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NK_1 Receptors Mediated Release of 6-Keto-$PGF_{1\alpha}$ from the *Ex Vivo* Perfused Canine Ileum[1]

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ABSTRACT

The purpose of this study was to determine the effects of tachykinins on prostanoid production by the dog ileum and to characterize the tachykinin receptor(s) responsible for the principal eicosanoid shown to be released, 6-keto-$PGF_{1\alpha}$. Substance P, the selective NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]substance P and neurokinin A caused concentration-dependent production of 6-keto-$PGF_{1\alpha}$; neurokinin A was least potent. The selective NK_2 agonist [Nle^{10}]neurokinin A(4–10) had no effect. The selective NK_1 antagonist CP-96,345 (10^{-7} M), blocked 6-keto-$PGF^{1\alpha}$ release from substance P (10^{-7} M), [Sar^9,$Met(O_2)^{11}$]-substance P (10^{-7} M) and neurokinin A (10^{-7} M). Although the putative NK_2 antagonist MEN 10207 (10^{-7} M) partially blocked the 6-keto-$PGF_{1\alpha}$ release induced by neurokinin A (10^{-7} M), we conclude that all these peptides acted primarily on NK_1 receptors to induce 6-keto-$PGF_{1\alpha}$. Additional experiments suggest that a major site of production of 6-keto-$PGF_{1\alpha}$ in the canine ileum may be the vasculature, but these experiments do not exclude other sources such as intestinal muscle for this prostanoid. Calcium-free Krebs' solution partially reduced the release of 6-keto-$PGF_{1\alpha}$ to substance P (10^{-7} M), implying that extracellular calcium helps support tachykinin-induced production of 6-keto-$PGF_{1\alpha}$. Blockade of synthesis of another vasoactive mediator, endothelium-derived relaxing factor (nitric oxide), by N^{ω}-L-arginine methyl ester) did not alter substance P-induced release of 6-keto-$PGF_{1\alpha}$. Thus, tachykinin-evoked release of 6-keto-$PGF_{1\alpha}$ in the canine ileum is mediated by NK_1 receptor and depends in part on extracellular Ca^{++} but not on synthesis of nitric oxide.

Recently, neural systems have been shown to participate in immunoinflammatory reactions in the gastrointestinal tract mediated by release of neuropeptides (Payan *et al.*, 1986). Thus, substance P injected intra-arterially into the canine ileum produces different concentration-dependent responses of the circular smooth muscle comprising at low doses a dose-related inhibition of phasic activity by the release of acetylcholine to inhibitory muscarinic receptors on muscle; at slightly higher doses, initial inhibition followed by excitation of the circular smooth muscle from release of acetylcholine to muscarinic receptors; and at higher concentrations, direct excitation of the circular smooth muscle (Daniel *et al.*, 1982; Fox *et al.*, 1983; Fox and Daniel, 1986; Milenov *et al.*, 1978). The direct contractile effects of substance P are mediated by membrane phosphoinositide hydrolysis with subsequent release of intracellular calcium to cause smooth muscle contraction (Brown, 1987). Linked to this is the possible activation of phospho-lipases to liberate arachidonic acid for eicosanoid synthesis (Kaiser *et al.*, 1990).

Marceau *et al.* (1989) showed that both substance P and neurokinin A significantly enhanced 6-keto-$PGF_{1\alpha}$ released from cultured human umbilical vein endothelial cells in a concentration-dependent manner *via* NK_1 and NK_2 receptors.

We therefore decided to investigate the mechanisms of effects of intra-arterially infused tachykinins on the release of eicosanoids in the perfused canine ileum *ex vivo*, a system in which the actions of some neuropeptides have been well defined (Fox-Threlkeld *et al.*, 1991; Manaka *et al.*, 1989).

Materials and Methods

The *ex vivo* perfused canine ileum. Adult mongrel dogs of either sex (weighing 15–30 kg) were fasted for 18 hr, anesthetized with α-chloralose (50 mg/kg i.v.) and urethane (500 mg/kg i.v.) and given artificial respiration. These procedures were approved by the McMaster Animal Research Ethics Board.

An ileal segment (15–20 cm in length and 20–30 g in weight) was surgically prepared as described (Fox-Threlkeld *et al.*, 1991; Manaka *et al.*, 1989). The segments were perfused free of red blood cells, and where there were areas containing stagnant blood or if high resistance to perfusion occurred (low-flow rate), these segments were discarded. As previously described, each segment had one or more strain gauges sewn to serosa to record circular muscle contractions, and subserosal silver electrode pairs were used to stimulate enteric nerves (Daniel *et al.*, 1994; Daniel and Kostolanska, 1989). They

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ABBREVIATIONS: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetra-acetic acid; L-NAME, N^{ω}-L-arginine methyl ester; LT, leukotriene; ANOVA, analysis of variance; RIA, radioimmunoassay; GC-MS, gas chromatography-mass spectrometry.

were placed on each side of the strain gauges, and one set was distal to one strain gauge and proximal to another. The methods for analysis of motor response are described in detail in Daniel *et al.* (1994). In brief, contractions were normalized in relation to the maximal response to intra-arterial acetylcholine, set at 100%. Data are expressed as percentage of this maximum.

The segments were equilibrated for about 20 min before initiating peptide infusions (Fox-Threlkeld *et al.*, 1991).

Solutions. The ileum was perfused with Krebs' solution (containing one-half normal concentration of glucose) comprising (in mM): NaCl 137.4, $MgSO_4$ 1.2, $NaHPO_4$ 1.2, $NaHCO_3$ 21.1, $CaCl_2$ 2.5 and glucose 5.5, and was gased with 95% O_2-5% CO_2 to maintained pH at approximately 7.4. Calcium-free Krebs' solution was prepared by omitting $CaCl_2$ and adding 100 μM EGTA.

Ileal perfusion. Substance P, neurokinin A, [Sar^9,$Met(O_2)^{11}$]substance P, [Nle^{10}]neurokinin A(4–10), EGTA, L-NAME and aprotinin were obtained from Sigma Chemical Co., (St. Louis, MO). The selective NK_1 receptor antagonists CP-96,345 was donated by Pfizer, Inc., (Groton, CT), and the NK_2 receptor antagonist MEN 10207 was from A. Menarini Pharmaceuticals, (Firenze, Italy). All of the drugs were dissolved in distilled water, stored at −70°C (at which they were stable) and subsequently diluted in Krebs' solution (40°C).

GC-MS assay of perfusate. The reagents were all of analytical grade and comprised absolute alcohol (Consolidated Alcohol Ltd., Toronto, Ontario), phosphoric acid (Fisher Scientific, Nepean, Ontario), citric acid, toluene, pyridine, acetonitrile, hexane, dichloromethane (BDH Chemicals, Toronto, Ontario), petroleum ether, anhydrous sodium sulfate (J.T. Baker Inc., Phillipsburg, NJ), methoxyamine hydrochloride, diisopropyl ethyl amine, methyl formate, pentafluorbenzyl bromide, diethyl ether, PGE_2, 6-keto-$PGF_{1\alpha}$, thromboxane B_2 and bis-trimethylsilyl-trifluoroacet-imide (Sigma Chemical Co.). C-18 reverse phase and silica gel mini-columns were purchased from Scientific Products and Equipment (Concord, Ontario).

The deuterated internal standards (PGE_2-d_4 and 6-keto-$PGF_{1\alpha}$-d_4 and LKs B_4, C_4, D_4 and E_4 were donated by Dr. A.W. Ford-Hutchinson, Merck-Frosst Institute for Therapeutic Research (Pointe-Claire, Dorval, Quebec), and these and the other prostanoids were dissolved in absolute ethanol and stored at −70°C.

Infusion of tachykinins. The tachykinin (freshly diluted in Krebs' solution) were injected into the main Krebs' perfusate at a rate of 0.23 mL/min. They were infused from lower to higher concentrations for periods of 9 min followed by a 48-min recovery period. The perfusate collected before, during and after tachykinin infusion was analyzed for eicosanoids, including 6-keto-$PGF_{1\alpha}$.

