Characterization of the capsaicin-sensitive component of cyclophosphamide-induced inflammation in the rat urinary bladder


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1 Cyclophosphamide (CYP) (150 mg kg⁻¹, i.p. 0.5–48 h before) caused a time-dependent plasma protein extravasation in the rat urinary bladder with the maximal extravasation occurring at between 2 and 4 h after administration of the drug.

2 Prior capsaicin desensitization of capsaicin-sensitive primary afferent neurones (CSPANs) (50 mg kg⁻¹, s.c., 4 days before) resulted in approximately 50% inhibition of the magnitude of the extravasation response at the 2 h time-point.

3 Intraperitoneal (i.p.) pretreatment with the tachykinin NK₂ receptor antagonist, RP 67,580 (0.44 mg kg⁻¹) or the bradykinin B₂ receptor antagonist, Hoe 140 (0.13 mg kg⁻¹) had significant inhibitory effects, giving responses of 56 ± 6% and 39 ± 4% of the control extravasation response to CYP treatment after 2 h. Pretreatment with the tachykinin NK₂ receptor antagonist, SR 48,968 (0.3 mg kg⁻¹, i.p.), the histamine H₁ receptor blocker, chlorpheniramine (10 mg kg⁻¹, i.p.), the 5-HT receptor blocker, methysergide (6 mg kg⁻¹, i.p.) or the cyclo-oxygenase inhibitor indomethacin (5 mg kg⁻¹, i.p.) had no significant effect upon the development of the extravasation response at this same time-point.

4 In rat isolated urinary bladder strips, the active metabolite of CYP, acrolein (1–300 μM) produced a concentration-dependent contraction that was significantly reduced by in vitro capsaicin desensitization (10 μM for 15 min) indicating direct stimulation of CSPANs. CYP was without appreciable effect.

5 The effect of acrolein in vitro was significantly reduced by pretreatment of the bladder with a combination of tachykinin NK₂ and NK₁ receptor antagonists, RP 67,580 (3 μM) and SR 48,968 (1 μM). The dose-response curve to acrolein was also significantly inhibited by treatment with indomethacin (10 μM) and slightly affected by Hoe 140 (1 μM).

6 These findings demonstrate the contribution of CSPANs to the development of CYP-induced cystitis. Plasma protein extravasation involves activation of tachykinin NK₁ and bradykinin B₂ receptors. Activation of CSPANs in the urinary bladder is likely to be due to the conversion of CYP into its active metabolite, acrolein, and not to a direct effect of CYP upon these nerve-ends.

Keywords: Cyclophosphamide; plasma protein extravasation; capsaicin-sensitive bladder afferents; rat urinary bladder; cystitis

Introduction

Cyclophosphamide (CYP) is an effective form of treatment for a variety of malignant and non-malignant disease states. Its activity is based upon conversion of CYP into its active oxidized metabolite of phosphamidate mustard in the liver. CYP therapy has several toxicity complications (for review see Fraiser et al., 1991) including bladder cystitis, which is due primarily to another CYP metabolite, acrolein. Acrolein has profound effects upon the urinary bladder causing painful voiding symptoms in the mildest of cases to haemorrhagic cystitis in the most severe. It has been shown that CYP-induced cystitis is due to the direct contact of acrolein with the urothelium (Phillips et al., 1961; Cox, 1979); however, the exact mechanism of the ensuing inflammatory response is little understood.

Normal vesicourethral function is controlled by a complex interplay between the innervation provided by sympathetic, parasympathetic, somatic and sensory nerves. The latter includes a subpopulation of primary afferent neurones that are sensitive to the actions of capsaicin (Maggi et al., 1986), the pungent ingredient from red peppers (for review of actions see Holzer, 1991). These capsaicin-sensitive primary afferent neurones (CSPANs) have been attributed a dual, sensory and efferent function (Maggi, 1990). The efferent activity of these neurones is achieved by the release of several neuropeptides (Maggi et al., 1987; 1988a,b) many of which possess inflammatory activity, producing what is termed neurogenic inflammation (for reviews see Chahl, 1988; Maggi, 1991). In turn, various mediators of inflammation, such as bradykinin (Maggi et al., 1989; 1993) and formylmethionyl-leucyl phenylalanine (Giuliani et al., 1991) have been shown to induce release of neuropeptides from sensory nerve endings in the urinary bladder. Further evidence for the inflammatory activity of sensory neuropeptides has been demonstrated by the fact that the acute administration of capsaicin itself will result in a plasma protein extravasation (PPE) (Maggi et al., 1987) and that a variety of stimuli for sensory nerves in the bladder, such as xylene and hypertonic solutions, produce inflammatory effects that are dependent upon the existence of an intact sensory afferent system, the response being modified by capsaicin treatment (Maggi et al., 1988b).

