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Contractile mechanisms coupled to TRPA1 receptor activation in rat urinary bladder

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ABSTRACT

TRPA1 is a member of the transient receptor potential (TRP) channel family present in sensory neurons. Here we show that vanilloid receptor (TRPV1) stimulation with capsaicin and activation of TRPA1 with allyl isothiocyanate or cinnamaldehyde cause a graded contraction of the rat urinary bladder in vitro. Repeated applications of maximal concentrations of the agonists produce desensitization to their contractile effects. Moreover, contraction caused by TRPA1 agonists generates cross-desensitization with capsaicin. The TRP receptor antagonist ruthenium red (10–100 μM) inhibits capsaicin (0.03 μM), allyl isothiocyanate (100 μM) and cinnamaldehyde (300 μM)-induced contractions in the rat urinary bladder. The selective TRPV1 receptor antagonist SB 366791 (10 μM) blocks capsaicin-induced contraction, but partially reduces allyl isothiocyanate- or cinnamaldehyde-mediated contraction. However, allyl isothiocyanate and cinnamaldehyde (10–1000 μM) completely fail to interfere with the specific binding sites for the TRPV1 agonist [^{3}H]-resiniferatoxin. Allyl isothiocyanate or cinnamaldehyde-mediated contractions of rat urinary bladder, which rely on external Ca^{2+} influx, are significantly inhibited by tachykinin receptor antagonists as well as by tetrodotoxin (1 μM) or indomethacin (1 μM). Allyl isothiocyanate-induced contraction is not changed by atropine (1 μM) or suramin (300 μM). The exposure of urinary bladders to allyl isothiocyanate (100 μM) causes an increase in the prostaglandin E_2 and substance P levels. Taken together, these results indicate that TRPA1 agonists contract rat urinary bladder through sensory fibre stimulation, depending on extracellular Ca^{2+} influx and release of tachykinins and cyclooxygenase metabolites, probably prostaglandin E_2. Thus, TRPA1 appears to exert an important role in urinary bladder function.

1. Introduction

There is compelling experimental evidence indicating that members of the transient receptor potential (TRP) family of non-selective cation channels are involved in several physiological and pathological states [1,2]. The search for the molecular targets for naturally occurring substances, especially those derived from plants, has allowed the recent characterization of many TRP channels. In fact, attempts to understand the heat- and pain-generating action of the vanillyl group containing the compound capsaicin (isolated from *Capsicum* sp.) and its ultrapotent analogue resiniferatoxin (from *Euphorbium* sp.) has led to the pharmacological characterization and cloning of the vanilloid receptor (TRPV1) [3].

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TRPV1 is found mainly in sensory fibres and is gated by different painful stimuli, including the action of protons, heat, and also some lipid mediators [3–6]. Emerging evidence implies that the TRPV1 channels are probably involved in most relevant physiological and pathophysiological processes, including painful diseases, such as arthritis, diabetic neuropathy and overactive bladder [7,8]. It is now well recognized that the TRPV1-expressing sub-population of primary afferents is extremely abundant in mammalian urinary bladder [9] where they are involved in the regulation of both normal and pathological voiding reflex [10,11]. In addition, TRPV1 is found expressed in basal and superficial urothelial cells [12]. TRPV1-expressing neurons include the majority of small- and medium-size cell bodies that synthesize neuropeptides, such as substance P and calcitonin gene-related peptide [9].

Recently, novel members of TRP channels have been cloned. The TRPA1 (formerly named ANKTM1) channel was first identified as an ankyrin-like protein, with transmembrane domains, that is lost after oncogenic transformation of human fibroblasts [13]. TRPA1 has been further characterized as a thermoreceptor activated by noxious cold [14]. Besides their reaction to cold stimuli and their similarity to TRPV1, some plant-derived substances, namely allyl isothiocyanate, cinnamaldehyde and allicin isolated from mustard, cinnamon and garlic, respectively, are also capable of stimulating TRPA1 [15–18]. TRPA1 is expressed in subsets of sensory neurons where it is co-expressed with TRPV1 [14]. Recently, it has been shown that TRPA1 is located in the sensory terminals of target organs, including the urinary bladder [19], but so far is still unknown whether TRPA1 is found in non-neuronal cells. However, the functional role of TRPA1 in the urinary bladder has so far eluded definition. Therefore, the present study aims to investigate some of the mechanisms through which TRPA1 agonists mediate contractile responses in the rat urinary bladder in vitro. Since TRPA1 and TRPV1 are found co-expressed in subsets of sensory neurons, we have also compared, the contractile response caused by TRPA1 agonists with that elicited by the selective TRPV1 agonist capsaicin.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 350–400 g were used throughout the experiments. All animals were housed in a room maintained at a constant temperature of 22 ± 2 °C under a 12 h light/12 h dark cycle at 60–80% humidity with food and water available ad libitum. The experiments were carried out in accordance with the international current guidelines for the care of laboratory animals and were approved by the local ethics committee (process numbers 262/CEUA and 23080.035334/2003-16/UFSC).

2.2. Preparation of urinary bladder strips

Animals were killed by intraperitoneal barbiturate overdose (100 mg/kg pentobarbital sodium). The urinary bladder was rapidly but carefully removed. Vertical halves were excised and cleaned from connective tissue and adherent fat. Bladder strips about 10 mm long by 4 mm wide (usually four for each bladder) were placed in a Petri-dish containing Krebs–Henseleit solution (composition mM: NaCl 119.0; KCl 4.7; $MgSO_4$ 1.5; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; $KHPO_4$ 1.2 and glucose 11.0; pH 7.4). Thereafter, the bladder strips were carefully mounted in 5 ml organ baths continuously aerated with 95% O_2 and 5% CO_2 and maintained at 37 °C. Isotonic tension changes were recorded by means of a polygraph (TRI-201-Letica Scientific Instruments, Spain). Preparations were submitted to a basal tension of 1 N, followed by an equilibration period of at least 60 min. During the equilibration period, the Krebs solution was changed every 15 min and the basal tension adjusted for 1 N in the first 10 min and 15 min before carbachol application. In all experiments, the tissues were first contracted with carbachol (0.1 μM) used as the standard stimulus. After reaching the point of stability of the tonic responses, the Krebs solution was changed three times followed by a new equilibration period of at least 15 min.

Following the equilibration period, complete cumulative concentration–response curves were plotted for capsaicin (0.001–0.3 μM), allyl isothiocyanate (0.001–3000 μM) or cinnamaldehyde (0.001–3000 μM). In another set of experiments, complete non-cumulative concentration–response curves were also plotted for the same agonists, at 30 min intervals between concentrations. Only one agonist was tested in each preparation.