Infusion of tachykinin antagonists. Tachykinin agonists were initially infused as above followed by addition of an antagonist to the reservoir containing the Krebs' solution during the recovery period (48 min). The second tachykinin agonist infusion was performed 30 min after exposure to the antagonist.

Infusion of L-NAME. Experiments with L-NAME involved a slightly different infusion protocol in which substance P (10^{-7} M) was infused; then, during the recovery period, L-NAME ($3 \cdot 10^{-4}$ M) was infused approximately 12 min before the second substance P (10^{-7} M) infusion. To ensure that nitric oxide synthesis was blocked with L-NAME, electrical field stimulation of enteric nerves (both 1 pps and 3 pps at 20 V/cm, 0.5-msec pulse and 2-min train) was applied before and after L-NAME infusion so we could observe that distal inhibitory motor responses induced by field stimulation were abolished and that proximal and local excitatory responses were enhanced after L-NAME administration (Daniel *et al.*, 1994; Daniel and Kostolanska, 1989).

Perfusion of canine ileum with calcium-free krebs' solution. To investigate the role of extracellular calcium in the release of 6-keto-$PGF_{1\alpha}$ three consecutive infusions of substance P (10^{-7} M) (48-min recovery period between each infusion) were performed. The first infusion of substance P was in regular Krebs' solution, whereas the second was in calcium-free Krebs' solution (with 100 μM EGTA) 12 min before, and during substance P (10^{-7} M) infusion. The third infusion followed 15 min later with perfusion with regular Krebs' solution. During the use of calcium-free Krebs' solution, electrical field stimulation (5 pps) of enteric nerves (Daniel *et al.*, 1994; Daniel and Kostolanska, 1989) was applied to establish a lack of a motor response suggesting that extracellular calcium was depleted. The motor response to electrical field stimulation returned to normal when the tissue was once again perfused with normal Krebs' solution.

Collection and analysis of perfusate. Perfusate was collected as one fraction for 3-min periods in plastic vials stored on ice. It was then transferred to 20-mL scintillation vials on ice and bubbled with nitrogen to eliminate the possibility of eicosanoids being oxidized. The samples collected for analysis contained 500 KIU/mL aprotinin to inhibit possible enzyme activity. Analysis of perfusate samples was performed initially by GC-MS and then routinely by RIA.

Analytical Methods of Eicosanoid Measurement in Perfusate

Perfusate preparation for GC-MS analysis. Eicosanoids were extracted from the perfusate and concentrated in toluene, using the procedure adapted from Powell (1980). The eicosanoids were then derivatized by methoximation, pentafluorobenzyl and trimethylsilylation using methods adapted from Mayer *et al.* (1986) and Leis *et al.* (1987).

The standards and samples were assayed by scanning and direct quantitation using a VG Masslab TRIO II guadrapole mass spectrometer (VG, Masslab, Manchester, UK) with negative ion chemical ionization mode coupled to a Hewlett Packard 5890GC (VG Instruments Canada, Pointe Claire, Quebec). Aliquots of 1 to 2 μl of the derivatized mixture were injected onto the column *via* a telescopic injector on a 15-m DB-1 fused silica column (0.23 mm i.d.; J&W Scientific, Rancho Cordova, CA) coated internally with 0.25-μm polymethyl siloxane. The outlet of this column was inserted directly into the ion source. The initial column temperature was 250°C and was then increased to 300°C at 8°C/min. The transfer line and ion source were maintained at 300°C and 200°C, respectively. The gas used for ionization by dissociative electron capture was methane and was maintained at a pressure of 5×10^{-3} Pa. The electron current was 980 μA, and the energy was 70 eV.

The area under the curve detected for the internal standard was used to account for loss of sample during the extraction and derivatization procedures. The calibration curves for each standard (*i.e.*, deuterated PGE_2, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2) were constructed to determine the linear range for each eicosanoid. The calibration curves were then used in conjunction with internal standards of d-6-keto-$PGE_{1\alpha}$ or d-PGE_2 to calculate the concentrations of PGE_2, 6-keto-$PGF_{1\alpha}$ and PGE_2 thromboxane B_2 (ng/ml) in each perfusate sample.

RIA of perfusate for 6-keto-$PGF_{1\alpha}$. RIA kits with tritiated standards for 6-keto-$PGF_{1\alpha}$ were purchased from Advanced Magnetics (Cambridge, MA) through Cedarlane Laboratories (Hornby, Ontario). The standard protocol outlined in the enclosed instruction booklet was followed for analysis of the perfusate samples. With the standard curve, the concentration of 6-keto-$PGF_{1\alpha}$ for each perfusate sample (ng/ml) was calculated.

HPLC assay. Eluates (1 ml) were purified on reverse-phase minicolumns (as above) except methanol was used in the final election (Powell, 1980). Dried fractions were dissolved in methanol and applied to the HPLC system through a Micro-Bondapac precolumn, washed with 50% methanol in 0.2% ortho-phosphoric acid, eluted onto a C-18 Novopack column with 62.5% acetonitrile in 1.0 mM trifluoroacetic acid and then isocratically eluted in the same solvent. HPLC was performed with Waters 501 and 510 pumps coupled to a WISP 700 autoinjector and monitored with Waters 484 and 480 Lamdamax UV detectors set at 280 and 235 nM, respectively (columns and equipment from Waters, Mississauga, Ontario). Limits of detection for LTs B_4, C_4, D_4 and E_4 were approximately 1 ng. PGB_2 (10 ng) was added to each sample as an internal standard.

Statistical Analysis. Data are presented as the arithmetic mean ± S.E.M. The number of perfused ileal segments, each from an individual dog used in each study, is indicated by *n*.

The concentration of 6-keto-PGF$_{1\alpha}$ determined in the perfusate is presented as units of ng/ml. Responses before, during and after tachykinin infusion were statistically compared using ANOVA and the two-tailed Student's t test. Levels of significance were established (see figure legends).

Results

Quantitation of prostanoids by GC-MS. Scanning of the perfusates by GC-MS revealed that the main prostanoids released in the perfusate of the canine ileum were 6-keto-PGF$_{1\alpha}$, PGE$_2$ and thromboxane B$_2$. LK levels in the perfusate were barely detectable. The most abundant prostanoid was 6-keto-PGF$_{1\alpha}$, assaying at about 6 to 10 times more so than the other prostanoids (fig. 1). During the extraction procedure, there was approximately 84% recovery of prostanoids from the perfusate. Sample loss, however, was corrected for by the use of an internal standard (deuterated PGE$_2$).

HPLC assay of LTs. No significant increase was observed in the release of LTs in response to substance P or neurokinin A (10^{-7} M) infusions during the same time course as shown in the experiments in figures 1 to 3.

Release of eicosanoids from the canine ileum during neuropeptide infusion. On infusion of substance P (10^{-7} M), the release of 6-keto-PGF$_{1\alpha}$ was shown to increase significantly, whereas the relative concentrations of PGE$_2$ and thromboxane B$_2$ did not change.

Neuropeptide-induced release of 6-keto-PGF$_{1\alpha}$ was further investigated in the canine ileum using tachykinins selective for NK$_1$ or NK$_2$ receptors. RIA was subsequently used to quantitate 6-keto-PGF$_{1\alpha}$ in the perfusate from the canine ileum during tachykinin infusion.

Quantitation of 6-keto-PGF$_{1\alpha}$ by RIA: Comparison to GC-MS. To confirm that the amount detected by the indirect RIA method corresponded to that detected by GC-MS, a parallel analysis was performed on the same perfusate samples using both GC-MS and RIA. Perfusate samples were analyzed before and during neurokinin A (10^{-7} M) infusion. The quantity of 6-keto-PGF$_{1\alpha}$ detected by RIA and GC-MS yielded similar results (fig. 2). For each sample analyzed, the concentration of 6-keto-PGF$_{1\alpha}$ was approximately 15% higher than that detected by GC-MS. We have not adjusted the RIA values for this difference. It seemed consistent over the concentration range studied, but our data are too limited to establish its origin.

