JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 617–622

Received November 8, 2004
Accepted January 11, 2005
DOI 10.1211/0022357055920
ISSN 0022-3573

Flavonoid galangin prevents smooth muscle fatigue of pig urinary bladder

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Abstract

There is increasing evidence that the generation of free radicals plays a role in the development of bladder dysfunction. Flavonoids are a group of polyphenolic compounds with broad pharmacological activity. In the present study, the protective effects of the flavonoid galangin on the progressive decrease of bladder smooth muscle contractile responses during repetitive field stimulation (RFS; a model for muscular fatigue) were demonstrated. Pig detrusor strips were mounted for tension recording in organ baths and were subjected to RFS for 90 min at 32 Hz for 15 s every 5 min. The strips were then washed four times with fresh buffer and allowed a period of recovery for 90 min. The 90 min of RFS caused a progressive decrease in maximal contractile response to electrical field stimulation and to muscarinic agonist-induced contractions (34% and 46% decrease, respectively). Galangin (10^{-7} M) prevented the decrease in contractile smooth muscle response of strips to electrical field stimulation during RFS compared with untreated tissues. The antioxidant activity of galangin was assessed by measuring its ability to inhibit the lipid peroxidation induced by iron and ascorbate in rat liver microsomes (IC50 $1.7 + 0.12 \times 10^{-6}$ M). If the data are confirmed in-vivo, exogenously administered galangin may be a new approach in the prevention and/or treatment of bladder dysfunction.

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Funding: Supported in part by grants from the European Community, Joint co-operation in REBEC (project no. QLG5-CT-2001-00822) and the WAMU foundation Maastricht, Netherlands.

Introduction

Overactivity of the bladder adversely affects the quality of life in a significant proportion of the elderly population. Worldwide, the cost of treating bladder dysfunction exceeds a billion dollars each year (Debruyne & Heesakkers 2004). Concerning the basic mechanisms of bladder overactivity, there is increasing evidence that the generation of free radicals plays a role in the development of this pathology. Results of a previous study showed that repetitive field stimulation (RFS) of smooth muscle strips isolated from the urinary bladder could be used as a model for muscular fatigue (Ohnishi et al 1998). This model has been demonstrated to produce exhaustion of synaptic stores of acetylcholine and direct neuronal damage, leading to increased lipid peroxidation and impaired smooth muscle contractility in the ischaemic and hypoxic media as well as in the normal physiological media (Ohnishi et al 1998). In that study, the extent of contractile impairment correlated with the level of lipid peroxidation, suggesting the involvement of oxidative stress in bladder smooth muscle dysfunction. Furthermore, recent studies have introduced the concept that reactive oxygen species may be a major factor in the progressive deterioration of bladder contractility induced by benign prostatic hyperplasia (partial outlet obstruction in animals) (Masick et al 2001).

Bladder dysfunction is frequently treated pharmacologically, but the side-effects associated with the commonly used agents can be uncomfortable and significantly influence patient compliance. During the last few years, research has stimulated the development of new therapeutic approaches for the overactive bladder. It is important to focus on the development of pharmacological agents that can suppress the symptoms without altering normal voiding function.

Flavonoids comprise a large group of naturally occurring polyphenolic compounds widely distributed throughout the plant kingdom. These natural products possess a broad spectrum of physiological and pharmacological effects, such as inhibitory effects

on both intestinal and thoracic aorta smooth muscle contraction (Ajay et al 2003; Gharzouli & Holzer 2004), but they are not equally physiologically active because of differences in chemical and physical properties (van Acker et al 1996). The pharmacological effects can be explained by their inhibition of certain enzymes and their antioxidant activity. Despite the large amount of data available on the various effects of flavonoids, little is known about the effect of flavonoids on bladder smooth muscle.