2.3. Desensitization to agonist-induced urinary bladder contraction

To test the possible desensitization to the contractile responses evoked by the agonists following the equilibration period, the preparations were repeatedly exposed to maximal concentrations of capsaicin (0.3 μM), allyl isothiocyanate (1 mM) or cinnamaldehyde (3 mM) with a 30 min interval between exposures. After, the preparations were exposed to the different concentrations of the agonists in order to evaluate any possible cross-desensitization among them.

2.4. Mechanism involved in the allyl isothiocyanate- and cinnamaldehyde-induced contraction

To further explore the mechanisms through which allyl isothiocyanate and cinnamaldehyde-induced contractions in the urinary bladder, preparations were pre-incubated for 20 min with one of the following drugs after the equilibration period: ruthenium red (non-selective TRP inhibitor, 10–100 μM), SB 366791 (TRPV1 antagonist, 10 μM), FK 888 (NK_1 receptor antagonist, 1 μM), SR 48968 (NK_2 receptor antagonist, 1 μM), SR 142801 (NK_3 receptor antagonist, 0.1 μM), tetrodotoxin (Na^+ channel blocker, 1 μM), indomethacin (non-selective COX inhibitor, 1 μM). The muscarinic receptor antagonist atropine (1 μM) and the non-selective purinergic receptor antagonist suramin (300 μM) were tested only against the allyl isothiocyanate-mediated contraction. In some experiments, we evaluated the participation of the TRPV1 receptor in prostaglandin E_2- or substance P-induced contraction. To do this, we contracted rat urinary bladder with prostaglandin E_2 (3 μM) or substance P (0.01 μM) in the presence or absence of

TRPV1 antagonist SB 366791 (10 μM). All experiments on the urinary bladder with exogenous substance P were carried out in the presence of peptidase inhibitors (3 μM captopril and 1 μM phosphoramidon) to inhibit peptide degradation. The choice of the concentration of each drug and the time of pre-incubation were selected on the basis of previous data from the literature [20–24].

The influence of extracellular Ca^{2+} on allyl isothiocyanate- and cinnamaldehyde-induced contraction of rat urinary bladder was investigated as previously described [25]. After a 60 min equilibration period, the preparations were transferred to Krebs–Henseleit solution without Ca^{2+} containing 1 mM of EGTA for 20 min, during which the bath solution was renewed every 5 min. Responses to allyl isothiocyanate and cinnamaldehyde were subsequently obtained in Ca^{2+}-free medium. After washout, the preparations were transferred to normal Krebs solution, and after 30 mins' equilibration the new contractions were recorded to assess the recovery of the allyl isothiocyanate and cinnamaldehyde contractile responses.

2.5. *Prostaglandin E_2 release in response to allyl isothiocyanate*

In order to investigate further the role of prostaglandin E_2 in the allyl isothiocyanate-induced contraction in rat urinary bladder, the levels of this prostanoid were measured before and after allyl isothiocyanate incubation in the preparations. The protocol used was identical to that employed in functional studies, and samples (500 μl) of the Krebs–Henseleit nutritive solution were collected from the cubes with a micropipette before stimulation with allyl isothiocyanate and also at peak of the contraction obtained after addition of allyl isothiocyanate (100 μM). Samples were frozen in liquid nitrogen and stored at −70 °C until assay. The prostaglandin E_2 levels were assessed using a specific enzyme immunoassay according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, USA). The results were expressed in picograms of prostaglandin E_2 per millimeter.

2.6. *Substance P release in response to allyl isothiocyanate*

In order to investigate further the role of substance P in the allyl isothiocyanate-mediated contraction in rat urinary bladder, the levels of this neuropeptide were measured before and after allyl isothiocyanate incubation. The protocol used was identical to that employed in functional studies, and samples (500 μl) of the Krebs–Henseleit nutritive solution were collected from the cubes with a micropipette before stimulation with allyl isothiocyanate and also at peak of the contraction obtained after addition of allyl isothiocyanate (100 μM). Samples were frozen in liquid nitrogen and stored at −70 °C. After, the samples were lyophilized to achieve concentration, and stored again at −70 °C until assay. On the day of the assay, samples were diluted with enzyme immunoassay buffer and the substance P levels were assessed using a specific enzyme immunoassay according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, USA). The results were expressed in picograms of substance P per milliliter.

2.7. *Binding of [^{3}H]-resiniferatoxin*

Binding assays were carried out as described previously [26,27]. To obtain membranes for the binding studies, spinal cords of rats were removed and disrupted with the aid of a tissue homogenizer in ice-cold buffer A (in mM, KCl 5; NaCl 5.8; $MgCl_2$ 2; $CaCl_2$ 0.75; sucrose 137 and HEPES 10, pH 7.4). The homogenate was first centrifuged for 10 min at 1000 × *g* at 4 °C; the low speed pellets were discarded; the supernatants were further centrifuged for 30 min at 35,000 × *g* at 4 °C; and the resulting high speed pellets, re-suspended in buffer A, were stored at −70 °C until assay.

Binding assays were carried out in duplicate with a final volume of 500 μl, containing buffer A, supplemented with 0.25 mg/ml bovine serum albumin, membranes (100 μg protein/ml), 50 pM of [^{3}H]-resiniferatoxin in the presence or absence of capsaicin (10 μM), allyl isothiocyanate (10–1000 μM), cinnamaldehyde (10–1000 μM) or their vehicles (ethanol 0.01% and tween 80 0.01%). Non-radioactive resiniferatoxin (100 nM) was included in some of the tubes to measure non-specific binding.