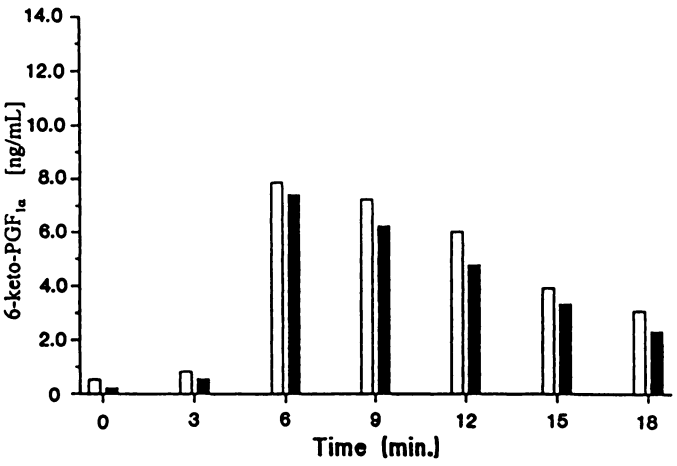

Fig. 2. Parallel analysis of perfusate for 6-keto-PGF$_{1\alpha}$ by RIA and GC-MS during neurokinin A infusion. The same perfusate samples were simultaneously analyzed by RIA (□) and GC-MS (■) for 6-keto-PGF$_{1\alpha}$ during neurokinin A (10^{-7} M) infusion. The values determined for 6-keto-PGF$_{1\alpha}$ release by RIA were approximately 15% higher than those determined by GC-MS. Therefore, the values obtained from subsequent RIA analysis of perfusate for this prostanoid were adjusted by a reduction of 15% to compare with that of GC-MS. Infusion was 0 to 9 min.

Quantitation of 6-keto-PGF$_{1\alpha}$ by RIA: Standard addition. A standard addition experiment was performed to further validate the measurement of 6-keto-PGF$_{1\alpha}$ by RIA. Perfusate was analyzed by RIA both before and after the addition of increasing quantities of 6-keto-PGF$_{1\alpha}$.

The quantity of 6-keto-PGF$_{1\alpha}$ added to the perfusate reflected the amount detected by RIA up to the addition of 100 pg 6-keto-PGF$_{1\alpha}$ (fig. 3). Thus, within this range the actual quantity of 6-keto-PGF$_{1\alpha}$ detected in the perfusate by RIA corresponded to the theoretical amount present in the assay

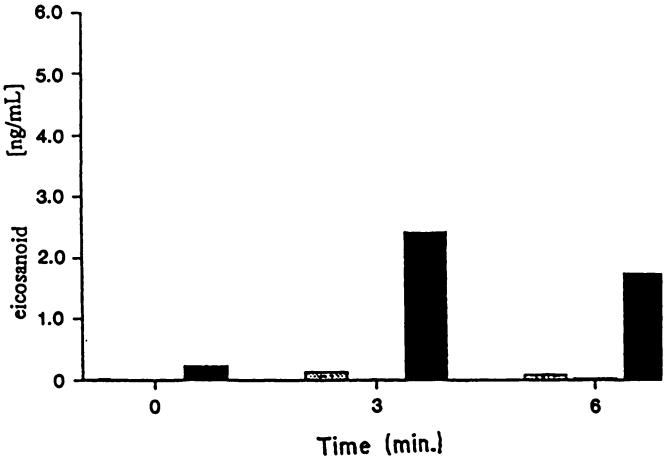

Fig. 1. Detection of eicosanoids from the perfused canine ileum by GC-MS. Perfusate was collected and analyzed by GC-MS for PGE$_2$ (▨), thromboxane B$_2$ (□) and 6-keto-PGF$_{1\alpha}$ (■) immediately before and 3 to 6 min during substance P (10^{-7} M) infusion. Increase in 6-keto-PGF$_{1\alpha}$ release was observed during infusion of substance P. Similar results were obtained with infusion of neurokinin A (10^{-7} M). Data are mean of 2 experiments.

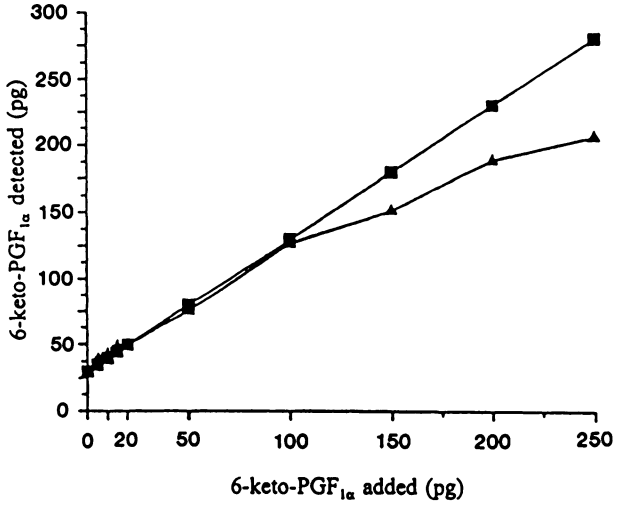

Fig. 3. Release of 6-keto-PGF$_{1\alpha}$ during two consecutive infusions of substance P. Increasing concentrations of 6-keto-PGF$_{1\alpha}$ were added to a perfusate containing 27 pg 6-keto-PGF$_{1\alpha}$ analyzed by RIA. The linear range of the standard addition curve is between 0 and 100 pg 6-keto-PGF$_{1\alpha}$. It is within this range that perfusate samples were analyzed by RIA for 6-keto-PGF$_{1\alpha}$ quantitation. Theoretical, ■; actual, ▲.

tube. Above 100 pg 6-keto-$PGF_{1\alpha}$ added to the perfusate, the amount detected by RIA was shown to be less than the theoretical amount added to the perfusate.

The volume of perfusate added to each assay tube was varied according to the concentration of 6-keto-$PGF_{1\alpha}$ present in each sample, so that the actual quantity of 6-keto-$PGF_{1\alpha}$ present in each assay tube was between 10 and 90 pg and within the linear range of the standard addition curve.

Control experiment: Substance P-induced release of 6-keto-$PGF_{1\alpha}$ from the canine ileum. The first initial infusion was performed under basal conditions without added pharmacological agents and was followed by the second in the presence of agonists/antagonists to determine their effects on 6-keto-$PGF_{1\alpha}$ release.

Preliminary experiments with a time period of 15 min between substance P (10^{-7} M) infusions showed a tachyphylactic response to 6-keto-$PGF_{1\alpha}$ release (fig. 4A). When a period of 48 min was allowed between two consecutive infusions of substance P (10^{-7} M), similar responses were observed for the release of 6-keto-$PGF_{1\alpha}$ (fig. 4B). Thus, the protocol with the longer period between infusions was used for the remainder of this study.

Characterization of receptors. Substance P caused a concentration-dependent increase in the release of 6-keto-$PGF_{1\alpha}$ from the canine ileum (fig. 5A). Release of 6-keto-$PGF_{1\alpha}$ was increased from a basal value of 0.77 ± 0.26 mg/ml and was maximal at 9.90 ± 2.09 mg/ml during the first 3 min of infusion of substance P (10^{-7} M); thereafter, it slowly returned to baseline.