The possible pathophysiological role that activation of CSPANs has to play in CYP-induced cystitis has been investigated previously (Maggi et al., 1992) in a rat model of CYP-induced cystitis described by Grinberg-Funes et al. (1990). This study demonstrated that, at 48 h after administration of CYP, extrinsic bladder denervation produced by bilateral removal of pelvic ganglia resulted in an attenuation of the plasma protein extravasation induced by CYP com-

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pared to animals with intact bladder innervation. However, 

prior depletion of sensory neurones with capsaicin poten-
tiated the plasma protein extravasation 48 h after CYP 
administration. Maggi et al. (1992) suggested that CSPANs 
may provide some protective effect at this time point, but 

speculated that an earlier timepoint in the development of 
the inflammation these nerves may subserve a pro-inflam-

matory role. To investigate this possibility we have used the 
same model to determine the development of extravasation 
induced by CYP from between 0.5 and 48 h after its adminis-

tration and the effect of capsaicin desensitization upon the 
development of this response. In this way any role that the 
sensory afferent system may have to play in CYP-induced 
plasma extravasation in the rat urinary bladder may be 
discerned. Secondly, we have addressed the question that 
CYP produces its effects via conversion to the metabolite, 
acrolein, by comparison of the effects of both CYP and 
acrolein upon smooth muscle activity of the rat bladder in 

vitro and the effects of capsaicin thereon.

Methods

In vivo

Plasma protein extravasation induced by CYP in the urinary 
bladder of male Wistar rats (Charles River, Italy) weighing 
350–400 g was measured by the Evan’s blue dye leakage 
technique (Saria & Lundberg, 1983). Rats were anaesthetized 
with urethane (1.2 g kg$^{-1}$, s.c.) and the left jugular vein 
canulated with PE 50 polyethylene tubing for i.v. adminis-

tration. Following cannulation Evan’s blue (50 mg kg$^{-1}$, i.v.) 

was administered and 15 min later the dye flushed out of the 

cardiovascular system by perfusion with warm (37°C) saline 
(25 ml min$^{-1}$) for 2 min. An intracardiac puncture, the 

urinary bladder was then excised, the extraneous tissue 

removed, washed in saline and blotted dry before weighing. 

The Evan’s blue content in each bladder was determined 

after extraction in formamide (24 h at 60°C) using a DB 

Beckman Spectrophotometer at 620 nm.

A time-course of the effect of CYP (150 mg kg$^{-1}$) was 
determined over a period spanning from 0.5 h to 48 h after 

CYP administration: the end point of the experiment was 

considered the start of the intracardiac perfusion procedure. 

To ascertain the involvement of activation of sensory nerves 
in the development of plasma extravasation in this system, 
animals were pretreated with a single s.c. injection of cap-

saicin (50 mg kg$^{-1}$) 4 days prior to the CYP treatment and 
the above experiment carried out alongside vehicle (10% 
ethanol, 10% Tween 80 in saline) controls. The capsaicin 
pretreatment has been repeatedly shown to produce >90% 
deposition of bladder content of sensory neuropeptides (Maggi 
et al., 1988a,b). From these results those time-points at which 
modulation by capsaicin occurred were further studied to 
characterize that part of the plasma extravasation response 
involving sensory afferent activation.

In a second series of experiments, performed 2 h after CYP 
administration, the effect of various drugs known to block 
specific receptors or prevent formation of mediators of 
inflammation was assessed and compared with vehicle-treated 
control rats carried out on the same day. The drug 
treatments and their relevant actions are described in Table 
I. The various in vivo treatments were selected from previous 

studies (referenced in Table I) in which effective blockade of 
the corresponding targets and selectivity has been assessed.