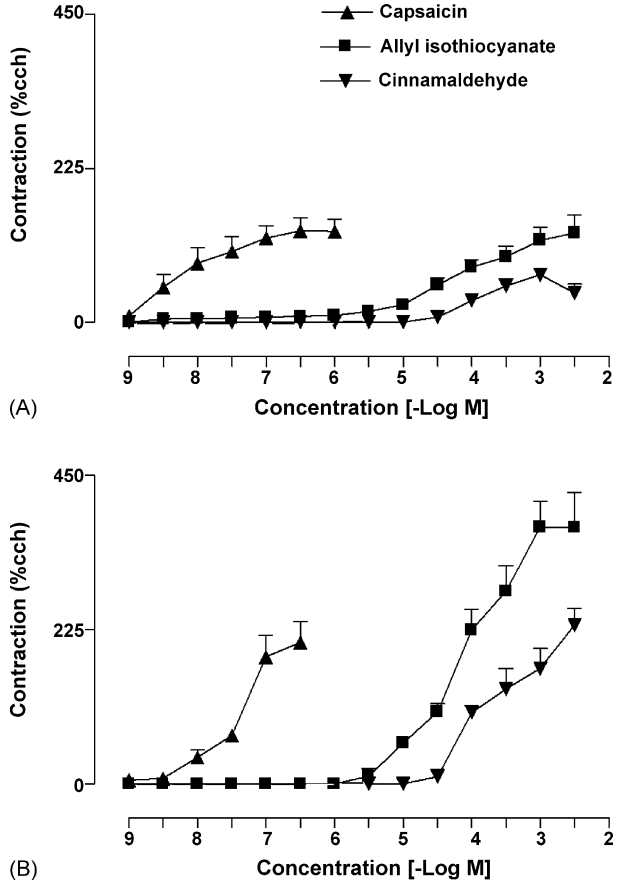

Fig. 1 – Mean cumulative (A) or non-cumulative (B) concentration–response curves for capsaicin, allyl isothiocyanate and cinnamaldehyde in the isolated rat urinary bladder. Results are expressed as the percentage of contraction induced by 0.1 μM of carbachol. Each point represents the mean and vertical lines indicate the S.E.M. of four to six experiments.

Assay mixtures were set up on ice and the binding reaction was then initiated by transferring the assay tubes to a 37 °C water bath. Following a 60 min incubation period, binding reaction was stopped by cooling the tubes on ice. Subsequently, 50 μl of α_1-acid glycoprotein (2 mg/ml) was added to each tube to reduce non-specific binding. Finally, separation of bound and free membranes [^{3}H]-resiniferatoxin was achieved by centrifuging for 15 min at 20,000 × *g* at 4 °C. The pellet was quantified by scintillation counting. Specific binding was calculated as the difference of the total and non-specific binding.

2.8. Drugs

The following drugs were used: capsaicin, allyl isothiocyanate, cinnamaldehyde, resiniferatoxin, tetrodotoxin, carbachol, atropine sulphate, EGTA (ethyleglycol-bis-(-aminoethyl aether) *N*,*N* (-tetraacetic acid)), ruthenium red (ammoniated ruthenium oxychloride), α_1-acid glycoprotein, substance P, captopril, phosphoramidon (all from Sigma, St. Louis, USA), [^{3}H]-resiniferatoxin (37 Ci/mmol, Perkin Elmer Life Sciences, Washington, USA), SB 366791 (4′-chloro-3-methoxycinnamanilide, Tocris Bioscience, Ellisville, USA), suramin sodic (Germanin®, Bayer, Leverkusen, Germany), prostaglandin E_2 (Cayman Chemical Company, Ann Arbor, MI, USA). The NK_2 receptor antagonist SR 48968 (*S*)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl]benzamide and the NK_3 receptor antagonist SR 142801 (*S*)-*N*-(1-3-(1-benzoyl-3-(3,4 dicholorophenyl) piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-*N*-methylacetamide were kindly supplied by Sanofi Recherche (Montpellier, France). The NK_1 receptor antagonist FK 888N^2-[(4R)-4-hydroxy-1-(1-methyl-1*H*-indol-3-yl)carbony-1-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-2-(2-naphtyl)-L-alaninamide was kindly supplied by Fujisawa Pharmaceutical (Osaka, Japan). The stock solutions of FK 888, SR 48968, SR 142801, SB 366791, prostaglandin E_2, capsaicin, tetrodotoxin and substance P were prepared in absolute ethanol, put into siliconized plastic tubes and kept in a freezer at −18 °C until use. Solutions of allyl isothiocyanate and cinnamaldehyde were made in ethanol and tween 80 and were diluted on the day of experiment just before use. Other drugs were dissolved in deionized water. The final bath concentrations of ethanol did not exceed 0.1% and tween 80 did not exceed 0.06%. In all experimental groups, at least one parallel control experiment was carried out in the presence of the vehicle used to dilute the drugs. The vehicles used had no pharmacological effects either on the tonus of preparations or on the agonist-induced contractions.

2.9. Statistical analysis

All values are expressed as mean ± S.E.M., except for the EC_{50} or IC_{50} values (i.e., the concentration of agonists necessary to produce 50% of the contractile response relative to the maximum effect, or the concentration of antagonists necessary to reduce agonist response by 50% relative to the control

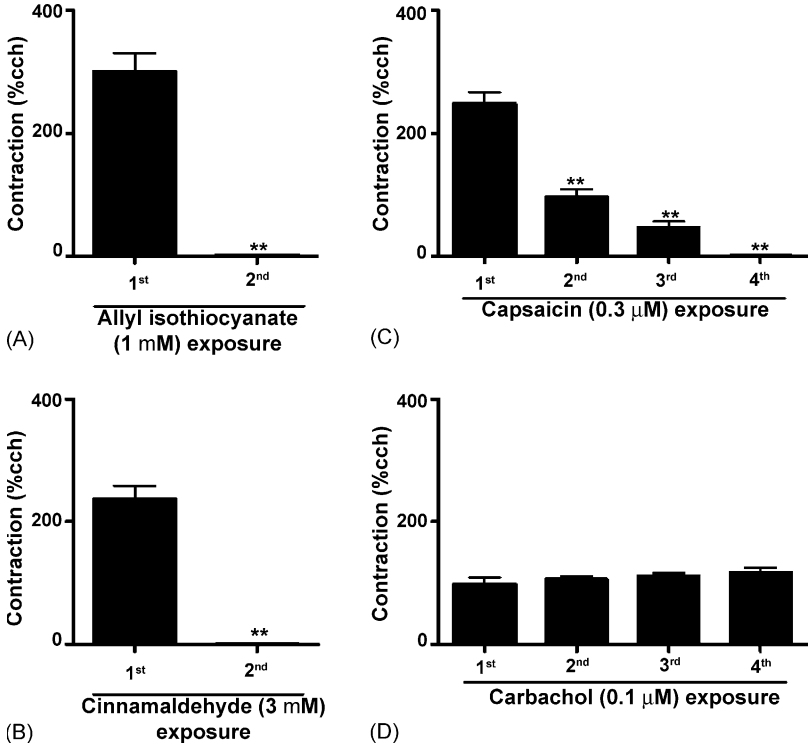

Fig. 2 – Desensitization to the contractile responses caused by capsaicin (0.3 μM, A), allyl isothiocyanate (1 mM, B) or cinnamaldehyde (3 mM, C), but not by carbachol (0.1 μM, D), in the isolated rat urinary bladder. Results are expressed as the percentage of contraction induced by 0.1 μM of carbachol. Each column represents the mean and vertical lines indicate the S.E.M. of four to six experiments. The asterisks denote the significance levels. **$P < 0.01$, compared with the first contraction (one-way ANOVA followed by Dunnett's test for C and Student's unpaired *t*-test for A, B).

value, respectively), which are given as geometric means accompanied by their respective 95% confidence limits. The EC_{50} and IC_{50} values were estimated by using four to six concentrations of each drug, between the minimum and maximum effect, using the linear regression for individual experiments with the GraphPad Prism software. The percentages of inhibition are reported as mean ± S.E.M. of inhibitions obtained in each individual experiment in relation to agonist response. Statistical significance was performed by Student's *t*-test or by one-way ANOVA followed by Dunnett's test. *P* values less than 0. 05 ($P < 0.05$) were considered to be statistically significant.