A similar concentration-dependent increase in 6-keto-$PGF_{1\alpha}$ was obtained with the infusion of NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]substance P (fig. 5B). As with substance P, concentrations of 10^{-8} and 10^{-7} M of this agonist produced a significant increase in 6-keto-$PGF_{1\alpha}$ output. The basal release of 6-keto-$PGF_{1\alpha}$ was 0.93 ± 0.46 ng/ml, which peaked to 10.41 ± 2.57 ng/ml during infusion of this agonist at 10^{-7} M. Fifteen minutes after infusion, 6-keto-$PGF_{1\alpha}$ re-

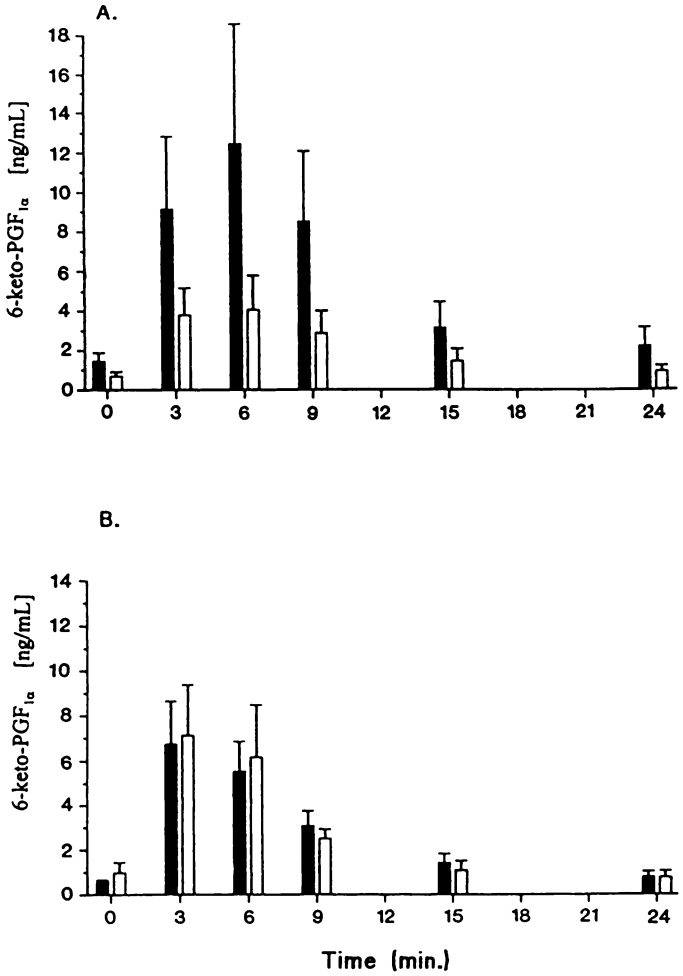

Fig. 4. Release of 6-keto-$PGF_{1\alpha}$ during two consecutive infusions of substance P. Experiments were performed to determine whether the induced release of 6-keto-$PGF_{1\alpha}$ was similar during two consecutive infusions of substance P (10^{-7} M). Substance P was infused for 9 min starting immediately after time 0. The time between infusions were 15 min and 48 min (fig. A and B, respectively). Tachyphylaxis occurred during the second infusion of substance P when 15-min recovery was allowed between infusions. Data are represented as mean ± S.E.M.; 10^{-7} M first infusion, ■; 10^{-7} M second infusion, □; n = 3 in A; n = 4 in B.

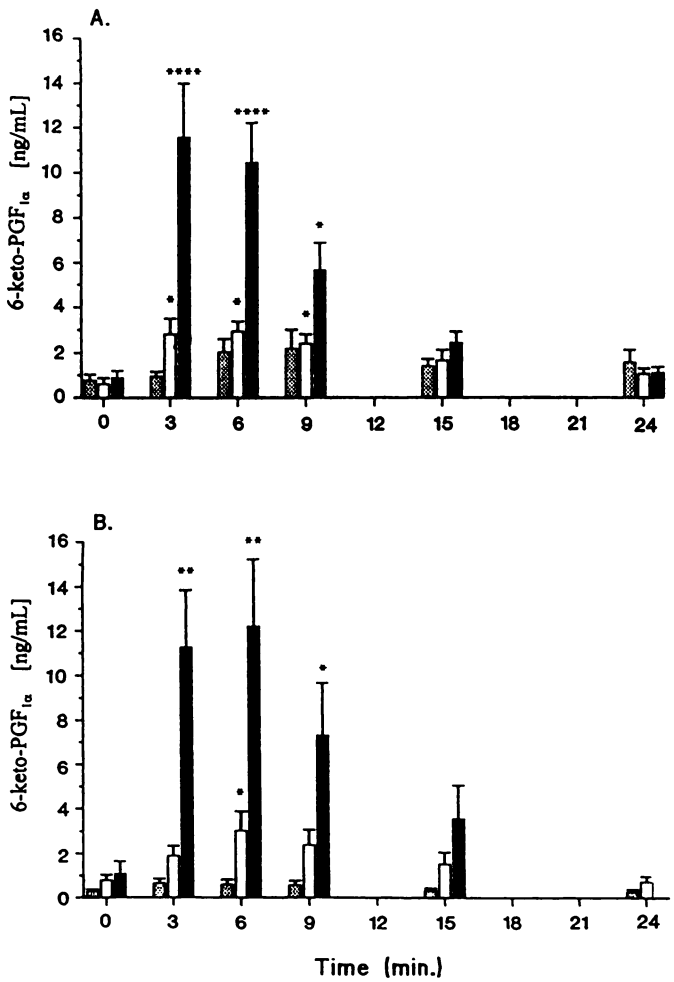

Fig. 5. Concentration-dependent release of 6-keto-$PGF_{1\alpha}$ during infusions of substance P and [Sar^9,$Met(O_2)^{11}$]-substance P: Substance P and [Sar^9,$Met(O_2)^{11}$]substance P caused a concentration-dependent release of 6-keto-$PGF_{1\alpha}$ from the canine ileum (A and B, respectively). Each agonist was infused for 9 min starting immediately after time 0. Data are represented as mean ± S.E.M. Statistical analysis was performed by comparing the concentration of 6-keto-$PGF_{1\alpha}$ at each time interval for a given concentration of agonist to that at time 0. Levels of significance were determined as ****P > .0001; ***P > .0005; **P > .005; *P > .05. A, 10^{-9} M ▨ n = 5; 10^{-8} M □ n = 4; 10^{-7} M ■ n = 15. Data are represented as mean ± S.E.M. B, 10^{-9} M ▨ n = 4; 10^{-8} M □ n = 5; 10^{-7} M ■ n = 7.

lease returned to values that were not significantly different from control.

To determine the role of NK_1 receptors in mediating release of 6-keto-$PGF_{1\alpha}$ the effects were compared with those of the selective NK_1 receptor antagonist CP-96,345 (10^{-7} M) in blocking the release of this prostanoid during NK_1 receptor activation with substance P (10^{-7} M) and [Sar^9,Met(O$_2$)^{11}]-substance P (10^{-7} M). CP-96,345 completely blocked the induced release of 6-keto-$PGF_{1\alpha}$ during infusion of substance P (10^{-7} M) (fig. 6A) as well as from infusion of 10^{-7} M [Sar^9,Met(O$_2$)^{11}]substance P (fig. 6B). Thus, the selective NK_1 receptor antagonist abolished the release of 6-keto-$PGF_{1\alpha}$ during NK_1 receptor activation.

Infusion of neurokinin A increased the release of 6-keto-$PGF_{1\alpha}$ in a concentration-dependent manner (fig. 7A) but with significant effects only at (10^{-7} M); the release of this prostaglandin increased from 0.77 ± 0.42 to 9.24 ± 2.01 ng/ml. The selective NK_2 receptor agonist [Nle^{10}]neurokinin A(4–10) did not significantly affect 6-keto-$PGF_{1\alpha}$ output under these conditions, even at 10^{-6} M (fig. 6B).

