Mechanisms Underlying the Relaxant Effect of Galetin 3,6-Dimethyl Ether, from *Piptadenia stipulacea* (Benth.) Ducke, on Guinea-Pig Trachea

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Galetin 3,6-dimethyl ether (FGAL), a flavonoid from the aerial parts of *Piptadenia stipulacea* (Benth.) Ducke, was found to exert a relaxant effect on carbachol (CCh)-pre-contracted guinea-pig trachea. Based on cumulative concentration-response curves to CCh, FGAL antagonized muscarinic receptors pseudo-irreversibly and noncompetitively, since it inhibited and shifted these curves towards higher concentrations in a nonparallel manner. In addition, FGAL was more potent in relaxing contractions induced by 18 mM as compared to 60 mM KCl ($pD_2 = 5.50 \pm 0.36$ and 4.80 ± 0.07, respectively), indicating the participation of K^+ channels. In the presence of 10 mM tetraethylammonium (TEA^+) chloride, a nonselective K^+ channel blocker, the relaxant potency of FGAL was reduced (from $pD_2 = 5.12 \pm 0.07$ to 4.87 ± 0.02). Among several selective blockers of K^+ channel subtypes, only apamin, an SK_{Ca} (small-conductance Ca^{2+}-activated K^+ channels) blocker, attenuated the relaxant potency of FGAL ($pD_2 = 4.85 \pm 0.06$), suggesting SK_{Ca} activation. FGAL was equipotent in relaxing trachea contracted by 60 mM KCl ($pD_2 = 4.80 \pm 0.07$) or 10^{-6} M CCh ($pD_2 = 5.02 \pm 0.07$), suggesting Ca_V (voltage-gated calcium channel), but not ROCs (receptor-operated calcium channels) participation. Furthermore, aminophylline-induced relaxation ($pD_2 = 4.12 \pm 0.06$) was potentiated around 4-fold ($pD_2 = 4.80 \pm 0.44$) in the presence of FGAL. Moreover, forskolin-induced relaxation ($pD_2 = 6.51 \pm 0.06$) was potentiated around 2.5-fold ($pD_2 = 6.90 \pm 0.05$) by FGAL. Conversely, sodium nitroprusside-induced relaxation was unaffected, indicating that the AC/cAMP/PKA pathway, but not the NO pathway, may be modulated by the flavonoid. These results suggest that, in guinea-pig trachea, FGAL induces relaxation by pseudo-irreversible noncompetitive antagonism on muscarinic receptors, modulation of K^+ and Ca^{2+} channels, as well as activation of the AC/cAMP/PKA pathway.

Key words: Airway Smooth Muscle, Relaxant Action, Ionic Channels, Galetin 3,6-Dimethyl Ether

Introduction

Piptadenia stipulacea (Benth.) Ducke (Fabaceae) is a tree of the Brazilian caatinga (Albuquerque and Andrade, 2002) popularly known as "Jurema Branca" (Fabricante and Andrade, 2007), "Jurema malícia-da-serra", "Carcará", and "Calumbi" (Florentino *et al.*, 2007). In folk medicine, the decoction or tincture of its bark and leaves are used to treat wounds (Albuquerque and Andrade, 2002) and as healing agents (Bezerra *et al.*, 2011).

The chloroform phase of the crude ethanolic extract from the aerial parts of *P. stipulacea* provides three flavonoids: santin, demethoxycentaureidin, and galetin 3,6-dimethyl ether (FGAL) (Fig. 1), whose structures were identified by ^1H and ^{13}C NMR (one-

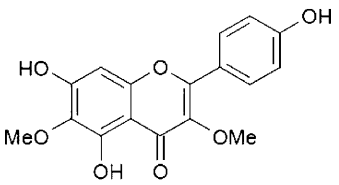

Fig. 1. Chemical structure of galetin 3,6-dimethyl ether (FGAL).

and two-dimensional) spectroscopy and by comparison with literature data (Queiroz *et al.*, 2010).