3. Results

Addition of capsaicin (0.001–0.3 μM), allyl isothiocyanate (0.001–3000 μM) or cinnamaldehyde (0.001–3000 μM) to the bath solution caused a concentration-dependent contraction of the rat urinary bladder (Fig. 1A and B). In the cumulative concentration–response curves, the obtained mean EC_{50} values were 0.01 (0.007–0.06), 41.1 (7.6–221.3) or 372.4 (46.2–2999.1) μM and maximal responses were 132.6 ± 17.4%, 130.5 ± 25.4% or 69.5 ± 8.4% (relative to contraction-induced by carbachol) for capsaicin, allyl isothiocyanate or cinnamaldehyde, respectively (Fig. 1A). When non-cumulative concentration–response curves were plotted, a marked increase in the efficacies was observed, but not in the potencies of the contractile effects induced by all the agonists. The estimated mean EC_{50} values for these effects were 0.04 (0.02–0.07), 63.1 (46.5–89.3) and 220 (162.2–299.2) μM, and the maximal effects (relative to carbachol) were 206.6 ± 30.6%, 374.1 ± 51% or 231.4 ± 24.1%, for capsaicin, allyl isothiocyanate or cinnamaldehyde, respectively (Fig. 1B). Comparison of cumulative and non-cumulative concentration–response curves suggests a rapid process of receptor

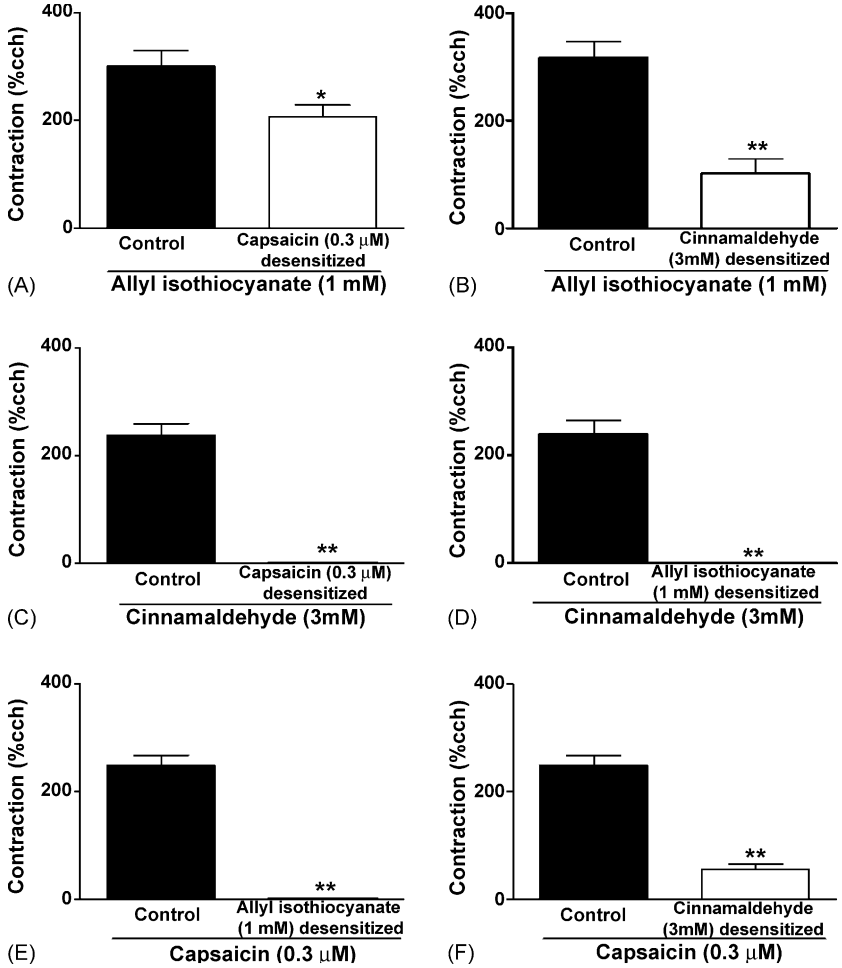

Fig. 3 – Effect of desensitization of preparations by capsaicin (0.3 μM) on the contractile effect induced by allyl isothiocyanate (1 mM) or cinnamaldehyde (3 mM) (A and C, respectively). Desensitization caused by allyl isothiocyanate (1 mM) on the contractile effect induced by cinnamaldehyde (3 mM) or capsaicin (0.3 μM) (D and E, respectively); or desensitization caused by cinnamaldehyde (3 mM) on the contractile effect induced by allyl isothiocyanate (1 mM) or capsaicin (0.3 μM) (B and F, respectively) in rat urinary bladder. Results are expressed as the percentage of contraction induced by 0.1 μM of carbachol. Each column represents the mean and vertical lines indicate the S.E.M. of four to six experiments. The asterisks denote the significance levels. *P < 0.05, **P < 0.01, compared with the agonist-induced contraction, without previous desensitization (Student's unpaired *t*-test).

desensitization, mainly for the allyl isothiocyanate- and cinnamaldehyde-mediated responses.

In fact, repeated applications of the maximal concentrations of capsaicin (0.3 μM), allyl isothiocyanate (1 mM) or cinnamaldehyde (3 mM), each at 30 min intervals, but not of carbachol (0.1 μM) (Fig. 2D), caused marked desensitization of their own contractile effects in the rat urinary bladder. However, complete desensitization to allyl isothiocyanate- or cinnamaldehyde-mediated contractions was obtained already in the second challenge of the TRPA1 agonists, while complete desensitization to capsaicin contraction was only observed in the fourth challenge of this TRPV1 agonist (Fig. 2A–C).