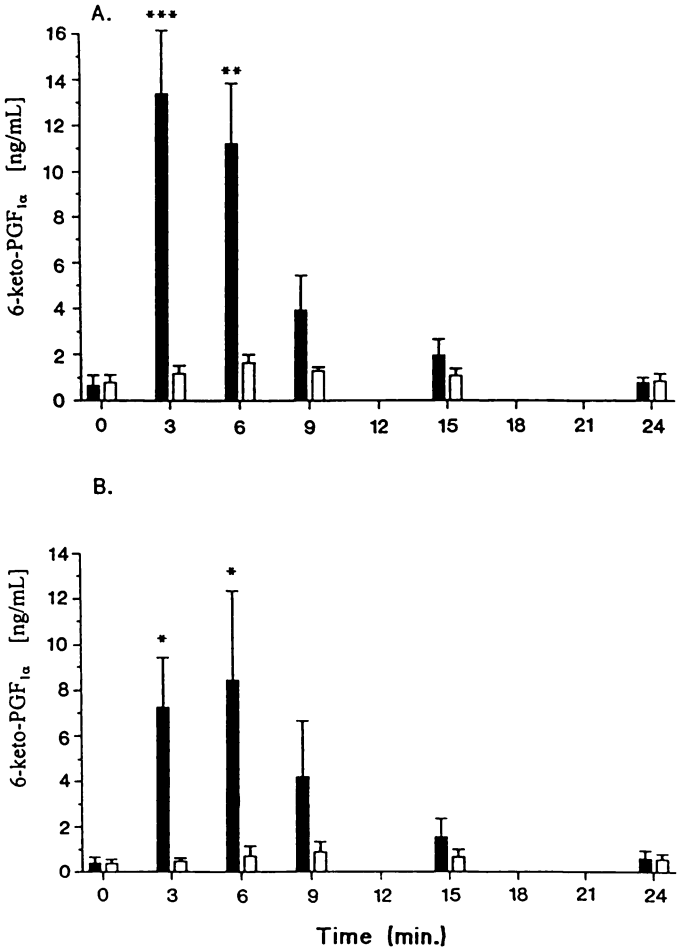

Fig. 6. The effect of CP-96,345 on the induced release of 6-keto-$PGF_{1\alpha}$ from the canine ileum during substance P and [Sar^9,Met(O$_2$)^{11}]substance P infusion. Substance P (10^{-7} M) and [Sar^9,Met(O$_2$)^{11}]substance P (10^{-7} M) were individually infused for 9 min both before and after exposure to the selective NK_1 receptor antagonist CP-96,345 (10^{-7} M) (A and B, respectively). The time interval between infusions was 48 min. CP-96,345 successfully blocked the induced release of 6-keto-$PGF_{1\alpha}$ during infusion of substance P and the selective NK_1 receptor agonist. Data are represented as mean ± S.E.M. Values that were significantly different from the value at the same time interval in the absence of the antagonist were determined as follows: ***P > .0005; **P > .005; *P > .05. A and B, 10^{-7} M ■; 10^{-7} M after CP-96,345 10^{-7} M □; n = 4.

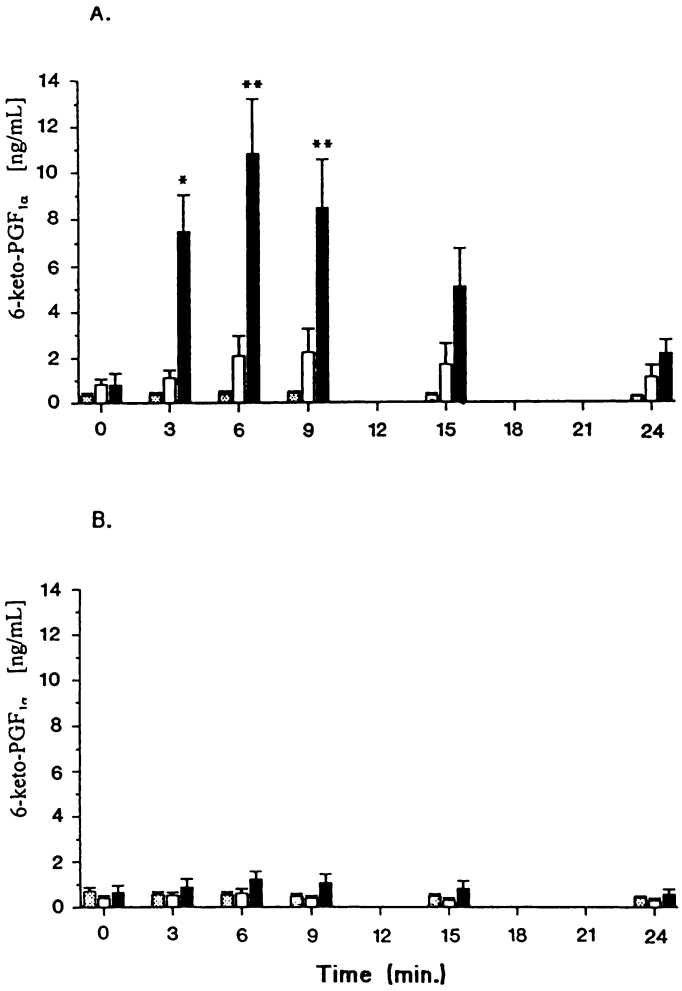

Fig. 7. Release of 6-keto-$PGF_{1\alpha}$ during infusions of neurokinin-A and [Nle^{10}]neurokinin A(4–10). Neurokinin A caused a concentration-dependent release of 6-keto-$PGF_{1\alpha}$ from the canine ileum (A). The selective NK_2 receptor agonist [Nle^{10}]neurokinin A(4–10) did not alter 6-keto-$PGF_{1\alpha}$ output (B). Each agonist was infused for 9 min starting immediately after time 0. Data are represented as mean ± S.E.M. Statistical analysis was performed by comparing the concentration of 6-keto-$PGF_{1\alpha}$ at each time interval for a given concentration of agonist to that at time 0. Levels of significance were determined as: **P > .005; *P > .05. A, 10^{-9} M ▨ n = 4; 10^{-8} M □ n = 4; 10^{-7} M ■ n = 9. B, 10^{-9} M ▨ n = 4; 10^{-8} M □ n = 4; 10^{-7} M ■ n = 4.

The selective NK_2 receptor antagonist MEN 10207 (10^{-7} M) partially blocked the increase in 6-keto-$PGF_{1\alpha}$ induced by neurokinin A (10^{-7} M) infusion (fig. 8A), whereas the NK_1 selective antagonist CP-96,345 (10^{-7} M) abolished the release of this prostaglandin by neurokinin A (10^{-7} M) (fig. 8B).

The concentration-dependent release of 6-keto-$PGF_{1\alpha}$ by substance P, neurokinin A and their analogs (fig. 9) shows that all except the NK_2 agonist [Nle^{10}]neurokinin A(4–10) have comparable maximal responses.