Flavonoids exert several pharmacological effects: antioxidant and anti-inflammatory (Hämäläinen *et al.*, 2007), antineoplastic (Izzo, 1996), vascular protective (Beretz and Cazenave, 1988), and relaxant on ileum (Macander, 1986; Lima *et al.*, 2005; Verspohl *et al.*, 2013), rat aorta (Ajay *et al.*, 2003; Lapa *et al.*, 2011; Avila-Villarreal *et al.*, 2013), and guinea-pig trachea (Lima *et al.*, 2011; Liu *et al.*, 2008). Some pharmacological activities have been described for FGAL, such as antiviral (Edwards *et al.*, 2010), used in the treatment of skin (Rosenbloom, 2003) and peripheral vascular and neural diseases (Rosenbloom, 2004), as well as anticancer activity (Rosenbloom, 2006), and antinociceptive and anti-inflammatory activities in mice (Queiroz *et al.*, 2010).

Recently, it was demonstrated that FGAL exhibits a nonselective spasmolytic activity on guinea-pig ileum and trachea and rat uterus and aorta (Macêdo *et al.*, 2011). Therefore, in the present study we aimed to investigate the mechanism underlying the FGAL-relaxant activity in guinea-pig trachea thereby contributing to the discovery of natural compounds of medicinal interest.

Materials and Methods

Drugs and chemicals

FGAL was obtained as previously described (Queiroz *et al.*, 2010). Briefly, aerial parts of the species were milled and extracted with ethanol at room temperature, obtaining the crude ethanol extract, which yielded the *n*-hexane, chloroform, ethyl acetate, and methanol phases after liquid/liquid extraction. The chloroform phase was subjected to column chromatography in normal phase providing three flavonoids: santin, demethoxycentaureidin, and galetin 3,6-dimethyl ether (FGAL). 4-Aminopyridine (4-AP), aminophylline, apamin, $BaCl_2$, Cremophor EL™, forskolin, glibenclamide, sodium nitroprusside (SNP), and tetraethylammonium (TEA^+) chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbamylcholine hydrochloride (carbachol, CCh) was purchased from Merck (New York, NY, USA). All substances were dissolved in distilled water, except glibenclamide, which was dissolved in ethanol (95 %), and FGAL, that was solubilized in Cremophor EL™ (3 %) and distilled water at the concentration of 10^{-2} M, being again diluted in distilled water as required for each experimental protocol. The final content of Cremophor EL™ in the organ solution never exceeded 0.01 % (v/v).

Animal and tissue preparation

Adult guinea-pigs (*Cavia porcellus*) from the Bioterium Prof. Thomas George of the Centro de Biotecnologia (CBiotec)/UFPB (João Pessoa, Brazil), weighing 300 – 500 g, were used. Actions on reducing pain, stress, and any suffering have been taken in accordance with the local ethical guidelines for animal usage. All experimental procedures had been approved and were performed in accordance with the Animal Research Ethic Committee of CBiotec/UFPB guidelines (protocol CEPA 0203/11). The trachea was dissected and immediately removed from the animals, cleaned of connective tissue, cut into transverse strips of three adjacent cartilage rings (3 – 4 mm wide), and suspended under an 1-g load in organ baths at 37 °C containing Krebs solution (in mM) [NaCl (118.0), KCl (4.6), $MgSO_4 \cdot 7H_2O$ (5.7), $KH_2PO_4 \cdot H_2O$ (1.1), $CaCl_2 \cdot H_2O$ (2.5), $NaHCO_3$ (25.0), and glucose (11.0)]. The rings were continuously bubbled with carbogen mixture (95 % O_2 and 5 % CO_2).