Galangin, a member of the flavonol class of flavonoids, is present in high concentrations in honey and *Alpinia officinarum* and has been demonstrated to possess several biological properties such as anti-mutagenic (Wall et al 1988) and radical scavenging actions (Imamura et al 2000). More recently, its inhibitory effect on rat bladder contractility has been investigated (Capasso & Tavares 2002). The present study was conducted to investigate the efficacy of galangin to counteract the detrusor damage caused by the smooth muscle of the urinary bladder being exposed to RFS.

Material and Methods

Compounds

Galangin and methacholine chloride were obtained from Sigma Chemical Co (Netherlands). $FeSO_4$ was obtained from Merck (Netherlands). Galangin was dissolved in dimethylsulfoxide (DMSO; the final concentration of DMSO in the bathing solution was less than 0.05%). All solutions were freshly prepared. All other chemicals were of the highest grade of purity available.

Tissue preparation

Experiments were performed on pig urinary bladders obtained from the slaughterhouse approximately 30 min after slaughter. Strips, 2×2 cm, were dissected from the dorsal side of the bladder dome and transported to the laboratory in oxygenated Krebs solution (mM: NaCl 118, KCl 5.6, $NaHCO_3$ 25, NaH_2PO_4 1.3, CaCl 2.5, $MgSO_4$ 1.2, glucose 6.1, pH 7.4, aerated with 95% O_2/5% CO_2). One or two strips were taken from each bladder. The mucosa and the submucosal fat layer were removed using a binocular microscope, and strips of 1 mm diameter and length of between 3 and 5 mm were excised. To facilitate diffusion, the thin layer covering the muscle fibre was opened and for the greater part removed. Care was taken to ensure that the muscle fibres were running longitudinally.

Each strip was mounted in a separate 20-mL organ bath containing Krebs buffer solution that was continuously gassed with 95% O_2/5% CO_2 at 37°C. An initial tension of 20 mN was placed on each strip. Measurements were started after an equilibration period of 60 min with four intermediate changes of buffer solution.

To establish the frequency of stimulation that had to be used to optimally stimulate nerves, a frequency–response curve (5-s train pulses, 1 ms duration, 7.5 V voltage and 0.25–32 Hz frequency) was determined. The maximum contraction was observed at 32 Hz. Accordingly, a frequency of 32 Hz was used for the entire experimental procedure. When the tissue was pre-incubated with 10^{-6} M tetrodotoxin for 10 min, the responses to 0.25–8-Hz stimuli were totally abolished and the responses to 16 and 32 Hz were inhibited by 93% and 83%, respectively, which demonstrated their predominant neurogenic origin.

Contractions were measured isometrically using mechanoelectrical transducers connected to the BAM4C amplifier (Scientific instruments, Heidelberg, Germany). The electrical field was generated between two platinum electrodes connected to the HM8130 Function Generator (Hameg Instruments, East Meadow, NY, USA).

Effects of galangin on detrusor muscle tone under normal conditions

After a 60-min equilibration period, detrusor strips were electrically stimulated at 32 Hz and submitted to a methacholine concentration–response curve (MCRC) (10^{-8} to 10^{-5} M), after which the strips were washed for up to 30 min. Then, galangin at different concentrations was added to the perfusing Krebs solution for a period of 180 min to reproduce the same time period of both RFS and the recovery period. Its effect on the muscle contractility was observed after 90 and 180 min.

Effect of RFS on detrusor contractility

After the equilibration period, detrusor strips were stimulated at 32 Hz (single stimulus) and submitted to a MCRC. Then, eight strips were submitted to RFS for 90 min (5-s train pulses, 1 ms duration, 7.5 V, 32 Hz, applied for 15 s every 5 min). At the end of the period of RFS, responses to electrical field stimulation (EFS) at 32 Hz (single stimulus) and methacholine were re-assessed. After that, all strips were washed four times with fresh buffer and the strips were allowed a 90-min recovery period (non-repetitive stimulation). At the end of this period, the strips were again submitted to EFS (single stimulus) and methacholine-induced contraction in order to detect the effect of the recovery period on muscle contractility.