In vitro

Male Wistar rats (Charles River, Italy) weighing 350–400 g 
were killed by cervical dislocation and the whole urinary 
bladder removed and cleared of extraneous tissue. Longi-
tudinal strips of the bladder (approximately 2–3 mm wide) 

were mounted in 5 ml organ baths containing Krebs solution 
gassed with 5% CO$_2$ in O$_2$, maintained at 37°C and under a 

constant load of 0.5 g. The composition of the Krebs solu-

tion was as follows: (mM) NaCl 119, NaHCO$_3$ 25, KCl 4.7, 

MgSO$_4$ 1.2, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2 and glucose 11. The 

contractile activity of the bladder was measured with Basile 
isotonic transducers and recorded by means of a penrecorder 
(Unirecord 7050, Basile, Italy). An incubation period of 
37°C was allowed before presentation. The concentration-
trate response to neurokinin A (NKA, 0.1 μM) was a gift from 
Rhone-Poulenc (Vitry, France). Hoe 140 (D-Arg$_2$-[Hyp$_2$ 

Thi$_2$, D-Tic$_2$, Oic$_4$]-bradykinin) was a gift from Hoechst 
(Frankfurt, Germany) and SR 48,968 (SN-methyl-N-[4-

(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl) 
butyl]benzamido) was a gift from Sanofi (Montpellier, 
France). Methysergide maleate was a gift from Sandoz 
(Basel, Switzerland).

Materials

Cyclophosphamide, capsaicin, indomethacin and chlorphen-

iramine maleate were purchased from Sigma Chemical Co, 
Italy. Evan’s blue was obtained from EGA chemie (Stein-

heim, Germany). RP 67,580 ([3α,7αR]-7,7-diphenyl-2-[1-

imino-2-[2-[(2-methoxyphenyl)-[3,4-dihydroxy-iodo-4-one] 
as a gift from Rhone-Poulenc (Vitry, France). Hoe 140 (D-Arg$_2$-[Hyp$_2$ 

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(Basel, Switzerland).

Cyclophosphamide, methysergide maleate, chlorphen-

iramine maleate were all made up freshly in saline on day of 
use. Indomethacin was dissolved in equimolar sodium hy-

droxide (0.1 N) and diluted in saline for in vivo pretreatment 
and in dimethylsulphoxide for in vitro experiments. Hoe 140 
was dissolved in distilled water and stored as frozen aliquots 
at $-20^\circ$C and RP 67,580 was dissolved in hydrochloric acid 
and adjusted to pH 5 on day of use. Capsaicin was dissolved 
in 10% ethanol and 10% Tween 80 in saline for in vivo 
treatment and in ethanol for in vitro experiments. Ethanol 
(final concentration in the bath 0.1%) had no significant 
effect on the rat isolated urinary bladder.

Table 1 Different drug treatments used against cyclophosphamide-induced plasma protein extravasation in the urinary bladder of the 

rat in vivo

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mode of action</th>
<th>Pretreatment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP 67,580</td>
<td>NK$_2$ receptor blocker</td>
<td>0.44 mg kg$^{-1}$, i.p., 5 min</td>
<td>Santicioli et al., 1993</td>
</tr>
<tr>
<td>SR 48,968</td>
<td>NK$_2$ receptor blocker</td>
<td>0.3 mg kg$^{-1}$, i.p., 5 min</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Hoe 140</td>
<td>B$_2$ receptor blocker</td>
<td>0.13 mg kg$^{-1}$, i.p., 5 min</td>
<td>Maggi et al., 1993</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Cyclo-oxgenase inhibitor</td>
<td>5 mg kg$^{-1}$, i.p., 5 min</td>
<td>Maggi et al., 1993</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
<td>H$_2$ receptor blocker</td>
<td>10 mg kg$^{-1}$, i.p., 5 min</td>
<td>Eglezos et al., 1992</td>
</tr>
<tr>
<td>Methysergide</td>
<td>5-HT receptor blocker</td>
<td>6 mg kg$^{-1}$, i.p., 15 min</td>
<td>Maggi et al., 1988b</td>
</tr>
</tbody>
</table>
Data and statistical analysis

Plasma protein extravasation is expressed as the content of Evan’s blue in nanogram per milligram of bladder weight. All of the results are expressed as mean ± s.e.mean. Effect of drug treatment upon the extravasation response is expressed as a percentage of the response obtained in vehicle control treated animals. Statistical significance was determined by Student’s t test for unpaired data (two-tailed analysis). Contractile responses to CYP and acrolein in the absence or presence of drug pretreatment are expressed as a percentage of the control response to NKA. Analysis of variance, corrected for multiple comparisons with the Bonferroni test, was used to determine the levels of significance with a P<0.05 being considered as statistically significant.