Measurement of contractile responses

Isometric tensions were recorded using an isometric transducer coupled to an amplifier (World Precision Instruments, Sarasota, FL, USA). Before the experiments started, the tracheal ring preparations were stabilized for 60 min, while the bathing solution was removed and replaced with fresh Krebs solution every 20 min; changes in resting tension were adjusted back to 1 g weight. In order to verify antagonism of muscarinic receptors, the effect of FGAL was evaluated in cumulative concentration-response curves to CCh. To evaluate the participation of K^+ channels, a contraction was induced with 18 or 60 mM KCl and, at

the tonic phase of the contraction, FGAL was cumulatively added to obtain a concentration-response relaxation curve. The relaxant effect induced by FGAL was expressed as the reverse percentage of the initial contraction elicited by 18 or 60 mM KCl. To investigate the K^+ channel subtypes, the following blockers were used: TEA^+ (10 mM), a nonselective K^+ channel blocker (Niu *et al.*, 2008); TEA^+ (1 mM), a selective blocker of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) (Niu *et al.*, 2008); glibenclamide ($3 \cdot 10^{-6}$ M), a selective blocker of ATP-sensitive K^+ channels (K_{ATP}) (Mishra and Aaronson, 1999); 4-AP ($3 \cdot 10^{-3}$ M), a selective blocker of voltage-gated K^+ channels (K_V) (Cole *et al.*, 1996); $BaCl_2$ (10^{-4} M), a selective blocker of inward rectifier K^+ channels (K_{ir}) (Orie *et al.*, 2006); and apamin ($5 \cdot 10^{-8}$ M), a selective blocker of small-conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) (Van-Der-Staay *et al.*, 1999). To verify the participation of voltage-dependent calcium channels (Ca_V) and/or receptor-operated calcium channels (ROCs) in the relaxant action of FGAL, its relaxant potency was compared with that achieved when the trachea was contracted by 10^{-6} M CCh and by 60 mM KCl. The involvement of cyclic nucleotide phosphodiesterases (PDEs) was investigated using aminophylline, a nonselective inhibitor of PDEs (Hirsh *et al.*, 2004). In addition, forskolin was employed as an adenylate cyclase activator (Seamon and Daly, 1981) and SNP as a nitric oxide (NO) donor (Murad *et al.*, 1978).

Statistical analysis

All data are expressed as means ± SEM. The negative decadic logarithm of the FGAL concentration that produced a half-maximal response (pD_2, also denoted as pEC_{50}) was used as the parameter for the potency, and the maximum effect (E_{max}) was the parameter of efficacy. Statistical comparisons were made by Student's *t*-test, for single comparisons, and one-way ANOVA followed by Bonferroni's test, for multiple comparisons. The differences were considered significant when $p < 0.05$.

Results

Effect of FGAL on cumulative concentration-response curves to CCh

To verify the antagonism exerted by FGAL (Fig. 1) on muscarinic receptors, two similar cumulative concentration-response curves to CCh (10^{-9} to 10^{-4} M) were obtained (control). Then, after a 15-min incubation of different preparations with increasing FGAL concentrations, a third cumulative concentration-response curve to CCh was obtained. Each preparation was exposed to a single FGAL concentration. The maximum amplitude was designated 100% (control), and all contractions in the presence of FGAL were related to it. The inhibitory effect of FGAL was evaluated based on the EC_{50} and the E_{max} values of CCh in both the absence (control) and presence of FGAL (Fig. 2, Table I).

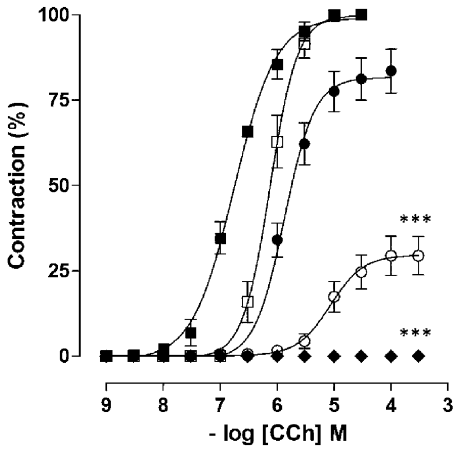

Fig. 2. Cumulative concentration-response curves to CCh in both the absence (■) and presence of FGAL: 10^{-5} (□), $3 \cdot 10^{-5}$ (●), 10^{-4} (○), and $3 \cdot 10^{-4}$ M (◆) ($n = 3$). Symbols and vertical bars represent the mean ± SEM, respectively; $^{***}p < 0.001$ (one-way ANOVA followed by Bonferroni's test: control *vs.* FGAL).

