Inflammation Modifies the Role of Cyclooxygenases in the Contractile Responses of the Rat Detrusor Smooth Muscle to Kinin Agonists

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ABSTRACT

The contractile responses elicited by the selective kinin B1 and B2 receptor agonists [desArg9]-bradykinin ([desArg9]-BK) and [Hyp3, Tyr(Me)8]-bradykinin ([Hyp3, Tyr(Me)8]-BK) (1 nM–10 μM), respectively, were evaluated in control vs. inflamed bladders and likewise abolished that produced by [desArg9]-BK in both control and inflamed bladders. Piroxicam (30 μM) prevented the PGE2 production evoked by [Hyp3, Tyr(Me)8]-BK in both control and inflamed bladders and likewise abolished that produced by [desArg9]-BK (1 μM) reduced the PGE2 production elicited by [desArg9]-BK in control and inflamed bladders. When NS-398 was tested on the [Hyp3, Tyr(Me)8]-BK-induced PGE2 production, it inhibited PGE2 production in the inflamed bladders only, without significantly modifying the response obtained in controls. These findings demonstrate that 1) in normal bladders, the activation of B1 and B2 receptors evokes contraction that is largely mediated by COX-1 metabolites, whereas the COX-2 appears to be involved in PGE2 production, and 2) in inflamed bladders, the activation of B1 and B2 receptors still produce PGE2, but the contractile response is not reduced by COX inhibitors, a result that indicates that additional mechanisms play a compensatory role.

Kinis and their C-terminal desArg metabolites are formed from kininogen precursors after inflammation or tissue damage. Kinins exert their effects by activating two types of receptors termed B1 and B2 (Regoli and Barabé, 1980). Initially defined on the basis of pharmacological criteria, the existence of kinin B1 and B2 receptors has been confirmed with gene cloning and receptor expression studies (Hess, 1997); both receptors belong to the family of G protein-coupled receptors. Most pharmacological effects of kinins are mediated by the B2 receptor which is constitutively expressed by different cell types, whereas the B1 receptor is up-regulated or expressed de novo after injury (Marceau, 1995).

In the urinary bladder, kinins generated during inflammatory processes may be involved in the pathogenesis of cystitis (Maggi, 1997). BK strongly stimulates urinary bladder motility (Maggi et al., 1993; Lecci et al., 1995) and induces edema (Giuliani et al., 1993; Ahluwalia et al., 1994); it has been demonstrated that kinin B2 receptor

ABBREVIATIONS: BK, bradykinin; [desArg9]-BK, [desArg9]-bradykinin; [Hyp3, Tyr(Me)8]-BK, [Hyp3, Tyr(Me)8]-bradykinin; B 9858, H-Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-OH; Hoe 140, H-DArg-Arg-Pro-Hyp-Thi-Ser-DTic-Oic-Arg-OH; COX, cyclooxygenase.

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antagonists ameliorate chemically induced experimental cystitis (Giuliani et al., 1993; Maggi et al., 1993; Ahluwalia et al., 1994). It is also known that a relevant fraction of the contractile response of the normal rat bladder to B2 receptor agonists can be blocked by COX inhibitors (Marceau et al., 1980; Pinna et al., 1992; Lecci et al., 1995). COX enzyme exists in two different isoforms, the constitutive (COX-1) and the inducible (COX-2), the latter being up-regulated by cytokines, by lipopolysaccharide and during inflammation (Mitchell et al., 1995).

The aim of this study was to investigate whether different COX isoenzymes are involved in mediating the contractile responses induced by selective agonists of the B1 and B2 receptor: [desArg9]-BK and [Hyp3, Tyr(Me)8]-BK, respectively (Khaleel et al., 1990; Regoli et al., 1991), in control conditions as compared with a cystitis model. Cystitis was induced by the i.p. administration of cyclophosphamide in rats (Grinberg-Funes et al., 1990; Maggi et al., 1992). The selectivity of [desArg9]-BK and [Hyp3, Tyr(Me)8]-BK in producing contractile responses in control and inflamed bladders was checked by using the B1 and B2 kinin receptor antagonists B9858 (Gera et al., 1996) and Hoe 140 (Lembeck et al., 1991), respectively.