Site of prostacyclin production in the canine ileum. Experiments were conducted to investigate the location of prostacyclin production (as measured by production of 6-keto-$PGF_{1\alpha}$) in the *ex vivo* perfused canine ileum. An additional cannula was reversely implanted in one of the side arterial branches just proximal to where the vessel enters the small intestine. With this approach, the release of 6-keto-$PGF_{1\alpha}$

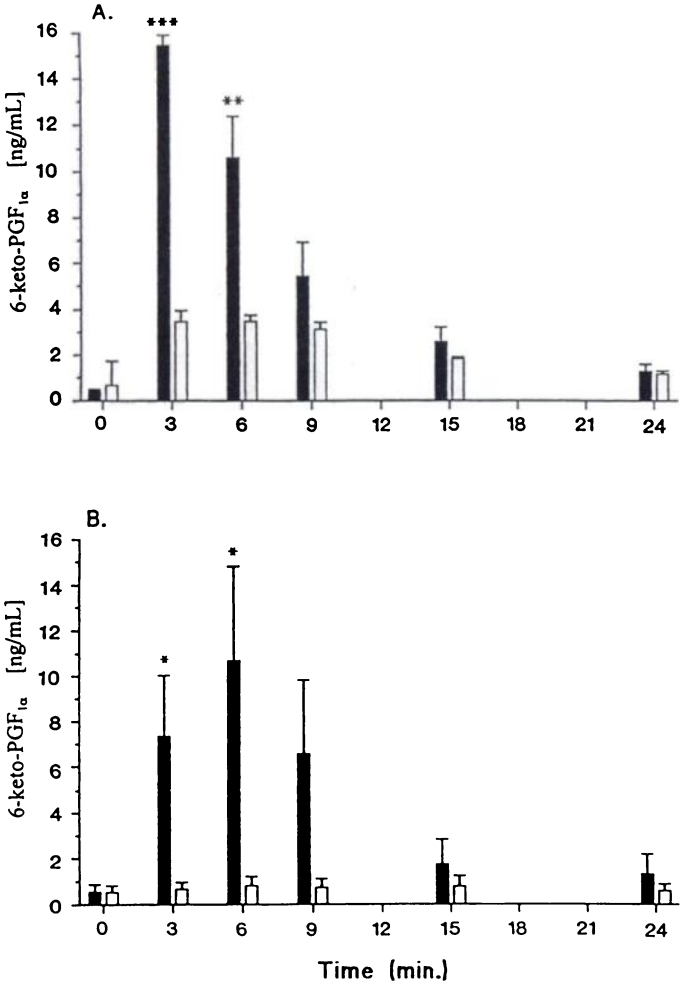

Fig. 8. The effect of MEN 10207 and CP-96,345 on neurokinin A-induced release of 6-keto-$PGF_{1\alpha}$ from the canine ileum. Neurokinin A (10^{-7} M) was infused for 9 min both before and after exposure to the NK_2 receptor antagonist MEN 10207 (10^{-7} M) and the selective NK_1 receptor antagonist CP-96,345 (10^{-7} M) (A and B, respectively). The time interval between infusions was 48 min. MEN 10207 was shown to partially block the induced release of 6-keto-$PGF_{1\alpha}$ during infusion of neurokinin A. CP-96,345 completely blocked neurokinin A-induced release of 6-keto-$PGF_{1\alpha}$. Data are represented as mean ± S.E.M. Values that were significantly different from the value at the same time interval in the absence of the antagonist were determined as follows: ***P > .0005; **P > .005; *P > .05. A, 10^{-7} M ■; 10^{-7} M after MEN 10207 10^{-7} M □; n = 4. B, 10^{-7} M ■; 10^{-7} M after CP-96,345 10^{-7} M □; n = 4.

was monitored from the arterial mesentery before entering the intestine.

Increase in 6-keto-$PGF_{1\alpha}$ release was observed from the arterial circulation during individual infusions of substance P, [Sar^9,$Met(O_2)^{11}$]substance P or neurokinin A at concentrations of (10^{-7} M) (fig. 10, A to C, respectively). This increase in 6-keto-$PGF_{1\alpha}$ release was successfully blocked by CP-96,345 (10^{-7} M). The concentration of this prostanoid detected from the arterial circulation was small in proportion to that detected in the venous outflow, reflecting the smaller area of vasculature perfused compared with that in the standard experiments.

To further assess whether the intestinal musculature could have been producing a prostanoid-like PGI_2, which inhibits intestinal muscle function (Sanders, 1984), we examined the effect of fluribiprofen at various concentrations on the motor responses of the intestine circular muscle to substance P. If an inhibitory prostanoid was being produced by substance P, inhibition of its synthesis by this cyclooxygenase inhibitor should augment contractile responses. However, this did not occur. After 9 min of preliminary perfusion, 1 and 10 μM fluribiprofen had little significant effect in motor responses to substance P (10^{-7} M), and at 100 μM (and after 9 min at 10 μM), it decreased them (see table 1). There was no trend showing an increase in contraction at any concentration of fluribiprofen. At each concentration of fluribiprofen, production of 6-keto-$PGF_{1\alpha}$ was reduced to levels below the detection limit, showing that effective block of cyclooxygenase had occurred. These observations suggested that the inhibition of contractions to substance P seen at higher concentrations were nonspecific effects. Thus, these data suggest that no inhibitory prostanoid was being produced by canine intestinal muscle under our conditions.

Interaction between prostacyclin and nitric oxide. Since prostacyclin is likely to be produced primarily from the arterial vasculature endothelium, there is a possibility for an interaction between this prostanoid and EDRF (nitric oxide) (DeNucci *et al.*, 1988). L-NAME was used to inhibit nitric oxide synthesis in the perfused canine ileum. Confirmation that nitric oxide release was blocked by L-NAME was observed by a loss of distal inhibition and an enhancement of proximal and local motor response to electrical field stimulation (1 pps and 3 pps). Substance P (10^{-7} M) was infused before and after the administration of L-NAME (3×10^{-4} M) to determine whether the inhibition of nitric oxide synthesis influenced substance P-induced release of 6-keto-$PGF_{1\alpha}$. The inhibition of nitric oxide synthesis did not influence the release of 6-keto-$PGF_{1\alpha}$ from the perfused canine ileum on NK_1 receptor activation by substance P (fig. 11).

Role of extracellular calcium in substance P-induced release of 6-keto-$PGF_{1\alpha}$. In the absence of extracellular calcium, a partial but significant inhibition of the substance P-induced release of 6-keto-$PGF_{1\alpha}$ was observed (fig. 12). On the replenishment of extracellular calcium, substance P-induced release of 6-keto-$PGF_{1\alpha}$ returned to normal. Thus, extracellular calcium is partially required for the induced release of 6-keto-$PGF_{1\alpha}$ from the canine ileum.

Discussion

The results show that the tachykinin-evoked release of 6-keto-$PGF_{1\alpha}$ from the canine ileum is mediated principally by NK_1 receptors. Thus, substance P, [Sar^9,$Met(O_2)^{11}$]substance P and neurokinin A induced a concentration-dependent release of 6-keto-$PGF_{1\alpha}$ from the canine ileum (figs. 4–9). The release of 6-keto-$PGF_{1\alpha}$ induced by NK_1 receptor activation was completely blocked by the selective NK_1 receptor antagonist CP-96,345 (fig. 6). The selective NK_2 receptor agonist [Nle^{10}]neurokinin A(4–10) did not alter the basal release of 6-keto-$PGF_{1\alpha}$ from the canine ileum (fig. 7B). Since neurokinin A effects were abolished by CP-96,345, the most parsimonious hypothesis is that it acted exclusively at the NK_1 receptor (Guard and Watson, 1991; Regoli and Nantel, 1991), a conjecture consistent with the high concentrations of this agonist required for effect. If this hypothesis is correct, the selective NK_2 antagonist used (MEN 1207) was also acting on NK_1 receptors under our experimental conditions. This compound at higher concentrations is known to inhibit

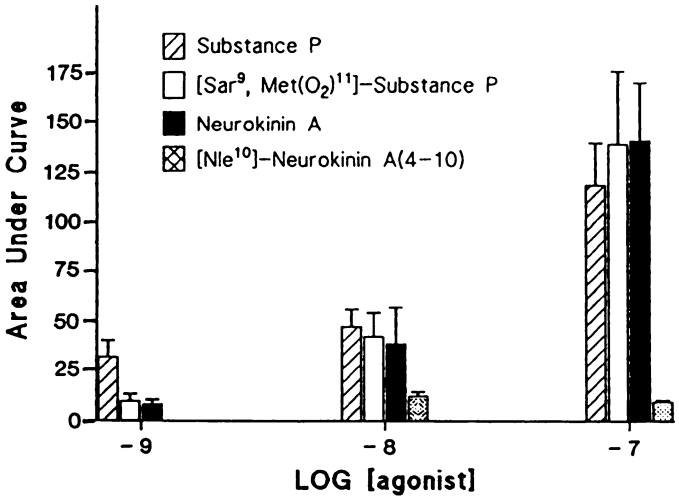

Fig. 9. Concentration-effect curve for the induced release of 6-keto-PGF$_{1\alpha}$ in the canine ileum by various tachykinin agonists. The total release of 6-keto-PGF$_{1\alpha}$ from the canine ileum induced by increasing concentrations of tachykinin agonists. The release of 6-keto-PGF$_{1\alpha}$ is represented by the area under the curve obtained from the temporal release of this prostanoid during the 9-min infusion period of each tachykinin agonist. Substance P □; [Sar^9,Met(O$_2$)^{11}]substance P □; neurokinin A ■; [Nle^{10}]neurokinin A(4-10) □.

activation of NK-1 receptors (Maggi *et al.*, 1990; Xie *et al.*, 1992). These results support the concept that tachykinin-induced release of 6-keto-PGF$_{1\alpha}$ from the canine ileum is mediated by NK$_1$ receptor activation. However, our studies do not exclude a possible role for NK$_2$ receptors, especially when high concentrations of selective agonists are used.