Table I. E_{max} and EC_{50} values of the effect of carbachol on guinea-pig trachea contraction in both the absence and presence of FGAL. $^{***}p < 0.001$ (one-way ANOVA followed by Bonferroni's test: control *vs.* FGAL).

FGAL [M]	EC_{50} [M]	E_{max} (%)
Absence	$1.8 \pm 0.2 \cdot 10^{-7}$	100
10^{-5}	$1.1 \pm 0.1 \cdot 10^{-6}$	100
$3 \cdot 10^{-5}$	$1.4 \pm 0.1 \cdot 10^{-6}$	83.6 ± 6.4
10^{-4}	$8.9 \pm 1.1 \cdot 10^{-6***}$	$29.6 \pm 5.6^{***}$
$3 \cdot 10^{-4}$	N/A	0^{***}

Effect of FGAL on guinea-pig trachea pre-contracted by 18 or 60 mM KCl

First, we determined whether FGAL activates K^+ channels or blocks Ca^{2+} channels. For this, the

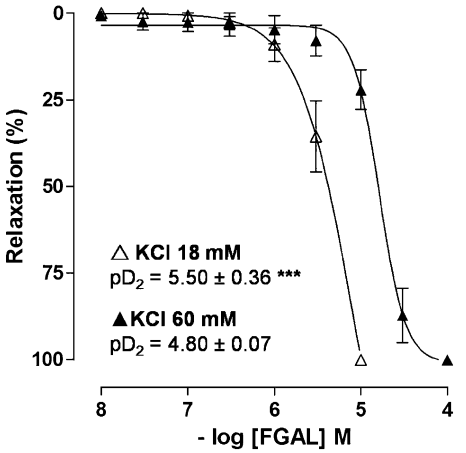

Fig. 3. Effect of FGAL on guinea-pig trachea pre-contracted by 18 mM (Δ) or 60 mM (▲) KCl ($n = 5$). Symbols and vertical bars represent the mean ± SEM, respectively; $^{***}p < 0.001$ (Student's *t*-test: 18 mM *vs.* 60 mM KCl).

FGAL-induced relaxation was observed for contractions induced by KCl. FGAL relaxed the trachea pre-contracted by 18 mM ($pD_2 = 5.50 \pm 0.36$) or 60 mM KCl ($pD_2 = 4.80 \pm 0.07$). The E_{max} (100%) was achieved at concentrations of $3 \cdot 10^{-5}$ and 10^{-4} M FGAL, respectively. FGAL was 2.3-fold more potent to relax the trachea pre-contracted by 18 mM KCl ($p < 0.001$) than by 60 mM KCl (Fig. 3).

Table II. pD_2 values of the relaxant effect of FGAL on guinea-pig trachea pre-contracted by 10^{-6} M CCh in both the absence and presence of K^+ channel blockers. $^*p < 0.05$ (control *vs.* blockers).