The COX inhibitors used were S-(−)-ketoprofen, piroxicam and NS-398. Experiments with different COX inhibitors were carried out to determine whether different COX isoenzymes may be involved in prostanooid generation induced by kinins in the inflamed vs. control bladders. Attempts to classify the available nonsteroidal antiinflammatory drugs as selective for one COX isoenzyme have been made by several authors, but discrepancies that depend on the model used have been recognized (Battistini et al., 1994; Frölich, 1997). Piroxicam and ketoprofen have been classified as being either COX-1-prefering or equipotent inhibitors (Mitchell et al., 1995; Carabaza et al., 1996; Cromlish and Kennedy, 1996), whereas NS-398 is claimed to be a selective COX-2 inhibitor (Futaki et al., 1993, 1994; Masferrer et al., 1994). PGE2 production was assayed in response to kinin agonists as a positive control for COX inhibition, and the effect of PGE2 itself on the contractility of the detrusor smooth muscle was evaluated.

**Materials and Methods**

**Functional experiments.** Experiments were performed in isolated urinary bladder from male albino rats of Wistar strain (320–350 g) normal (controls) and animal pretreated (48 h before) with cyclophosphamide 150 mg kg−1 i.p. The animals were stunned and bled, and the whole urinary bladder was removed and placed in gassed (95% O2, 5% CO2) Krebs solution. As a positive control on the effectiveness of induced cystitis, controls and cyclophosphamide-inflamed bladders were weighed; they were found to weigh 109 ± 4 mg (n = 31) and 254 ± 10 mg (n = 35), respectively (P < .01). Four longitudinal strips of detrusor muscle were excised from each bladder. The composition of the Krebs solution was as follows (in mM): NaCl 118, KCl 4.7, CaCl2 2.5, MgCl2 0.5, NaH2PO4 1.0, NaHCO3 25 and glucose 11. The strips were transferred to organ baths (5 ml) and prepared for isometric recording (load 5 mN) of mechanical activity (Basile transducers), which was displayed on a Basile 7050 pen recorder. After an equilibration period of 45 min, the preparations were exposed to KCl (80 mM) to check their contractility. After washout, test drugs were administered, and after a 2 h incubation period, a cumulative concentration-response curve to [desArg9]-BK or [Hyp3, Tyr(Me)8]-BK (1 nM–10 μM) was constructed. At the end of the curve, a contractile response to KCl (80 mM) was obtained. Both B1 and B2 receptor agonists were tested, in the presence or absence of test drugs on different strips from the same bladder, and studied in parallel.

For experiments with antagonists, a contact time of 15 min was used before the concentration-response curve was constructed. Peptidase inhibitors (thiopran, bestatin and captopril, 1 μM) were added 15 min before determination of the agonist concentration-response curves or 15 min before administration of the antagonist.

It has already been shown by Maggi et al. (1992) that the bladder strips from cyclophosphamide-treated animals develop less tension than matched strips from vehicle-treated rats in response to carbachol, KCl and electrical stimulation at 20 Hz. However, when contractions were expressed as a percentage of the response produced in each bladder by 10 μM carbachol, taken as an internal standard, no significant difference was evident between strips from vehicle- and cyclophosphamide-treated animals. The same method was followed throughout this study: contractile effects to agonists were expressed as a percentage of the KCl concentration that evoked a maximal contraction of bladders (both control and inflamed) determined at the end of the experiment.

The data were analyzed by two-way analysis of variance for repeated measures followed by Fisher’s LSD (least significant difference) test for multiple comparisons (performed when the appropriate F of the ANOVA had P < .05). EC50 values were calculated by linear regression.

**Prostaglandin determination.** The release of PGE2 from urinary bladder strips into the medium was measured with a specific enzyme immunoassay (EIA) according to the manufacturer’s instructions (Amersham International plc, Buckinghamshire, UK). The same protocol as that used for contractility studies was followed. The total collection time was 15 min both for basal and stimulated samples. Each kinin receptor agonist, [desArg9]-BK or [Hyp3, Tyr(Me)8]-BK (1 μM each), was left in contact with the tissue for 5 min before the medium was collected. Samples were frozen in liquid nitrogen and stored at −70°C until assay. The bladder strips were gently dried by blotting paper and weighed. Results were expressed in picograms per milligram of tissue. The net PGE2 production induced by the kinin B1 or B2 receptor agonist was calculated by subtracting the respective basal value from the total amount produced in the stimulated samples (the change in the picograms per milligram of tissue).

The data were analyzed by two-way analysis of variance for randomized measures, followed by Student’s t test for unpaired data.