We also observed a cross-desensitization among the tested agonists. Allyl isothiocyanate- or cinnamaldehyde-induced contractions were significantly attenuated in capsaicin-desensitized urinary bladder preparations, with reductions of 31.1 ± 7.3% and 100% when compared with naive preparations (Fig. 3A and C). Likewise, capsaicin-induced contraction was reduced by 100% and 65.5 ± 7.0% in allyl isothiocyanate- or cinnamaldehyde-desensitized tissues, respectively (Fig. 3E and F). The probable cross-desensibilization between the TRPA1 agonists was also analysed. The allyl isothiocyanate-induced contraction was reduced by 68.1 ± 8.8% in cinnamaldehyde-desensitized tissues, while cinnamaldehyde-induced contraction was abolished (100%) in allyl isothiocyanate-desensitized tissues (Fig. 3B and D). Since all of the agonists showed desensitization after repetitive challenges, we used a single sub-maximal concentration of capsaicin (0.03 μM), allyl isothiocyanate (100 μM) or cinnamaldehyde (300 μM) to characterize further the mechanisms involved in their contractile effects in the rat urinary bladder.

The results in Fig. 4 show that the non-selective TRP antagonist ruthenium red (1–100 μM) blocked, in a graded manner, both the capsaicin- and the allyl isothiocyanate-induced urinary bladder contraction. However, ruthenium red was about 10-fold less potent in antagonizing the allyl isothiocyanate in comparison with capsaicin-mediated contraction. The estimated mean IC_{50} values for these effects were 2.2 (1.8–2.8) and 22.1 (17.8–27.5) μM, respectively. Moreover, ruthenium red (100 μM) greatly reduced cinnamaldehyde-induced contraction by 85.7 ± 14.3% (result not shown).

To assess the contribution of external Ca^{2+} to the contractile responses elicited by allyl isothiocyanate (100 μM) and cinnamaldehyde (300 μM), some experiments were carried out in Ca^{2+}-free medium containing EGTA (1 mM). Similar to those described for capsaicin [28], the contractile responses elicited by allyl isothiocyanate and cinnamaldehyde were totally abolished in Ca^{2+}-free medium containing EGTA (1 mM) (Fig. 5A and B). When preparations were transferred to normal Krebs solution (containing Ca^{2+}, 2.5 mM), the contraction caused by allyl isothiocyanate was rapidly recovered (results not shown). In contrast to capsaicin [28], the addition of tetrodotoxin (1 μM) to the preparations significantly reduced the contractile response induced by allyl isothiocyanate and cinnamaldehyde (27.9 ± 6.9% and 38.1 ± 8.8%, respectively) (Fig. 5C and D). The selective antagonists of tachykinin NK_1 (FK 888, 1 μM), NK_2 (SR 48968, 1 μM) or NK_3 (SR 142801, 0.1 μM) receptors, or the non-selective cyclooxygenase inhibitor indomethacin (1 μM), also caused significant inhibition of allyl isothiocyanate-mediated contraction in rat urinary bladder (with inhibitions of 29.4 ± 5.8%, 35.9 ± 7.5%, 35.2 ± 8.3% and 31.6 ± 6.5%, respectively) (Fig. 6A and C). However, only FK888, SR 48968 and indomethacin reduced cinnamaldehyde-induced contraction (with inhibitions of 36.2 ± 7.1%, 36.8 ± 6.7% and 48.9 ± 9.0%, respectively) (Fig. 6B and D). The antagonists of muscarinic (atropine, 1 μM) or purinergic (suramin, 300 μM) receptors did not alter allyl isothiocyanate-induced contraction (Fig. 5E and F).

Next, we assessed whether prostaglandin E_2 could be the cyclooxygenase metabolite involved in the allyl isothiocyanate-mediated contractile response by measuring the levels of this prostanoid in the nutrient solution. Even in basal conditions, there was a substantial production of prostaglandin E_2 in the rat urinary bladder. Following stimulation with allyl isothiocyanate (100 μM), a significant increase in prostaglandin E_2 levels was observed (Fig. 6F). Moreover, we also confirmed the participation of substance P in the allyl isothiocyanate-mediated contractile response by measuring the levels of this neuropeptide in the nutrient solution. In basal condition, there was only a small release of substance P in the rat urinary bladder. Following stimulation with allyl isothiocyanate (100 μM) caused a significant increase in substance P levels (Fig. 6E).

The pre-incubation with the selective TRPV1 antagonist SB 366791 (10 μM) abolished the capsaicin-induced contraction (inhibition of 100%, Fig. 7A), but only partially reduced (inhibition of 38.2 ± 4.9% and 34.3 ± 4.8%, respectively) the allyl isothiocyanate- and cinnamaldehyde-mediated urinary bladder contractions (Fig. 7B and C). The inhibition of the contractile effect of allyl isothiocyanate and cinnamaldehyde caused by SB 366791 seems to be unrelated with a direct

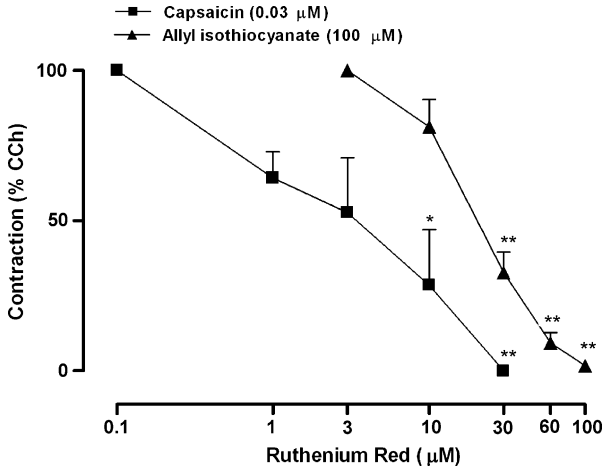

Fig. 4 – Mean inhibitory concentration–response curves for ruthenium red in the contraction induced by capsaicin (0.03 μM) or allyl isothiocyanate (100 μM) in the rat urinary bladder. Results are expressed as the percentages of the contraction induced by 0.1 μM of carbachol. Each point represents the mean with vertical lines showing the S.E.M. for four to six experiments. Significant differences from respective control values $^{*}P < 0.05$, $^{**}P < 0.01$ (one-way ANOVA followed by Dunnett's test).