Blocker	pD_2
Control	5.12 ± 0.07
TEA^+ 10 mM	$4.87 \pm 0.02^*$
TEA^+ 1 mM	5.31 ± 0.05
Glibenclamide	5.25 ± 0.03
4-AP	5.04 ± 0.06
$BaCl_2$	5.28 ± 0.06
Apamin	$4.85 \pm 0.06^*$

Effect of FGAL on guinea-pig trachea pre-contracted by CCh in both the absence and presence of K^+ channel blockers

To assess whether K^+ channels participate in FGAL-mediated relaxation of the guinea-pig trachea, preparations were pre-incubated for 20 min in TEA^+ (10 mM), a nonselective K^+ channel blocker, and FGAL was then cumulatively added. The relaxant potency of FGAL was attenuated in a significant manner ($p < 0.05$), according to the pD_2 value that changed from 5.12 ± 0.07 (control) to 4.87 ± 0.02 in the presence of the blocker. Other K^+ channel subtypes were investigated using their respective selective blockers: TEA^+, at 1 mM, a BK_{Ca} blocker; glibenclamide, a K_{ATP} blocker; $BaCl_2$, a K_{ir} blocker; and 4-AP, a K_V blocker. However, the relaxant potency of

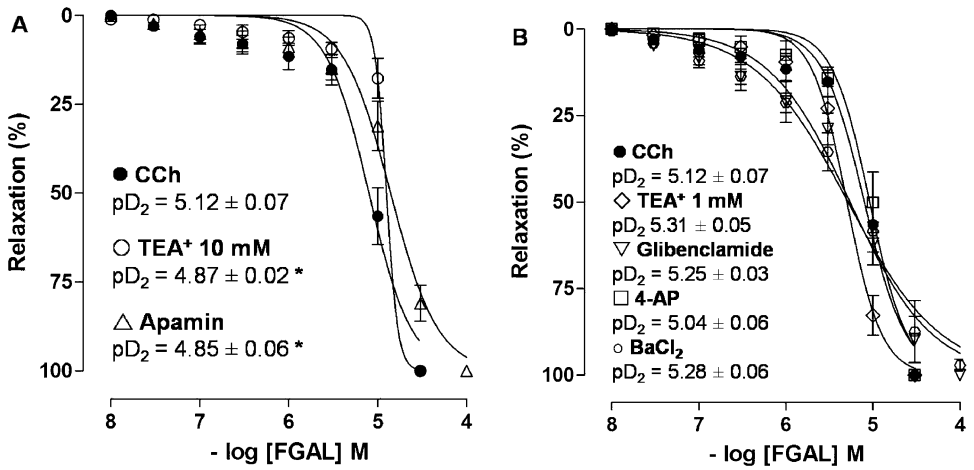

Fig. 4. Effect of FGAL on guinea-pig trachea pre-contracted by 10^{-6} M CCh in both the absence (●) and presence of (A) 10 mM TEA^+ (○) or $5 \cdot 10^{-8}$ M apamin (Δ), (B) 1 mM TEA^+ (◇), $3 \cdot 10^{-6}$ M glibenclamide (∇), $3 \cdot 10^{-3}$ M 4-AP (□) or 10^{-4} M $BaCl_2$ (○) ($n = 5$). Symbols and vertical bars represent the mean ± SEM, respectively; $^*p < 0.05$ (Students *t*-test: control *vs.* blockers).

FGAL was not significantly altered in the presence of any of the blockers ($pD_2 = 5.31 \pm 0.05$, 5.25 ± 0.03, 5.28 ± 0.06, and 5.04 ± 0.06, respectively). But, interestingly, in the presence of apamin, a SK_{Ca} blocker, the relaxant potency of FGAL was significantly attenuated ($p < 0.05$) ($pD_2 = 4.85 \pm 0.06$) (Fig. 4 and Table II).

Effect of FGAL on guinea-pig trachea pre-contracted by CCh or 60 mM KCl

To verify the role of Ca_V and/or ROCs in the induction of relaxation by FGAL, the flavonoid was added cumulatively after pre-contraction of the trachea by either 10^{-6} M CCh or 60 mM KCl. The relaxant effects of FGAL were comparable ($pD_2 = 5.12 \pm 0.07$ and $pD_2 = 4.80 \pm 0.07$, respectively) (Fig. 5).