**Drugs and solutions.** Drugs used were bestatin [desArg9]-BK, (Peninsula Laboratories Europe, Cheshire, UK), captopril, cyclophosphamide, PGE2 (Sigma, Dorset, UK), [Hyp3, Tyr(Me)8]-BK (Novasbiochem, Läufelfingen, Switzerland), NS-398, piroxicam (Tocris Cookson, Bristol, UK) and thiorphan (Bachem, Essex, UK). All salts used were purchased from Merck (Darmstadt, Germany). S(−)-ketoprofen was synthesized at Laboratorios Menarini (Barcelona, Spain). The kinin B2 antagonist Hoe 140 and the kinin B3 antagonist B9858 were synthesized by Dr. L. Quartara at Menarini Ricerche (Florence, Italy). NS-398 and piroxicam were dissolved in dimethylsulfoxide (10 mM) and stored at −28°C. All other compounds and peptides were dissolved in distilled water (10 mM) and stored at −28°C. Cyclophosphamide was made up freshly in saline (3 ml kg−1) on the day of its use.

**Results**

**Effect of selective B1 and B2 receptor agonists.** Bladder muscle strips from normal rats contracts in a concentration-dependent manner in response to the B1 and B2 receptor-selective agonists [desArg9]-BK (1 nM–10 μM) and [Hyp3, Tyr(Me)8]-BK (1 nM–10 μM), respectively (fig. 1, A and B) with EC50 values of 58 ± 14 and 27 ± 9 nM (n = 10 each),
Effect of B$_1$ and B$_2$ kinin receptor antagonists. The contractile responses to [desArg$_9$]-BK were shifted to the right by the B$_1$ receptor antagonist B 9858 (1 µM) (fig. 2, A and C) in both control and inflamed bladders. The B$_2$ receptor antagonist Hoe 140 (1 µM) did not modify the response to [desArg$_9$]-BK.

The concentration-response curves to [Hyp$_3$, Tyr(Me)$_8$]-BK in control bladders were unaffected by B 9858 (1 µM) and were consistently shifted to the right by Hoe 140 (1 µM) (fig. 2 B). In inflamed bladders, the curve to [Hyp$_3$, Tyr(Me)$_8$]-BK was shifted to the right by Hoe 140 and also by B 9858, though to a lesser extent; B 9858 also reduced the maximal effect evoked by [Hyp$_3$, Tyr(Me)$_8$]-BK in inflamed bladder strips (fig. 2D).

Hoe 140 did not exert agonist activity in either normal or inflamed bladders, whereas B 9858 produced a slowly developing and sustained contraction amounting to 13 ± 1% (n = 8) of the maximal response to KCl in inflamed bladders.

Effect of S-(-)-ketoprofen and piroxicam. In control bladder strips, S-(-)-ketoprofen (10 µM) markedly depressed the concentration-response curves to both [desArg$_9$]-BK and [Hyp$_3$, Tyr(Me)$_8$]-BK, the maximal effects produced being reduced by about 80% and 62%, respectively (fig. 1, C and D). On the other hand, S-(-)-ketoprofen did not significantly affect the response to either receptor agonist in strips from inflamed bladders (fig. 1, E and F). Comparable results were obtained with another COX inhibitor, piroxicam (30 µM). Piroxicam inhibited the concentration-response curves to both [desArg$_9$]-BK and [Hyp$_3$, Tyr(Me)$_8$]-BK in normal bladders by about 61% and 47%, respectively (fig. 3, A and B), respectively. The maximal effect averaged 28 ± 6% and 57 ± 7% (n = 10 each) of the maximal response to KCl (80 mM) for [desArg$_9$]-BK, and [Hyp$_3$, Tyr(Me)$_8$]-BK, respectively.

When concentration-response curves to the same agonists were produced in bladder strips from animals pretreated with cyclophosphamide (150 mg kg$^{-1}$ i.p., 48 h before the "in vitro" experiment), A and B. The agonists' cumulative concentration-response curves obtained in preparations from normal animals (○) and from animals pretreated with cyclophosphamide (□) (n = 10–13 each group), C and D. The effect of S-ketoprofen (10 µM, 2 h of contact time) (■) on the B$_1$ and B$_2$ agonist-elicited motor responses in normal rat urinary bladder (n = 4 each group). E and F. The lack of effect of S-(-)-ketoprofen (10 µM, 2 h of contact time) (●) on the B$_1$ and B$_2$ contractile responses elicited in cyclophosphamide-infamed rat isolated urinary bladder (n = 4 each group). Each value represents the mean ± S.E.M. * P < .05 vs. control.
was significantly abolished by previous incubation with piroxicam in both normal and inflamed bladder strips (fig. 5, B and C). In parallel experiments, NS-398 was used. The PGE$_2$ output evoked by [desArg$^9$]-BK was significantly reduced in both control and inflamed preparations in the presence of this inhibitor (fig. 4B); the PGE$_2$ production by [Hyp$^3$, Tyr(Me)$^8$]-BK did not significantly change in the presence of NS-398 in normal bladders, whereas it was significantly inhibited in the inflamed ones (fig. 5C).