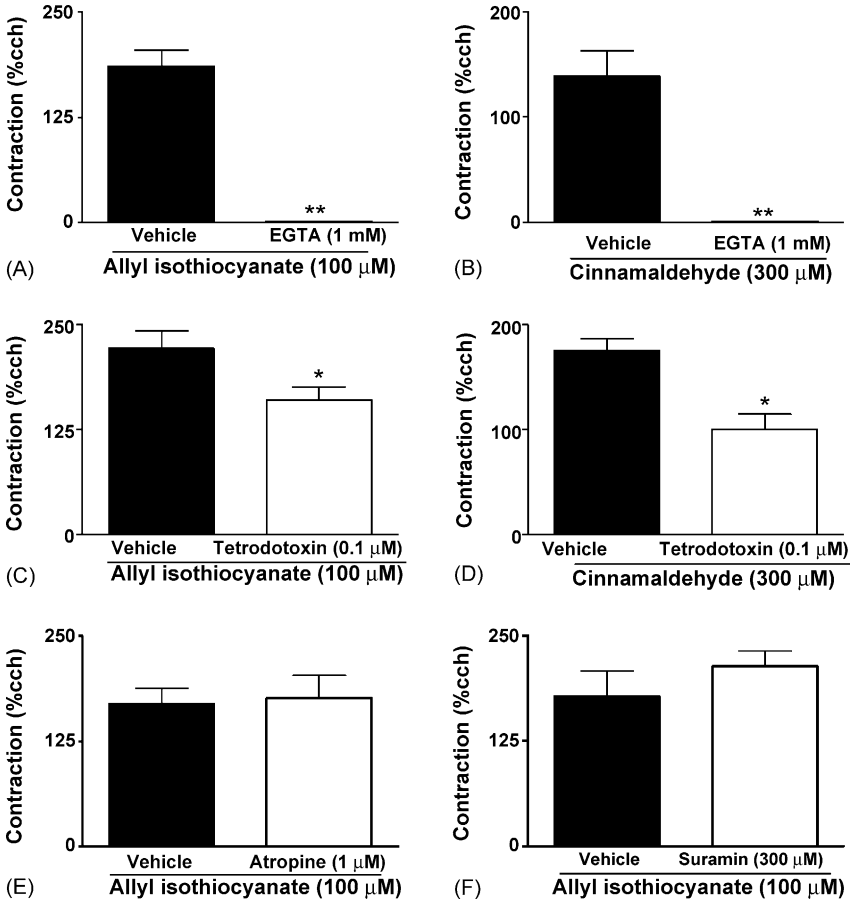

Fig. 5 – Mean contractile responses for allyl isothiocyanate (100 μM) or cinnamaldehyde (300 μM) obtained in the rat urinary bladder in Ca^{2+}-free solution plus EGTA (A and B, respectively), or in the absence or presence of tetrodotoxin (0.1 μM) (C and D, respectively). Mean contractile response induced by allyl isothiocyanate (100 μM) in the absence or presence of atropine (1 μM, E) or suramin (300 μM, F). In all the graphics (A–F) results are expressed as the percentages of contraction induced by 0.1 μM of carbachol. Each column represents the mean, with vertical lines showing the S.E.M. for four to six experiments. Significant differences from respective control values $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's unpaired *t*-test).

interaction of these TRPA1 agonists with TRPV1 receptors, as neither allyl isothiocyanate nor cinnamaldehyde (up to 1000 μM) were capable of interfering with the specific binding site of [^{3}H]-resiniferatoxin in the rat spinal cord membranes (Fig. 7F). Next, we investigated whether or not the putative TRPV1 stimulation could be indirectly mediated by activation of substance P or prostaglandin E_2 receptors. However, SB 366791 (10 μM) did not significantly interfere with prostaglandin E_2- or substance P-induced contractions (Fig. 7D and E).

4. Discussion

The main data of the present study is that the TRPA1 agonists allyl isothiocyanate and cinnamaldehyde, similar to the TRPV1 agonist capsaicin, elicited a concentration-dependent contraction of rat isolated urinary bladder. Allyl isothiocyanate was about three-fold more potent and 60% more efficacious than cinnamaldehyde in inducing rat urinary bladder contractile response, according to the analysis at EC_{50} and maximal effect levels when experiments were carried out by the non-cumulative method. Reinforcing the idea that these substances are acting on TRPA1 receptors to produce their contractile effects, the used concentrations of cinnamaldehyde and allyl isothiocyanate in our study were quite similar to that employed for intracellular Ca^{2+} increment in cells expressing TRPA1 [14]. However, different from our study, Bandell et al. [15] reported that both cinnamaldehyde and allyl isothiocyanate exhibited the same efficacy in the cited intracellular Ca^{2+} study. This discrepancy may be explained, at least in part, by the underestimated value of the cinnamaldehyde maximal effect obtained in our study, since concentrations of cinnamaldehyde higher than 3 mM could not be tested due to the limitation in solubility.

Comparing the TRPA1- and TRPV1-mediated contractile responses in rat urinary bladder, capsaicin was about 4000-fold more potent than allyl isothiocyanate in inducing contractile response, although allyl isothiocyanate was as efficacious as capsaicin in the induction of contraction when experiments were carried out through the cumulative method. These results further reinforce the previous findings [29] arising from the use of cumulative concentration–response curves, i.e., that allyl isothiocyanate was less potent, but had the same efficacy as capsaicin in eliciting contraction