Effect of galangin on detrusor contractility under RFS conditions

After the equilibration time and electrical and agonist stimuli had been applied, detrusor strips were subjected to 90 min of RFS followed by a 90-min recovery period. During this 180-min period, galangin at different concentrations was added to the perfusion medium. At the end of the RFS and the recovery period, the muscle response of the strips was tested by the application of a single electrical stimulus at 32 Hz and methacholine (10^{-8} to 10^{-5} M).

Lipid peroxidation

The present experimental model was developed to detect the antioxidant activity of galangin by measuring the inhibition of iron/ascorbate-induced lipid peroxidation.

Liver microsomes were prepared from male Wistar rats, 200–250 g (van Acker et al 1996). The Animal Protection Committee of Maastricht University approved the protocol. All animal experiments complied with British Home Office Regulations and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

Liver microsome pellet was heated at 100°C for 90 s to remove all enzymatic factors just before resuspending it in ice-cold Tris buffer. Stock solution of galangin was freshly prepared in oxygen-free DMSO and water (1:1) just before use.

All compounds were added in ice, after which the incubates were transferred to a water bath (37°C) and 50 μM ascorbate was added. The reaction was started by adding 10^{-5} M freshly prepared $FeSO_4$. Lipid peroxidation was assayed by measuring thiobarbituric acid reactive material in 'oxygen-free' pure water. The reaction in an aliquot of the incubation mixture was stopped by mixing with ice-cold thiobarbituric acid/trichloroacetic acid/HCl/butylhydroxytoluene solution. After heating (15 min, 80°C) and centrifugation (10 000 *g*, 5 min), the absorbance at 535 versus 600 nm was determined.

The IC50 of galangin was determined by measuring the percent lipid peroxidation inhibition of several concentrations and interpolating the 50% inhibition point on a straight line fitted through the concentrations, which resulted in 20–80% inhibition.

Data analysis

Concentration–response data on methacholine were evaluated by sigmoid curve fitting and −log EC50 values (pD_2) and maximum effect (E_{max}) were calculated from individual values by non-linear regression analysis using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Differences between mean values were statistically analysed.

The effect of RFS on contractions induced by EFS at 32 Hz, and the response following the recovery period are presented as the mean (± s.e.m.) percentage of the control response (response before RFS).

All isometric contractions induced by EFS were sampled with a computer and stored for further analysis. Phase plots, which represent the first derivative of force as a function of the force itself, were calculated. Normally, a straight line can characterize these phase plots of isometric smooth muscle contractions:

$$F = F_{iso}[1 - e^{-(t/C)}]$$

where F is the measured force, F_{iso} is the maximum extrapolated isometric force, t is time, and C is the negative reciprocal of the time constant for isometric force development. The time constant is an indicator of the rate limiting process in the excitation–contraction coupling and tells when 66% of the maximum force saturation level is reached (van Koeveringe & van Mastrigt 1991). The smaller the value of C, the faster the rate of force development.

C represents the limiting rate constant in the excitation–contraction coupling process.

Artefacts due to movement of fluid could be excluded from further analysis because they were recognized as irregular spikes. The value of EFS before RFS was used as reference for the time constant. Changes in time constant compared with the baseline measurements were determined. All calculations were processed using Matlab 12.1 (The Mathworks, Inc., Natick, MA, USA).

Statistical evaluation

Data are expressed as mean ± s.e.m. when appropriate. Statistical analyses were carried out using one-way analysis of variance followed by Dunnett's test. A value of $P < 0.05$ was regarded as significant. All analyses were performed using GraphPad Prism software (version 3.0).

Results

Effects of vehicle control

The vehicle for galangin (0.05% DMSO) had no protective effect on the muscle contractility after RFS (results not shown). The effect of DMSO on muscle contractility after RFS was determined because it is known that solvents such as DMSO possess good radical scavenging activities (van Acker et al 1996).