**Effect of PGE$_2$.** PGE$_2$ (1 nM–1 μM) induced a concentration-dependent contraction in normal bladders (fig. 6) that was unaffected by pretreatment with S-(-)-ketoprofen (10 μM), a result that excludes any nonspecific action by the COX inhibitor in preventing contractions of the urinary bladder. When PGE$_2$ was administered to inflamed bladders, the resultant contractile effect was depressed by about 50% compared with that found in normal bladders, whereas the EC$_{50}$ values were comparable (43 ± 4 nM vs. 29 ± 3 nM in normal bladders, n = 4 each group). The concentration-dependent contractile response to PGE$_2$ observed in inflamed bladders in the presence of S-(-)-ketoprofen was superimposable on that obtained in normal bladders in terms of both magnitude of effect and EC$_{50}$ value (28 ± 3 nM, n = 4) (fig. 6).

**Discussion**

Kinin B$_1$ and B$_2$ receptors both mediate contraction of the rat isolated bladder smooth muscle (Marceau et al., 1980). After setup of bladder strips, B$_2$ receptor-mediated contractile responses increase in a time-dependent manner, and they are magnified after induction of chemical cystitis (Marceau et al., 1980; Roslan et al., 1995). On the other hand,
the contraction due to stimulation of the B2 receptor does not show either time-dependent or inflammation-dependent changes. It is commonly held that B1 receptors are synthesized de novo after tissue injury, whereas B2 receptors are expressed constitutively (Marceau, 1995). In this study, we show that the contraction of the inflamed detrusor produced by the stimulation of B1 receptors is potentiated as compared with that recorded in normal tissue, whereas the contractile response due to stimulation of B2 receptors is unchanged. The estimate of the EC50 values of kinin receptor-selective agonists indicates that their affinities were unchanged by experimental inflammation. Experiments performed with selective antagonists confirmed agonist selectivity in normal bladders but also suggest that [Hyp3, Tyr(Me)8]-BK acts in part by stimulating the kinin B1 receptor in inflamed bladders. This result substantially confirms previous findings indicating that in tissues that exhibit a consistent B1 receptor-mediated contractile response, B2 receptor agonists such as BK and [Hyp3, Tyr(Me)8]-BK can also stimulate B1 receptors (Meini et al., 1996). Metabolic degradation of this latter agonist and generation of metabolites acting at B1 receptors can be ruled out because of the replacement of the peptide bonds Phe8-Arg9, which protects from kininase I and II activity (Rhaleb et al., 1990; Regoli et al., 1991) and because our experiments were performed in the presence of enzyme inhibitors (see “Materials and Methods”).

In keeping with previous observations (Marceau et al., 1980; Maggi, 1997), we show that the contractile responses induced by the B1 and B2 receptor agonists in the normal bladder were significantly reduced by the COX inhibitors (S)-(-)-ketoprofen and piroxicam, which indicates that the effects of either agonist are mediated through the production of arachidonic metabolites that induce smooth muscle contraction. Accordingly, both B1 and B2 receptor agonists are able to stimulate PGE2 production in normal bladders, and this effect is prevented by piroxicam. Neither (S)-(-)-ketoprofen nor piroxicam displays sufficient selectivity between different COX isoforms for us to evaluate their different contributions to the phenomenon under study. The COX-2-selective inhibitor NS-398 has been reported to induce the maximal relaxation of the guinea pig isolated trachea at 1 μM concentration (Charette et al., 1995). At this concentration, NS-398 did not inhibit the contraction of normal bladders to B1 and B2 receptor agonists, although it reduced the B1 (but not the B2) receptor-mediated PGE2 production. These data suggest that the B2 receptor-induced PGE2 production is mediated by COX-1 activation and that the B1 receptor is able to stimulate both COX isoforms; they also suggest that PGE2 is not the arachidonic acid metabolite involved in detrusor muscle contraction induced by the stimulation of B1 receptors. In this respect, thromboxanes could be likely candidates because, like PGE2, they have a potent contractile effect in the rat urinary bladder (Maggi et al., 1988).