Effects of aminophylline on guinea-pig trachea pre-contracted by CCh in both the absence and presence of FGAL

To investigate the participation of cyclic nucleotide PDEs, aminophylline ($10^{-10} - 10^{-3}$ M) was added cumulatively and found to relax the tracheal rings pre-contracted by 10^{-6} M CCh in both the absence ($pD_2 = 4.12 \pm 0.06$) and presence ($pD_2 = 4.80 \pm 0.44$) of 10^{-5} M FGAL that was added 20 min prior to the addition of aminophylline. FGAL potentiated ($p < 0.05$) the aminophylline-induced relaxation about 4-fold (Fig. 6).

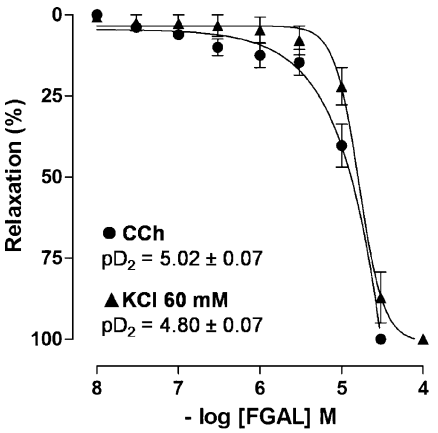

Fig. 5. Effect of FGAL on guinea-pig trachea pre-contracted by 10^{-6} M CCh (●) or 60 mM KCl (▲) ($n = 5$). Symbols and vertical bars represent the mean ± SEM, respectively.

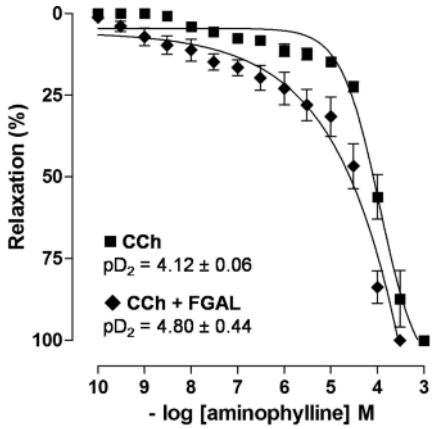

Fig. 6. Effect of aminophylline on guinea-pig trachea pre-contracted by 10^{-6} M CCh in both the absence (■) and presence (◆) of 10^{-5} M FGAL ($n = 5$). Symbols and vertical bars represent the mean ± SEM, respectively.

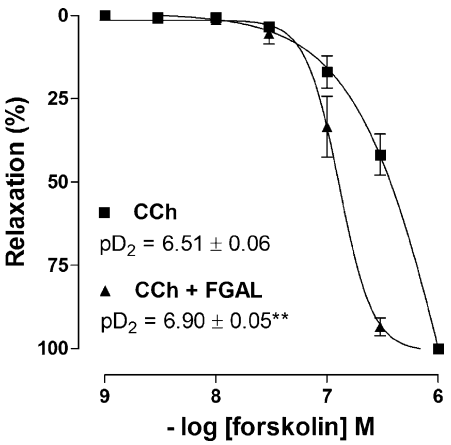

Fig. 7. Effect of forskolin on guinea-pig trachea pre-contracted by 10^{-6} M CCh in both the absence (■) and presence (▲) of 10^{-5} M FGAL ($n = 3$). Symbols and vertical bars represent the mean ± SEM, respectively; $^{**}p < 0.01$ (Students *t*-test: control *vs.* FGAL).

Effects of forskolin on guinea-pig trachea pre-contracted by CCh in both the absence and presence of FGAL

To investigate adenylate cyclase involvement, forskolin was cumulatively added ($10^{-9} - 10^{-6}$ M) and found to relax the tracheal rings pre-contracted by 10^{-6} M CCh in both the absence ($pD_2 = 6.51 \pm 0.06$) and presence ($pD_2 = 6.90 \pm 0.05$) of 10^{-5} M FGAL added 20 min prior to the addition of forskolin. FGAL potentiated ($p < 0.01$) the forskolin-induced relaxation about 2.5-fold (Fig. 7).