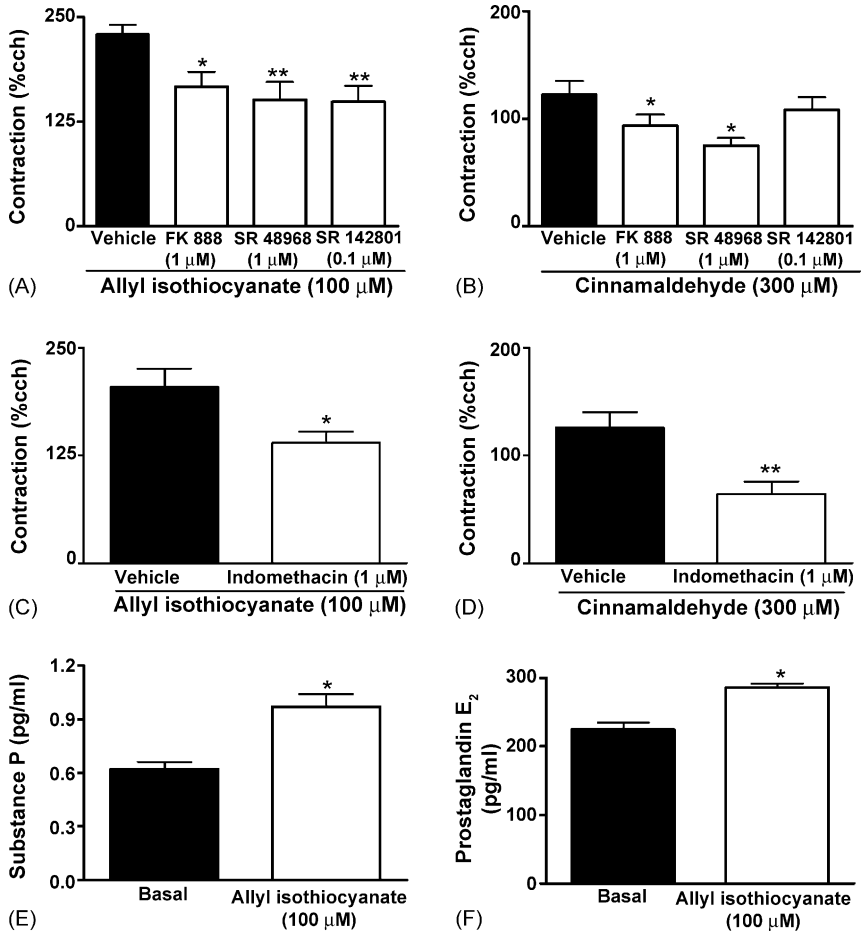

Fig. 6 – Mean contractile responses obtained for allyl isothiocyanate (100 μM) and cinnamaldehyde (300 μM) in the rat urinary bladder in the absence or presence of FK 888 (1 μM), SR 48968 (1 μM) or SR 142801 (0.1 μM) (A and B, respectively) or indomethacin (1 μM) (C and D, respectively). Results are expressed as the percentages of contraction induced by 0.1 μM of carbachol. Release of substance P (E) or prostaglandin E_2 (F) induced by allyl isothiocyanate (100 μM) in the isolated urinary bladder. The levels of substance P or prostaglandin E_2 were established by ELISA and are described in Section 2. Each column represents the mean, with vertical lines showing the S.E.M. for four to six experiments. Significant differences from respective control values $^{*}P < 0.05$, $^{**}P < 0.01$ (one-way ANOVA followed by Dunnett's test for A and B, Student's unpaired *t*-test for C and D and Student's paired *t*-test for E and F).

responses in the rat urinary bladder. When non-cumulative curves were plotted, our results revealed that the efficacy, but not the potency, of the tested compounds was greatly increased. It has been suggested that the reduction of the efficacy for certain agonists, when experiments are carried out through the cumulative concentration–response method, in comparison with non-cumulative curves, could probably be explained by the rapid receptor desensitization process [30–32].

It is now well recognized that prolonged or repeated stimulation with capsaicin results in a marked desensitization of recombinant or native TRPV1 receptor [33,4]. Nagata et al. [19] have shown that allyl isothiocyanate-induced current in HEK 293 cells expressing TRPA1 desensitize in hyperpolarized cells, but not in depolarized cells. Our results further extend the previous findings [29] which have demonstrated that both capsaicin and mustard oil produce desensitization in the urinary bladder contractile effect. Moreover, we found that capsaicin desensitized preparations were unresponsive to cinnamaldehyde, but were still partially responsive to allyl isothiocyanate. Another interesting piece of data in the present study was the complete desensitization to cinamaldehyde in allyl isothiocyanate exposed bladders and the incomplete desensitization to allyl isothiocyanate in cinnamaldehyde pre-treated preparations. These results suggest that cinnamaldehyde and capsaicin use the same subset of sensory fibres to produce their contractile effects, but that allyl isothiocyanate stimulates a distinct subset of fibres. Interestingly, Bandell et al. [15] demonstrated that cinnamaldehyde was more specific than allyl isothiocyanate in the stimulation of cold-activated dorsal root ganglion neurons. These results lead the authors to suggest that allyl isothiocyanate must have been acting via another receptor or even in a different TRPA1 receptor subtype. Recent studies carried out in cells expressing recombinant receptors have found that the allyl isothiocyanate and cinnamaldehyde were not able to stimulate TRPV1,

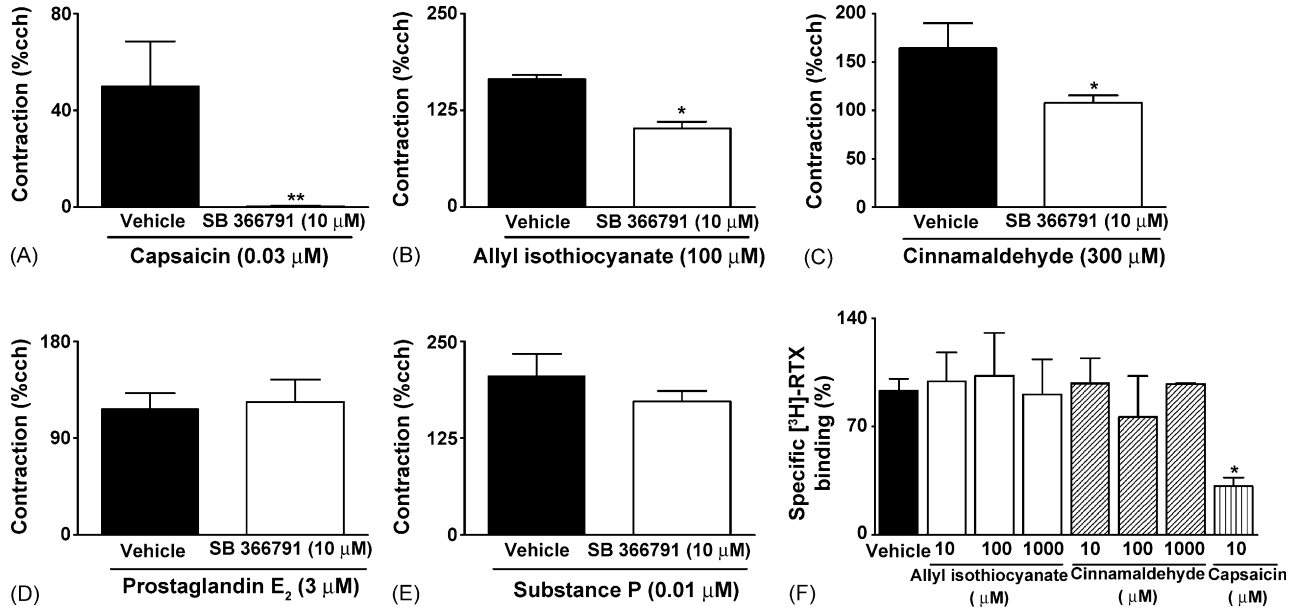

Fig. 7 – Mean contraction responses induced by capsaicin (0.03 μM (A), allyl isothiocyanate (100 μM (B), cinnamaldehyde (300 μM (C), prostaglandin E_2 (3 μM (D) or substance P (0.01 μM (E) in the rat urinary bladder obtained in the absence or presence of selective TRPV1 antagonist SB 366791 (10 μM). Results are expressed as the percentages of contraction induced by 0.1 μM of carbachol. Each column represents the means, with vertical lines showing the S.E.M. for four to six experiments. Assay of competition for [^{3}H]-resiniferatoxin binding to spinal cord membranes of rats by capsaicin (10 μM), allyl isothiocyanate (10–1000 μM) or cinnamaldehyde (10–1000 μM) (F). Each column represents the mean, with vertical lines showing the S.E.M. of three experiments conducted in duplicate. Results are plotted as percentage of control. Significant differences from respective control values $^*P < 0.05$, $^{**}P < 0.01$ (Student's unpaired *t*-test for A–E and one-way ANOVA followed by Dunnett's test for F).