Incubation in normal physiological medium and galangin-containing medium without RFS

Preliminary experiments without RFS in which isolated strips were incubated in either normal Krebs solution or in galangin solution (10^{-8} M, 5×10^{-8} M and 10^{-7} M) showed no degradation of the contractile responses to EFS ($P > 0.05$) and MCRC after a 3-h incubation period ($P > 0.05$) (data not shown).

Effects of 90 min of RFS followed by 90 min of recovery on bladder tissue strip contraction in response to EFS and MCRC in normal physiological medium

RFS for 90 min in the presence of normal Krebs solution significantly reduced bladder strip contractions in response to EFS (Figure 1) and methacholine (Figure 2) (34% and 46%, respectively). Furthermore, the RFS resulted in a significant increase in the time constant (i.e. a slower developing contraction) of contractions induced by EFS (Figure 3). Following the 90 min of recovery, there were no further differences in the contractile responses to EFS, the MCRC or the time constant ($P > 0.05$).

Effects of 90 min of RFS followed by 90 min of recovery on bladder strip contractions in response to EFS and MCRC in galangin medium

The responses of detrusor muscle strips to EFS in the galangin-treated groups are shown in Figure 1. Galangin

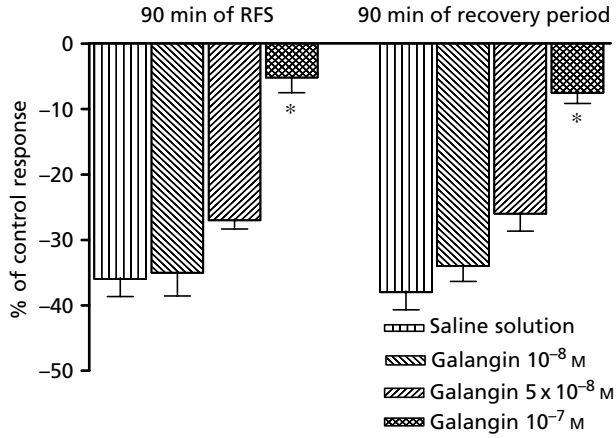

Figure 1 Electrical field stimulation-induced contractile responses of pig detrusor strips subjected to 90 min of repetitive field stimulation (RFS) to induce smooth muscle fatigue and a subsequent 90-min recovery period. Experiments were carried out in the absence or presence of galangin at different concentrations. The values are presented as percentages of the initial measured force values before RFS and expressed as mean ± s.e.m. *$P < 0.05$, significantly different compared with the control group.

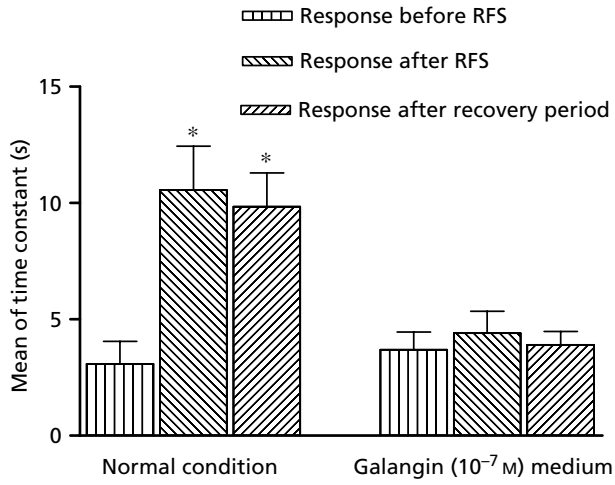

Figure 3 Effect of 90 min of repetitive field stimulation followed by a 90-min recovery period on the time constant of contractions induced by electrical field stimulation (32 Hz) in different media. Each bar is the mean ± s.e.m for six to eight strips. *$P < 0.05$, significantly greater than the response before RFS.