Results

Plasma protein extravasation

The urinary bladder content of Evan’s blue in control rats was 12 ± 2 ng mg⁻¹ (n = 9) of tissue.

CYP (150 mg kg⁻¹) caused a time-dependent plasma extravasation (n = 7–12 for each time-point) which reached a maximal peak at between 2 and 4 h followed by a decline over the following 24 h (Figure 1). Haemorrhage was apparent from the 24 h timepoint onwards. Capsaicin pretreatment (n = 7–14 for each time-point) resulted in a significant inhibition of the plasma extravasation response presented at the 2 h time-point only (P<0.01) by approximately 45% (n = 13) as shown in Figure 1. The 2 h time-point was then used in all following in vivo experiments to characterize further the capsaicin-sensitive part of the inflammatory response to CYP. As shown in Figure 2, RP 67,580 (0.44 mg kg⁻¹, n = 11), Hoe 140 (0.13 mg kg⁻¹, n = 16) and capsaicin pretreatment all significantly inhibited the plasma extravasation response whereas methysergide (n = 7), chlorpheniramine (n = 7), SR 48,968 (n = 7) and indomethacin (n = 7) had no significant effect. The combined administration of RP 67,580 and Hoe 140 failed to produce an inhibitory effect greater than that achieved by the combined antagonist alone (Figure 2).

Isolated urinary bladder strips

Acrolein (1–300 μM, n = 7) caused a concentration-related, slowly developing, tonic contraction of rat isolated urinary bladder strips, whereas CYP (n = 9) in the same concentration range had little or no effect as shown in Figures 3a and 4. Prior incubation of the preparation with capsaicin (10 μM) (n = 7) resulted in a suppression of the contractile effects of acrolein, significant differences being achieved at the upper part of the concentration-response curve (Figures 3a and 4). Administration of RP 67,580 (3 μM) or SR 48,968 (1 μM), at concentrations producing effective blockade of NK₁ and NK₂ receptors respectively (Meini et al., 1993), inhibited the response to 300 μM acrolein by 62 ± 8% and 66 ± 5% respectively (n = 5). Since muscle contraction of the rat isolated bladder to endogenous tachykinins is mediated through both NK₁ and NK₂ receptors (Maggi et al., 1991), the two antagonists were combined to unravel a possible contribution of endogenous tachykinins in the response to acrolein. Co-administration of the two antagonists clearly induced a more pronounced inhibition on the response to 300 μM acrolein (−80 ± 7%, n = 9). For clarity, only the results showing the effect of the combined administration of RP 67,580 and SR 48,968 on acrolein (1–300 μM)-induced contraction are shown in Figure 3b. Indomethacin (10 μM, n = 6) suppressed the concentration-response curve to acrolein in the same extent as the tachykinin antagonists mixture (Figure 3b). The inhibitory effect of indomethacin or RP 67,580 plus SR 48,968 on acrolein-induced contractions was specific since they did not significantly modify the contractile response to 10–80 mM KCl (EC₅₀ = 24 mM (18–34 c.l.) and E₅₀ = 117 ± 4% of the response to 0.1 μM NKA, n = 5–8). Hoe 140 (1 μM, n = 5) (Figure 3b) significantly reduced the response to acrolein only at one point of the concentration-response curve (10 μM).

Discussion

CYP is an effective form of treatment for certain types of cancer; however, the toxicity complications raise problems when using this drug. One of the most pronounced of these complications is the development of cystitis. Previous studies
in the airways (Lee et al., 1992) and urinary bladder (Maggi et al., 1992) suggested that activation of sensory afferent fibres may be involved in the irritant effects of CYP and that the metabolite of this agent, acrolein, could be responsible for the complications which occur from treatment with CYP. In agreement with these suggestions we have found that capsaicin pretreatment, and hence the removal of the possible CSPANs component to the response to CYP, resulted in a modulation of the extent of plasma protein extravasation at the 2 h time-point. At this time point CYP, most likely through acrolein generation, stimulates the release of pro-inflammatory neuropeptides from CSPANs which contribute to the formation of oedema. Thus neurogenic inflammation takes place during CYP-induced cystitis, although the major part of PPE produced following CYP administration seems to be independent of an active contribution of CSPANs.

In the present series of experiments we did not demonstrate any significant potentiation of CYP induced plasma protein extravasation by capsaicin pretreatment at the 48 h time-point (Maggi et al., 1992). A possible explanation for this anomaly may be a different source of the animals used in each study, which itself can result in significant variability in the magnitude of plasma extravasation responses even in the same strain (Ahluwalia, unpublished observations).