TRPV3, TRPV4 or TRPM8 [15,16]. However, more studies must be conduced in order to verify the selectivity of allyl isothiocyanate and cinnamaldehyde on other targets, including some ion channels and G-protein coupled receptors.

Both TRPA1 and TRPV1 are channels that allow cation influx into the cell [16,3]. Our results show that allyl isothiocyanate-mediated contraction of rat urinary bladder was totally abolished by the removal of external Ca^{2+} from the medium, demonstrating that TRPA1 function is also largely Ca^{2+}-dependent. The mechanism for activation of the sensory fibres of guinea-pig bladder induced by capsaicin requires the presence of extracellular Ca^{2+} [28], demonstrating that TRPV1 function is also Ca^{2+}-dependent. The TRPV1 pore loop has negative charges that bind both divalent cations and the TRP inhibitor ruthenium red [34]. Ruthenium red is an inorganic polycationic dye that non-selectively blocks response to several members of the TRP channel family [35]. Our results with rat urinary bladder and previous findings observed in cells expressing TRPV1 or TRPA1 [34,19] show that ruthenium red inhibited both TRPV1 and TRPA1 agonists, being about 10-fold more potent in inhibiting capsaicin than the allyl isothiocyanate-mediated contractions. Together, these results suggest that both TRPV1 and TRPA1 agonists activate Ca^{2+} influx to induce urinary bladder contraction.

A great amount of evidence now indicates that capsaicin-induced contraction of rat isolated bladder is associated with primary sensory fibre stimulation in the bladder wall through a tetrodotoxin-resistant depolarization [36]. Previous findings [29] and presents results demonstrate that in contrast with capsaicin, allyl isothiocyanate-mediated contraction in this preparation exhibits both tetrodotoxin-sensitive and resistant components. Our data extends this observation to cinnamaldehyde-induced contraction. It has been shown that the activation of cholinergic and purinergic fibres contracts the urinary bladder through a tetrodotoxin-sensitive mechanism [37,38]. However, we have found that allyl isothiocyanate does not appear to stimulate these fibres in the rat urinary bladder, since muscarinic or purinergic receptor antagonists were not capable of altering its contractive effect. Another tetrodotoxin-sensitive component in the urinary bladder contraction seems to be the axon reflex arrangement [29]. It is well known that the axon reflex releases neuropeptides from sensory fibre branches and, in fact, we observed a role of neuropeptides in the allyl isothiocyanate- or cinnamaldehyde-mediated contractions in the rat urinary bladder.

Tachykinins are neuropeptides present in capsaicin-sensitive primary afferent nerves of the rat urinary bladder and other mammalian species. The peripheral release of these peptides may have important effects on detrusor function [39]. Our results show that in the rat isolated urinary bladder, allyl isothiocyanate produces a contraction that is significantly reduced by NK_1, NK_2 and NK_3 selective antagonists, suggesting that the release of substance P, neurokinin A and neurokinin B exerts an important role in the allyl isothiocyanate-induced contraction. Confirming the participation of tachykinins in this process, the application of allyl isothiocyanate to rat

urinary bladder preparations induces a significant increase in the levels of substance P in the organ bath. Our results are in line with those of a recent study by Bautista et al. [17] who demonstrated that allyl isothiocyanate and allycin (another TRPA1 agonist from garlic) induces relaxation of mesenteric arterial segments in vitro by releasing neuropeptides from capsaicin-sensitive nerve fibres that innervate vascular smooth muscle. However, different from allyl isothiocyanate, the NK_3 antagonist is unable to alter cinnamaldehyde-mediated contraction. As discussed above, these results again suggest that cinnamaldehyde is unlikely to stimulate all subsets of sensory fibres activated by allyl isothiocyanate.

Besides tachykinins, we also investigated whether the cyclooxygenase metabolites could be involved in allyl isothiocyanate-induced contraction. Prostanoids are important modulators of bladder function and micturition, and are potent spasmogens of the urinary bladder [40]. Present data shows that the inhibition of cyclooxygenase pathways significantly reduces the TRPA1 agonist-mediated contraction. Of note, the application of allyl isothiocyanate to rat urinary bladder induces a fast increase in the levels of prostaglandin E_2 in the organ bath, an action that seems to correlate well with the allyl isothiocyanate-mediated contractile effect in these preparations.

Our results also demonstrated that capsaicin or TRPA1 agonist-induced contraction of the urinary bladder was abolished or reduced by antagonist TRPV1 SB366791, respectively. There are at least two explanations for these results. First, allyl isothiocyanate or cinnamaldehyde might interact directly with TRPV1. However, this idea seems to be improbable, since we observed that both compounds at concentrations up to 1000 μM completely failed to interfere with the specific binding site of [^{3}H]-resiniferatoxin. Moreover, literature data has shown that neither allyl isothiocyanate nor cinnamaldehyde is capable of activating Ca^{2+} influx into cells transfected with rat TRPV1 [15]. Nevertheless, our binding study cannot exclude the possibility that allyl isothiocyanate or cinnamaldehyde could interact with TRPV1 in a site different from that recognized by [^{3}H]-resiniferatoxin. Second, some mediators released by TRPA1 agonists might stimulate TRPV1 indirectly. In fact, it is well established that several metabotropic receptors activate TRPV1 through indirect mechanisms, such as phosphorylation or phosphatidylinositide removal [2]. Again, it seems not to be the case, at least for prostaglandin E_2 or NK_1 receptors, since we observed that SB36791 was not capable of altering either prostaglandin E_2- or substance P-induced rat urinary bladder contractions. Finally, SB36791 might interact directly with TRPA1 receptors to inhibit allyl isothiocyanate or cinnamaldehyde responses. Thus, additional studies using recombinant cells expressing this receptor need to be carried out to clarify this point.

Collectively, the results of the present study have shown that TRPA1 stimulation with agonists produces graded contraction of the rat urinary bladder through the stimulation of sensory neurons and neuropeptide and prostanoid release. The contractile effect of allyl isothiocyanate was found to be more efficacious than that of capsaicin, suggesting a relevant role for TRPA1 in normal urinary bladder function. It is tempting to speculate that TRPA1 could play a role in bladder disorders, since sensory afferent dysfunction has been involved in some of the pathologies.

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