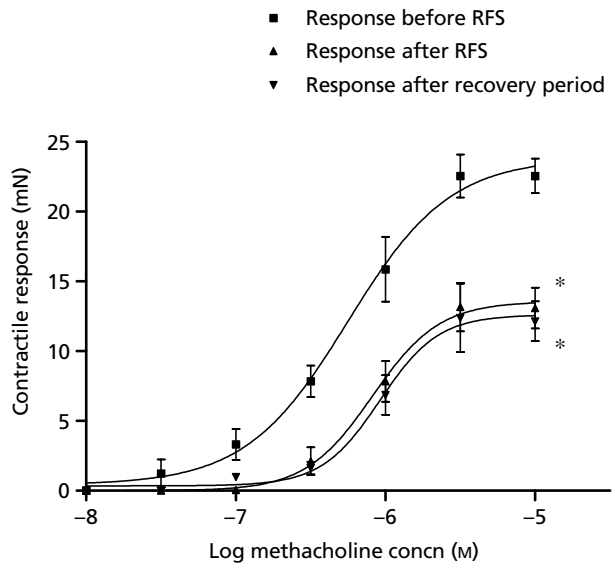

Figure 2 Effect of repetitive field stimulation (RFS) on the contractile response to methacholine in normal physiological medium. Each point is the mean ± s.e.m. for six to eight individual preparations. The $pD_2 \pm$ s.e.m. values of contraction induced before RFS, after 90 min of RFS and after a 90-min recovery period were 6.25 (0.06), 6.08 (0.03) and 6.0 (0.04), respectively ($P < 0.05$ versus contraction induced before RFS). The maximal effect (mN) was 23.81 (0.11), 13.42 (0.03) and 13.23 (0.009) before RFS, after RFS and after the recovery period, respectively. *$P < 0.05$, contractions after RFS and recovery period compared with the initial response before RFS.

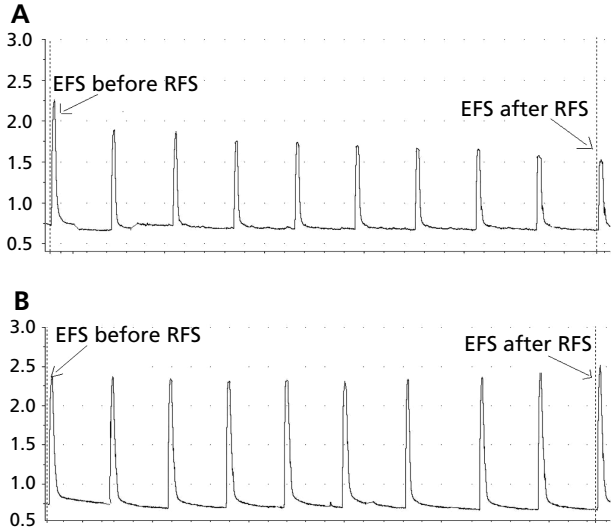

Figure 4 A typical trace showing the effects of 90 min of repetitive field stimulation (RFS) on detrusor contractility induced by electrical field stimulation (EFS). A. Saline medium: the figure shows a control contraction (before RFS), the last 45 min of RFS and a final contraction (after 90 min of RFS). B. Galangin 10^{-7} M medium (control contraction), the last 45 min of RFS and a muscle contraction after a period of RFS.

at 10^{-7} M significantly prevented RFS-induced muscle fatigue (Figure 4), reaching 94.80 + 3.25% as compared with 64 + 2.65% for the control (RFS in saline medium) ($P < 0.05$). At 10^{-8} M and 5×10^{-8} M, galangin did not exert any significant protective effects on muscle contractility (65.00 + 3.56% and 73.00 + 1.34%, respectively) compared with control (RFS in saline medium) ($P < 0.05$).