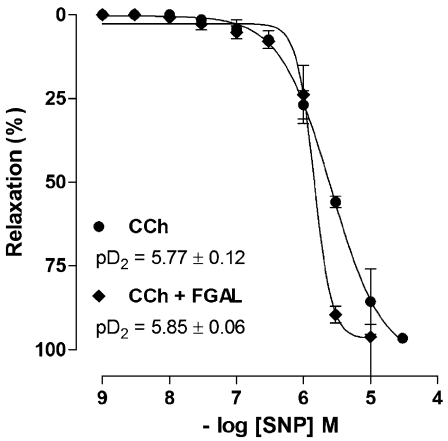

Fig. 8. Effect of sodium nitroprusside (SNP) on guinea-pig trachea pre-contracted by 10^{-6} M CCh in both the absence (●) and presence (◆) of 10^{-5} M FGAL ($n = 3$). Symbols and vertical bars represent the mean ± SEM, respectively.

Effects of sodium nitroprusside on guinea-pig trachea pre-contracted by CCh in both the absence and presence of FGAL

To investigate the involvement of the NO pathway, sodium nitroprusside (SNP) was cumulatively added ($10^{-9} - 3 \cdot 10^{-5}$ M) and found to relax the tracheal rings pre-contracted by 10^{-6} M CCh in both the absence ($pD_2 = 5.77 \pm 0.12$) and presence ($pD_2 = 5.85 \pm 0.06$) of 10^{-5} M FGAL in an equipotent manner (Fig. 8).

Discussion

The relaxant effect of the flavonoid galetin 3,6-dimethyl ether (FGAL) on guinea-pig trachea appears to involve a positive modulation of K^+ channels, in particular of the SK_{Ca} subtype, as well as a block of Ca_V, with the participation of the cyclic nucleotide PDE pathway.

In a preliminary pharmacological screening, we had previously found that FGAL causes relaxation of the CCh-pre-contracted guinea-pig trachea in an equipotent manner in denuded as well as intact epithelium (Macêdo *et al.*, 2011), indicating that its effect is independent of the release of epithelium-derived relaxing factors. Thus, all experiments in the present work were performed with denuded epithelium. Since FGAL caused relaxation of trachea pre-contracted by CCh, we hypothesized that the flavonoid could be acting by antagonizing muscarinic receptors. To probe this hypothesis, we examined the effect of FGAL on cumulative contractions to CCh. FGAL shifted the response curves to CCh towards higher concentrations in a nonparallel manner, resulting in reduction of E_{max} (Fig. 2). According to May *et al.* (2007), in pseudo-irreversible noncompetitive antagonism, the dissociation of the drug from the receptor occurs so slowly that its action is prolonged after its removal. In the presence of a slowly dissociating antagonist, the maximum effect of the agonist is reduced and finally abolished at higher concentrations of the antagonist. Thus, according to the curves' profile in Fig. 2, FGAL exhibited the pharmacological profile of a pseudo-irreversible noncompetitive antagonist on muscarinic receptors.

In the guinea-pig trachea, extracellular and intracellular sources of Ca^{2+} are important for the development of the muscular tonus, and Ca^{2+} entry into smooth muscle cells occurs by two subtypes of Ca^{2+} channels, *i.e.* Ca_V and ROCs (Evangelista *et al.*, 2007). Furthermore, K^+ channels regulate the membrane potential and cellular excitability, thereby participating in the contraction and relaxation of the smooth muscle. To determine whether FGAL could activate K^+ channels or block Ca^{2+} channels, the trachea was pre-contracted by both a moderate and a high elevation of the extracellular K^+ concentration ($[K^+]_e$), 18 and 60 mM, respectively. It is known that K^+ channel activators are more relaxant at moderate $[K^+]_e$, while Ca^{2+} channel blockers are more active at high $[K^+]_e$ (Raeburn and Giembycz, 1995). FGAL was 2.3-fold more relaxant in tracheal rings pre-contracted by 18 mM KCl, indicating that it is possibly a K^+ channel activator (Fig. 3).