With the use of the selective, non-peptide NK1 receptor antagonist, RP 67,580 (Garret et al., 1991), it would seem that at least part of the response to CYP at the 2 h time-point is mediated through activation of these receptors by endogenous tachykinins, the most probable candidate for this being substance P. In fact, studies investigating the inflammatory effects of direct application of capsaicin to the urinary bladder have demonstrated that the ensuing PPE is due to activation of tachykinin NK1 receptors (Eglezos et al., 1991). Pretreatment with Hoe 140, the selective, potent bradykinin B1 receptor antagonist (Lembeck et al., 1991; Wirth et al., 1991), also resulted in a significant inhibition of the oedema response. This is congruent with findings in a
xylene-induced model of cystitis, where the PPE response was sensitive to both B2-receptor blockade, by Hoe 140, and NK1-receptor blockade (Giuliani et al., 1993). However, contrary to the xylene-induced cystitis, the combination of both Hoe 140 and RP 67,580, did not produce an additive inhibitory effect on CYP-induced cystitis, suggesting that the CSPAN-sensitive part of this response is dependent upon the activity of bradykinin at B2 receptors, rather than an unlinked involvement of these inflammatory mediators. In accordance with these results, it has been demonstrated, also using Hoe 140, that bradykinin is involved in the induction of detrusor overactivity and increased micturition frequency during CYP-induced cystitis (Maggi et al., 1993), events also known to be sensitive to capsaicin pretreatment.

As indicated earlier, CYP is believed to produce its toxic effects via conversion in the liver by P450 cytochrome enzymes into its active metabolite, acrolein. From the present results it would appear that CYP produces at least part of its inflammatory effects by activating CSPANs. Since it is known that capsaicin produces contractile effects upon urinary bladder through the release of tachykinins (Maggi et al., 1988c) we used this in vitro preparation to investigate whether conversion to acrolein was in fact a prerequisite for the apparent activation of CSPANs by CYP. This was achieved by comparing the contractile effects of CYP and acrolein, on both muscle strips of the rat urinary bladder in vitro. The present studies demonstrate quite clearly that CYP has no direct stimulatory effects upon CSPANs, whereas acrolein produced a concentration-dependent contractile response which was attenuated to similar extents by both capsaicin pretreatment and blockade of tachykinin receptors. These antagonists had no effect upon the response curve to potassium, suggesting that the inhibitory effects of these agents on the contractile responses to acrolein do not involve a non-specific effect on bladder smooth muscle contractility.

Contrary to the in vivo experiments, in which Hoe 140 blocked CYP-induced PPE, this bradykinin B2 receptor antagonist was substantially ineffective towards acrolein on the isolated bladder. The latter finding could be explained by the fact that the neuroactive components of the kallikrein system are not available for de novo bradykinin formation in vitro. Therefore, while acrolein can activate CSPANs without inducing bradykinin generation in vitro, the in vivo findings, point to bradykinin generation as a necessary intermediate step in the activation of CSPANs following CYP-induced cystitis since combination of both RP 67,580 and Hoe 140 led to no greater inhibitory effect than when either was given alone, i.e. no summation. The acrolein-induced contraction of the rat isolated bladder was blocked by indomethacin, indicating that tachykinin release from CSPANs occurs via the generation of prostanoids. In contrast, indomethacin induced no effect on PPE produced by CYP administration. This second difference between the in vivo and in vitro findings may argue against the role of acrolein as activator of CSPANs during CYP-induced cystitis. At this stage we cannot clearly identify why this difference exists, however, one explanation for this anomaly could be that the amount of acrolein produced following conversion from CYP, in vivo, is much less than those concentrations used for the in vitro experiments, and that relatively low concentration of acrolein induce bradykinin generation. However, at present there exists no information concerning the ability of acrolein to induce bradykinin formation in vivo. Alternatively CYP itself or other metabolites may be capable of inducing bradykinin formation in vivo.

In conclusion, the present findings demonstrate an active role of CSPANs via tachykinin release in the early phase of the urinary bladder inflammation produced by CYP. Bradykinin generation seems an important intermediate step for CSPANs activation in in vivo conditions. Acrolein, a major metabolite of CYP, is capable of activating CSPANs in the isolated bladder; however, the mechanisms of action of acrolein in vitro appear to differ from those responsible for the in vivo inflammatory response induced by CYP.

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References


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