The responses of detrusor muscle strips to methacholine in the galangin media (10^{-8} M, 5×10^{-8} M and 10^{-7} M) are shown in Figure 5. Following the period of recovery, the contractility of isolated strips and the time constant were not statistically different compared with the responses

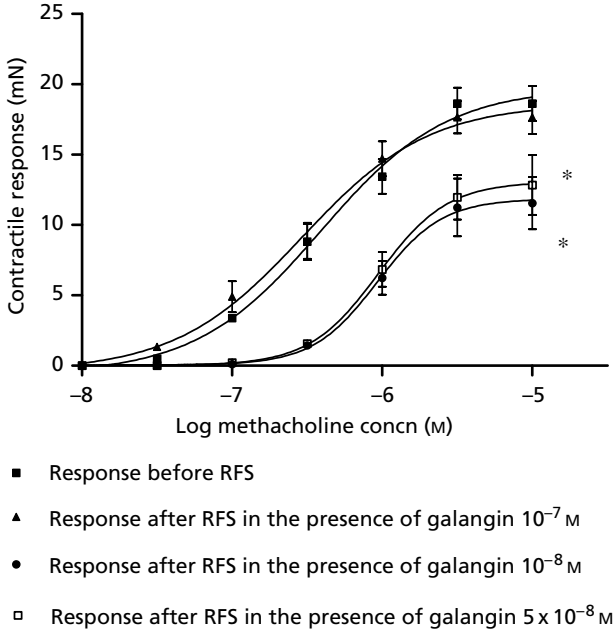

Figure 5 Effect of repetitive field stimulation (RFS) on the contractile response to methacholine in galangin medium at different concentrations. Each point is the mean ± s.e.m. for six to eight individual preparations. The $pD_2 \pm$ s.e.m. value of the contraction induced before RFS was 6.40 ± 0.07. After 90 min of RFS in the presence of galangin at 10^{-7} M, 5×10^{-8} M and 10^{-8} M, the $pD_2 \pm$ s.e.m. values were 6.53 ± 0.07, 6.03 ± 0.02 and 6.02 ± 0.01, respectively. $^*P < 0.05$, significantly less than the response before RFS.

obtained immediately before the recovery period in all groups ($P < 0.05$).

Antioxidant activity of galangin

Galangin exhibited remarkable antiperoxidant activity, with an IC50 value of $1.7 + 0.12 \times 10^{-6}$ M (Figure 6).

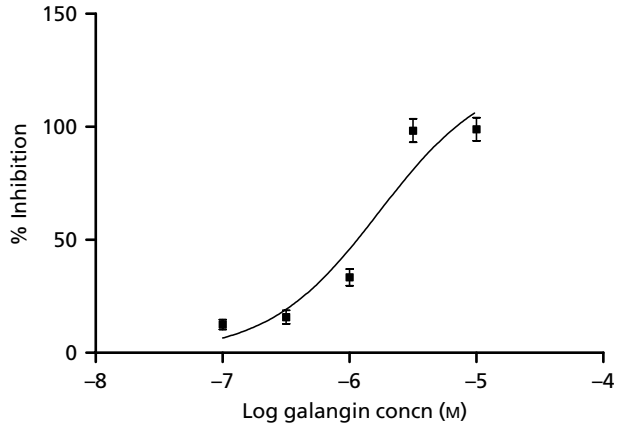

Figure 6 Dose–response curve for the inhibition of iron/ascorbate-induced lipid peroxidation of galangin.

Discussion

There is increasing evidence that flavonoids have beneficial effects and can be used for the treatment and prevention of specific diseases related to reactive oxygen species damage (Tokalov et al 2004). Although many of these effects have been linked to their potent antioxidant activity, other mechanisms of action may also contribute, such as vasodilatation and inhibition of the activity of various ATPases (So et al 1997). We studied the effects of the flavonoid galangin on pig detrusor contractility after a period of RFS.