FGAL seems to positively modulate K^+ channels, resulting in hyperpolarization of the plasma membrane with subsequent Ca_V blockade and then relaxation of the tracheal smooth muscle. A nonselective K^+ channel blocker attenuated the FGAL-relaxing effect about two-fold, supporting the participation of K^+ channels. Since several subtypes of these channels are expressed in the tracheal smooth muscle, such as K_{ATP} (Teramoto, 2006), K_V (Gordienko *et al.*, 1999), SK_{Ca}, BK_{Ca} (Wei *et al.*, 2005), and K_{ir} (Oonuma *et al.*, 2002), we used channel-selective blockers to obtain evidence for their possible involvement. Interestingly, only apamin attenuated the relaxant potency of FGAL, suggesting that the flavonoid selectively modulates SK_{Ca} positively, thereby leading to tracheal smooth muscle relaxation (Fig. 4).

Ca_V and ROCs are the channels most responsible for Ca^{2+} influx into the smooth muscle, which then leads to contraction. Contraction elicited by elevated $[K^+]_e$ is due to the Ca^{2+} influx mainly mediated by Ca_V, while agonist-induced contraction is mainly the result of ROCs activation, with a minor participation of Ca_V (Noguera *et al.*, 1997; Morello *et al.*, 2006). Since FGAL relaxed guinea-pig trachea contracted by both elevated $[K^+]_e$ (60 mM KCl) and by an agonist (10^{-6} M CCh) in a comparable manner, the flavonoid does not appear to activate ROCs, but rather Ca_V, since the common step in the action of these contractile agents is Ca_V activation. If ROCs were involved, one would expect that FGAL relaxes the CCh-induced contraction more potently, since this agonist promotes contraction by activating both ROCs and Ca_V (Evangelista *et al.*, 2007) (Fig. 5). An important pathway involved in smooth muscle relaxation is the cyclic nucleotide PDE pathway. cAMP and cGMP activate the protein kinases PKA and PKG, which, in turn, phosphorylate several intracellular targets, among them K^+ channels, eventually leading to smooth muscle relaxation. PDEs inactivate cAMP and cGMP by hydrolysis, thus interrupting this cell signaling mechanism (Lugnier, 2006), and PDE inhibitors raise the cyclic nucleotide level and thus potentiate smooth muscle relaxation (Lugnier, 2006; Bender and Beavo, 2006). Several flavonoids have been shown to inhibit PDEs (Rahimi *et al.*, 2010), *e.g.* isoliquiritigenin, a flavonoid (chalcone) of *Glycyrrhiza glabra*, relaxed guinea-pig trachea by inhibition of PDEs (Liu *et al.*, 2008). Aminophylline enhanced the relaxant potency of FGAL around four-fold, indicating participation of cyclic nucleotide PDEs in the relaxant effect of FGAL on guinea-pig trachea (Fig. 6). Interestingly, FGAL potentiated the relaxant effect of forskolin, which activates adenylate cyclase thereby increasing the cytosolic levels of cAMP (Brooker *et al.*, 1983), providing further evidence for the involvement of cAMP in the relaxant activity of FGAL (Fig. 7). It is well established that SNP is a potent broncho-relaxant that stimulates soluble guanylyl cyclase and thus causes intracellular cGMP accumulation (Waldman and Murad, 1987). FGAL did not potentiate the relaxant effect of SNP on guinea-pig trachea, indicating that the NO pathway is not involved (Fig. 8).

Therefore, the relaxant action of FGAL on guinea-pig trachea involves activation of K^+ channels, blockade of Ca_V, and participation of the nucleotide PDE pathway, particularly the AC/cAMP/PKA pathway, which possibly reduce the cytosolic Ca^{2+} concentration leading to airway smooth muscle relaxation.

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