Both piroxicam and NS-398 reduced the basal levels of PGE2 outflow in the normal bladders, which indicates that the COX-2 isoform may have been basally activated during prolonged in vitro incubation. This basal activation, as well as the B1 receptor-mediated stimulation of COX-2, are magnified after induction of cystitis (as indicated by the reduction in basal PGE2 levels after NS-398), but this phenomenon...
cannot account for the observed increase in the B₁ receptor-mediated contractile response. In fact, although NS-398 and piroximac almost suppressed the B₁ receptor-stimulated production of PGE₂, none of the COX inhibitors reduced the functional response induced by [desArg⁹]-BK in the inflamed bladder. On the other hand, NS-398 enhanced the B₂ receptor-mediated contractile response of the inflamed bladder, which suggests that the inflammation-induced activation of the COX-2 isozyme is linked to the production of arachidonic acid metabolites that inhibit smooth muscle contraction. In this respect, it is interesting to observe that the treatment of smooth muscle with inflammatory agents (lipopolysaccharide or inflammatory cytokines) enhances the BK-stimulated production of PGI₂ (Wen et al., 1997).

Another striking difference between the normal and the inflamed tissue lies in the sensitivity of the contractile response induced by [Hyp³, Tyr(Me)⁸]-BK to COX inhibitors: these drugs were no longer able to inhibit this response in the inflamed bladder. It has been previously reported that traumatic stimuli decrease the potency of COX inhibitors to block the production of arachidonic acid metabolites in the rat bladder (Jeremy et al., 1990). However, concentrations of piroximac (and NS-398) that totally prevented [Hyp³, Tyr(Me)⁸]-BK-induced PGE₂ production did not at all affect the [Hyp³, Tyr(Me)⁸]-BK-induced contractile response in the inflamed bladder.

One possible explanation for the observed ineffective-ness of COX inhibitors in blocking B₁ or B₂ receptor-mediated contractile responses in inflamed bladders may depend on a change in the ability of COX products to generate a contraction after inflammation. Actually, PGE₂ produced in the inflamed bladder a concentration-dependent contraction that was depressed compared with the contraction produced in the normal bladder. This depression does not depend on the inability of the PGE₂ to produce a contraction, because in the presence of S(-)-ketoprofen, the concentration-response curve to PGE₂ in inflamed bladder was restored to control values (normal bladders), whereas a nonspecific effect by S(-)-ketoprofen on bladder contractility can be excluded, as indicated by the lack of effect on PGE₂-mediated contraction in the control bladder. These results show that in the inflamed bladder, prostanoids maintain a bladder tone (basal PGE₂ levels are higher in inflamed bladders) and that the addition of exogenous prostanoids results in a minor contractile response as compared with controls. The increased prostanoïd-mediated basal tone could hide the prostanoïd-dependent component of kinin agonist-mediated contraction in the inflamed bladder. Although kinin receptor agonists are able to stimulate prostanoid production, and COX inhibitors can block this production, the prostanoïd-independent part of the contraction is magnified after inflammation for both the B₁ and B₂ receptor. Quantitatively, the prostanoïd-independent component of the response averages the 30% of the KCl-induced contraction in normal bladder and around the 70% to 80% in the inflamed bladder. The induction of inflammation changes the contractile properties of bladder smooth muscle in such a way that other mechanisms of excitation, contraction or coupling, supplement the contributory role played by prostanoids in normal bladders.

Because ONO 1078, a selective leukotriene D₄ receptor antagonist (Tominaga et al., 1996), also failed to affect kinin B₁ or B₂ receptor-mediated contractions in the inflamed bladder (data not shown), the involvement of leukotriene D₄ in the contractile response induced by kinin B₁ or B₂ receptor agonists can be excluded.

It is worth noting that the [Hyp³, Tyr(Me)⁸]-BK-stimulated PGE₂ production is reduced by the COX-2 inhibitor NS-398 in inflamed bladders. Whether this inhibitory effect means that the B₂ receptor activates COX-2 after inflammation or represents the B₂-stimulating effect of [Hyp³, Tyr(Me)⁸]-BK cannot be decided at present.

In conclusion, our results suggest that persistent inflammation in the rat urinary bladder renders B₁ or B₂ receptor-mediated contractile responses resistant to COX inhibitors, whereas B₁ or B₂ receptor agonists are still able to stimulate PGE₂ production that is sensitive to COX inhibitors.

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