The possible role of oxidative stress in the pathophysiology of bladder dysfunction has not been clarified. It has been suggested that the urinary bladder may undergo cyclical ischaemia/reperfusion during overdistension or increased pressure due to outlet obstruction, which in turn may lead to oxidative stress and injury by free radicals (Zhao et al 1997). Results of previous studies showed that RFS of smooth muscle strips isolated from the urinary bladder can lead to exhaustion of the synaptic stores of acetylcholine and to direct neuronal damage that does not reverse upon cessation of the RFS (Ohnishi et al 1998). These studies demonstrated that RFS caused a significant increase in the accumulation of malondialdehyde (MDA) within the smooth muscle membrane components of the bladder strips, and that both the MDA concentration and level of contractile dysfunction are directly related to the calcium concentration: high calcium enhanced both the content of MDA in the tissue and the level of contractile dysfunction, whereas low calcium reduced both parameters (Levin et al 1998). Two recent studies demonstrated that dangerously elevated levels of cytosolic Ca^{2+}, or Ca^{2+} overload, and iron overload occur during ischaemia/reperfusion in cardiac myocytes (Oudit et al 2003; Eigel et al 2004). These studies indicated that the permeation of these ions likely occurs through the L-type voltage-dependent Ca^{2+} channels as well as rapid reactivation of the Na^+/Ca^{2+} exchanger. Therefore, it can be suggested that radical scavengers capable of limiting the Ca^{2+} influx into cells may be used as effective drugs for the prevention of tissue damage by ischaemia/reperfusion injury.

The present study is consistent with other studies and has shown that RFS in the presence of a normal physiological solution results in a decrease of the contractile responses of the bladder smooth muscle to both EFS and a muscarinic agonist (Ohnishi et al 1998). Additionally, we showed that a period of RFS resulted in a much slower contraction development induced by EFS, which was demonstrated by the significantly higher time constant (the time constant was $3.08 + 0.98$ s before RFS and $10.55 + 1.98$ s after RFS). We have previously demonstrated that an active IP3 pathway was necessary for the development of a contraction with a fast rate of force development in pig urinary bladder smooth muscle (unpublished data). In the present study, the force development of contractions was affected after 90 min of RFS. We hypothesize that RFS decreases bladder smooth muscle contractility by affecting, at least partially, the IP3 pathway. We have also demonstrated that galangin at

10^{-7} M is capable of avoiding the decrease of smooth muscle contraction amplitude and the rate of force development after a period of RFS. Capasso & Tavares (2002) have shown that galangin, over a higher concentration range, reduced the contraction amplitudes evoked by electrical stimulation of rat bladder in a concentration-dependent manner. To our knowledge, this is the first time the protective effects of galangin at a lower concentration on bladder smooth muscle contractility have been demonstrated.

Many attempts have been made to elucidate the structure–activity relationships of the antioxidant activity of flavonoids (Cotelle et al 1992; Heijnen et al 2002). However, this is hampered by the fact that antioxidant activity can be explained by several factors, of which lipophilicity, iron chelation and scavenging of free radicals are the most important. Furthermore, as flavonoids are known to be good transition metal chelators, most lipid peroxidation inhibition assays measure a combination of transition metal (usually iron) chelation and radical scavenging.

Some flavonoids have a biphasic effect on the contractile responses, with a potentiation at lower concentrations (10^{-6} M) and a relaxant effect at higher concentrations (10^{-5} M) (Herrera et al 1996). At low concentrations (sub-micromolar) the protective effect of galangin predominates, while at higher concentrations ($> 10^{-5}$ M) its effect on smooth muscle relaxation prevails (Capasso & Tavares 2002). It is likely that this effect can be explained, at least partially, by its structure.

The mechanism by which galangin protects the detrusor muscle contractility from RFS injury is a matter of speculation. For example, it may be suggested that galangin can act through L-type calcium channels (Capasso & Tavares 2002) and avoid the elevated levels of cytosolic Ca^{2+} that can occur during RFS (Levin et al 1998). Further elucidation of the mechanism of action of galangin is crucial for development of new approaches for the treatment of bladder dysfunction.

Conclusion

Galangin exerted a protective effect on bladder smooth muscle contractility. Although there is no proof that the results are reproducible in functional or dysfunctional human bladders, we suggest that if the data are confirmed in-vivo, exogenously administered galangin may have a possible role in treating bladder dysfunction.

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