The proposed site of tachykinin-induced production of prostacyclin (as measured by its degradation product 6-keto-PGF$_{1\alpha}$) from the canine ileum is the mesenteric vasculature (fig. 10). Substance P, [Sar^9,Met(O$_2$)^{11}]substance P and neurokinin A all equally induced the release of 6-keto-PGF$_{1\alpha}$ from the mesenteric vasculature. Thus, much of the 6-keto-PGF$_{1\alpha}$ release measured from the whole perfused ileal segment may have originated primarily from the mesenteric vasculature (figs. 10, A to C) as considered further below.

Prostacyclin is primarily produced by the vascular endothelium (Ingerman-Wojenski *et al.*, 1981). The release of prostacyclin by tachykinins has recently been investigated using freshly prepared cultured human umbilical vein endothelial cells (Marceau *et al.*, 1989). NK$_1$ receptors were shown to mediate tachykinin-induced release of 6-keto-PGF$_{1\alpha}$ from these cells (Marceau *et al.*, 1989). Although it was not possible to determine the specific cell type responsible for prostacyclin production used in the perfused canine ileum in the present studies, its findings are consistent with endothelial cells being one of the principal sources (Marceau *et al.*, 1989).

TABLE 1

Responses (percent maximum contraction) to 10^{-7} M substance P at various times of infusion

Concentration of fluribiprofen	3 min	6 min	9 min
Control	82±17	74±10	64±9
1 μM	75±13	55± 6	45±4
Control	69±18	81± 6	69±3
10 μM	67±19	56±12	40±5*
Control	87± 4	71± 7	55±1
100 μM	72± 9	48± 7*	31±1*

All values are mean ± S.E.M. n = 4 at all concentrations of fluribiprofen.
* P > .05 that fluribiprofen affected responses.

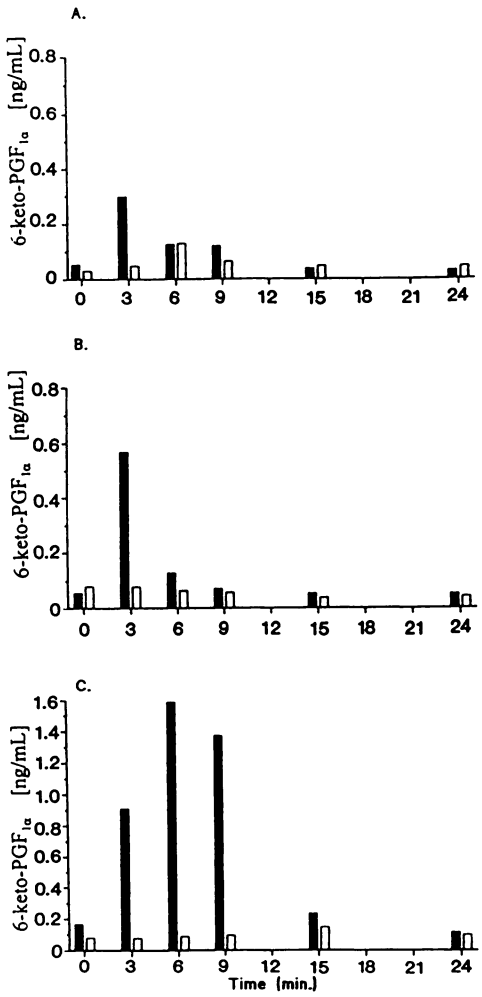

Fig. 10. Release of 6-keto-PGF$_{1\alpha}$ from the canine mesenteric arterial vasculature during tachykinin infusion. Substance P (10^{-7} M), [Sar^9,Met(O$_2$)^{11}]substance P (10^{-7} M) and neurokinin A (10^{-7} M) were individually infused for 9 min both before and after exposure to CP-96,345 (10^{-7} M) (A, B and C, respectively). The time interval between infusions was 48 min. Perfusate was collected from a mesenteric arterial branch at a location just before entering the ileum. All three tachykinins caused a increase in 6-keto-PGF$_{1\alpha}$ release from the arterial vasculature that was blocked by CP-96,345. This is a typical experiment from the four performed. A, B and C; 10^{-7} M ■; 10^{-7} M after CP-96,345 10^{-7} M.

Strain gauges sewn onto record circular muscle contraction recorded contractions in response to the tachykinins infused in this study. Analysis of those responses after blockade of 6-keto-PGF$_{1\alpha}$ production by 1 or 10 μM fluribiprofen revealed little or no effects on motor function (table 1). At 100 μM fluribiprofen, there was significant inhibition of the responses to substance P, and a significant reduction was seen at one time point when 10 μM was used. At no concentration was there a trend to increased response with cyclooxygenase inhibition (Sanders, 1984). This suggested that inhibitory prostanoid production by tachykinins in the muscle of the intestine was not modifying contractions. Since PGE$_2$ and PGI$_2$ can inhibit such contractions, (Sanders, 1984), the finding reinforces that the vasculature was the major source of

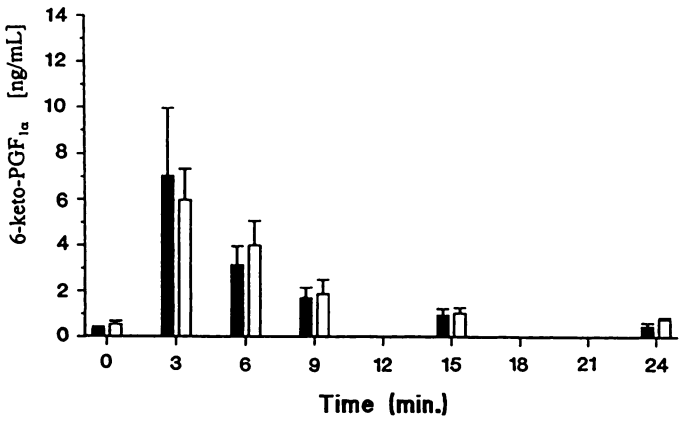

Fig. 11. The effect of L-NAME on substance P-induced release of 6-keto-$PGF_{1\alpha}$ from the canine ileum. Substance P (10^{-7} M) was infused for 9 min before and after exposure to the nitric oxide synthase inhibitor, L-NAME (3×10^{-4} M). The time interval between infusions was 48 min. L-NAME did not affect the induced release of 6-keto-$PGF_{1\alpha}$ during infusion of substance P. Data are represented as mean ± S.E.M. Statistical analysis was performed by comparing the release of 6-keto-$PGF_{1\alpha}$ induced by substance P at each time interval in the absence and presence of L-NAME. 10^{-7} M ■; 10^{-7} M after L-NAME (3×10^{-4} M) □, $n = 4$.

