evoked by Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

Acknowledgments

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