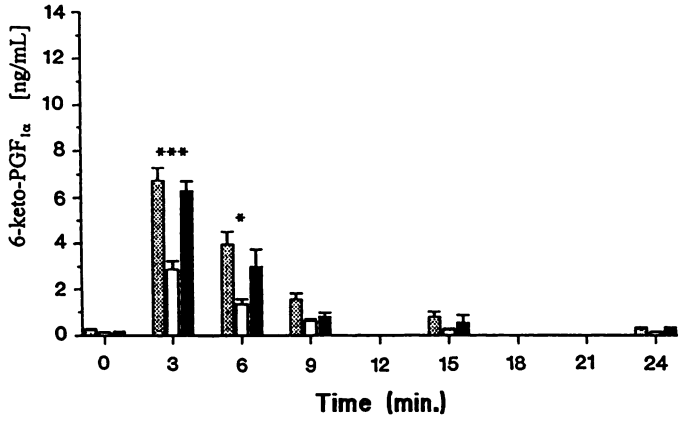

Fig. 12. The effect of calcium-free Krebs' solutions on the induced release of 6-keto-$PGF_{1\alpha}$ from the canine ileum during substance P infusion. Substance P (10^{-7} M) was infused for 9 min both before and after exposure to calcium-free Krebs' solution. Substance P was infused for a third time after replenishment of extracellular stores of calcium. The time interval between each infusion of substance P was 48 min. Calcium-free Krebs' solution significantly reduced substance P-induced release of 6-keto-$PGF_{1\alpha}$. The induced release of 6-keto-$PGF_{1\alpha}$ during substance P infusion returned to normal after re-infusion with normal Krebs' solution. Data are represented as mean ± S.E.M. Statistical significance was performed by comparing the concentration of 6-keto-$PGF_{1\alpha}$ at each time interval during the second and third infusions of substance P to that during the first infusion of substance P. Levels of significance were determined as ***$P > .0005$; *$P > .05$. 10^{-7} M □ $n = 4$; 10^{-7} M calcium-free Krebs' solution ▨ $n = 4$; 10^{-7} M ■ $n = 4$.

6-keto-PGF_2 contractions. Other sources such as the intestinal musculature could not be excluded by these data. Other possible sources, such as nerves, also seem unlikely since responses to electrical field stimulation of nerves were also unaffected by fluribiprofen at the two lower concentrations. Both PGE_2 and PGI_2 are inhibitors of mediator release in most cholinergic systems (Sanders, 1984; Serio and Daniel, 1989). However, conclusive proof that only endothelial cells produced PGI_2 cannot be provided by our data, and a contribution from the intestinal musculature is also possible.

The physiological significance of tachykinin-induced production of prostacyclin in the mesenteric vasculature of the canine ileum remains to be established. The possible physiological and pathophysiological functions of substance P and other tachykinins has received have been reviewed in depth (Otsuka and Yoshioka, 1993). This review summarized evidence that along with CGRP substance P often is located in nerves, mostly of sensory origin, that innervate blood vessels. There is a large body of evidence that substance released from these nerves in response to axon reflexes regulates local blood flow in an endothelium-dependent manner and can cause the "neurogenic inflammation" induced by capsaicin in endothelial cells and other noxious stimuli. NK_1 receptors appear to be the sites of initiation of these responses (Otsuka and Yoskioka, 1993). Since local vasodilation and edema are involved in these responses and they can be blocked by antibodies and antagonists to substance P, it appears likely that NK_1 receptor activation by substance P plays both physiological and pathophysiological roles in modulation of blood flow and inflammatory responses to vasculature. The relative importance of PGI_2 compared with EDRF release in responses to substance P is not entirely clear. Substance P is a potent vasodilator both *in vivo* and *in vitro*, whose action is mediated through NK_1 receptor activation (Bartho and Holzer, 1985; Regoli *et al.*, 1988). The vasodilatory effects of substance P are, however, resistant to indomethacin (Couture and Regoli, 1982) but require the presence of an intact endothelium (Regoli *et al.*, 1988). Thus, the relaxation of vascular smooth muscle was proposed not to be due to prostanoid release but rather due to the release of an EDRF (Regoli *et al.*, 1988) such as nitric oxide (Sanders and Ward, 1992). However, this does not rule out the possibility of an interaction between the release of prostacyclin and EDRF, both responses of which are mediated by NK_1 receptor activation (Marceau *et al.*, 1989; Regoli *et al.*, 1988).

DeNucci *et al.* (1988) have proposed that the induced release of both EDRF and PGI_2 from bovine aortic endothelial cells is coupled. In their studies, bovine aortic endothelial cells were grown on microcarrier beads and perfused with Krebs-Ringer solution. The vasodilatory effect of EDRF was bioassayed on strips of rabbit aorta, whereas PGI_2 was analyzed by RIA for 6-keto-$PGF_{1\alpha}$. The release of EDRF and prostacyclin was shown to be induced by bradykinin, ADP, ATP, arachidonic acid and phospholipase C activation (DeNucci *et al.*, 1988). Bradykinin and calcium ionophore A23187 were also previously shown to induce the release of PGI_2 and EDRF from porcine aortic endothelial cells using this same methodology (Gryglewski *et al.*, 1986).

It is suggested that the co-release of EDRF and PGI_2 from bovine endothelial cells is due to the activation of phospholipase C (DeNucci *et al.*, 1988). Exogenous phospholipase C was shown to induce the release of both EDRF and PGI_2 where as phospholipases B and D were ineffective (DeNucci *et al.*, 1988). Phospholipase A_2, however, was shown only to induce the release of PGI_2 from bovine endothelial cells (DeNucci *et al.*, 1988). In porcine endothelial cells, bradykinin activated both phospholipase A_2 and C (Hong and Deykin, 1982; Lambert *et al.*, 1986), leading to rapid production of inositol triphosphate with subsequent mobilization of calcium and liberation of arachidonic acid for PGI_2 synthesis (Lambert *et al.*, 1986).

The possibility of an interaction between substance P-induced prostacyclin production and EDRF release was in-

vestigated in the perfused canine ileum by the blockade of nitric oxide synthase with L-NAME. If the release of these two vasodilators during substance P infusion is coupled, one would expect to observe an alteration in substance P-induced release of 6-keto-PGF$_{1\alpha}$ during nitric oxide blockade. This study did not reveal an alteration in the induced release of 6-keto-PGF$_{1\alpha}$ by substance P during the inhibition of nitric oxide synthesis.

It therefore appears that in this present model, substance P activates phospholipase C to induce release of PGI$_2$ independent of any requirement for release of nitric oxide. It is also possible that substance P stimulates the activity of only phospholipase A$_2$ to induce the direct release of prostacyclin. Further investigations with specific phospholipase inhibitors would provide definitive proof of the mode of action of substance P-induced release of prostacyclin from the canine ileum.

Receptor-mediated release of prostanoids is dependent on the influx of extracellular calcium to activate phospholipase A$_2$ for arachidonic acid liberation (Smith, 1989). The role of extracellular calcium in substance P-induced release of 6-keto-PGF$_{1\alpha}$ in the canine ileum was investigated. The use of calcium-free Krebs' solution containing 100 μM EGTA partially (but significantly) reduced the release of 6-keto-PGF$_{1\alpha}$ induced by intra-arterial infusion of substance P (10^{-7} M). The release of 6-keto-PGF$_{1\alpha}$ was not totally abolished despite the absence of extracellular calcium, indicated by the lack of a motor response of the circular muscle during electrical field stimulation before substance P infusion. Thus, internal calcium stores as well as extracellular calcium must have been used to activate the release of this prostanoid during substance P infusion. This finding suggests that substance P activated phospholipase C in this study.

We conclude that tachykinins act, most likely on the intestinal vasculature, through NK$_1$ receptors to release PGI$_2$ and that this release uses both intracellular and extracellular calcium but is independent of activity of nitric oxide synthase. One major probable source of PGI$_2$ is the vascular endothelium, and activation of phospholipase C may be involved in its release in response to substance P.

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