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Functional characterisation of tachykinin receptors mediating ion transport in porcine jejunum

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Abstract

In the present study, tachykinin receptors (designated NK_1, NK_2 and NK_3) involved in regulation of ion transport in porcine jejunum were characterised. Stripped tissue preparations were mounted in Ussing chambers and short-circuited. Substance P produced a concentration dependent increase in short-circuit current, the relationship showing a double sigmoidal form. The non-peptide NK_1 receptor antagonist, CP 99,994 ((2*S*,3*S*)-3-(2-methoxybenzyl)amino-2-phenylpiperidine), caused a dextral shift of the first sigmoidal response, indicating the involvement of an NK_1 receptor. This was further supported by a concentration-dependent response of the NK_1 receptor agonist [$Sar^9Met(O_2)^{11}$]substance P with an EC_{50} value of 235.0 ± 53.9 nM. Increasing concentrations of CP 99,994 (0.1, 0.3 and 1 μM) produced a parallel dextral shift of the [$Sar^9Met(O_2)^{11}$]substance P curve with a slope of the Schild regression significantly different from unity (1.59). The neurokinin A concentration–response curve, with an EC_{50} value of 68.87 ± 16.23 nM, was not significantly changed by the non-peptide NK_2 receptor antagonist SR 48,968 ((*S*)-*N*-methyl-*N*-(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl)bezamide). In additional studies, the peptide NK_2 receptor antagonists, GR 94,800 (PhCO–Ala–Ala–DTrp–Phe–DPro–Pro–$NleNH_2$) and PD 147,714 ((2,3-diOMeZ)-(*S*)Trp(*S*)αMePheGlyNH_2), did not change the response to neurokinin A. However, CP 99,994 totally inhibited neurokinin A responses at 0.5 μM and above. The NK_2 receptor agonist, [β-Ala^8]neurokinin A-(4–10), caused only an increase in short-circuit current in μM concentrations, whereas the NK_3 receptor agonist, senktide, did not elicit a response. These results indicate, that substance P and neurokinin A mediate ion transport in porcine jejunum through NK_1 receptors. However, tachykinins seem to activate another receptor. Two active conformers of the NK_1 receptor might be present.

Keywords: Substance P; Neurokinin A; Tachykinin receptor; Porcine jejunum; Ion transport

1. Introduction

Tachykinins are a family of peptides sharing a common C-terminal sequence. In mammalian tissue, the family includes substance P, neurokinin A, neurokinin B and the two N-terminal extended forms of neurokinin A, neuropeptide K and neuropeptide γ (Mussap et al., 1993). Substance P and neurokinin A are the major tachykinin peptides present in the porcine small intestine (Schmidt et al., 1991). These peptides have been located in the submucosal and myenteric nerve plexus, indicating a role as neurotransmitters in the enteric nervous system (Furness and Costa, 1980; Otsuka and Yoshioka, 1993). The presence of substance P and neurokinin A in the submucosal plexus of porcine jejunum suggests a role in regulation of intestinal ion transport (Schmidt et al., 1991). Indeed, it was shown that tachykinins alter ion transport through substance P-preferring receptors associated with neural pathways and tachykinin receptors on target tissues (Parsons et al., 1992).

In both biochemical and functional assays on different species and tissues, it has been shown that substance P, neurokinin A and neurokinin B exhibit preferential affinity for three tachykinin receptors designated NK_1, NK_2 and NK_3. However, all tachykinins are able to act as full agonists at all three receptors, although with different affinities (Regoli et al., 1987). A more thorough characterisation of tachykinin receptors in target tissues has until recently been hampered by the lack of selective and high affinity antagonists. Within the last half decade a number of antagonists of both peptide and non-peptide nature have become available (Maggi, 1995).

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The aim of the present study was to extend our knowledge on the effects of substance P and neurokinin A on ion transport in porcine jejunum by further characterising the tachykinin receptors involved. The pig was chosen for this receptor characterisation, due to the close similarity to man with respect to gastrointestinal anatomy and physiology (Miller and Ullrey, 1987). Tachykinins seem to be involved in the secretory actions of *Clostridium difficile* toxin A, and in inflammatory bowel disease, in which alterations in ion transport constitute part of the pathophysiology (Mantyh et al., 1988; Pothoulakis et al., 1994; Keränen et al., 1995; Mantyh et al., 1996). Thus, tachykinin receptor antagonists might have a future therapeutic potential in diseases involving excessive intestinal secretion.

2. Materials and methods

2.1. Tissue preparation

Danish Landrace/Yorkshire crossbred 6–8 weeks (13–15 kg) fully weaned pigs of each sex on a commercial standard diet (N.A.G. svinefoder 5, Helsinge, Denmark) were fasted 12 h before experiment, but had free access to sterile drinking water containing D-glucose (55 g/l). Twenty minutes before the commencement of anaesthesia, pigs were sedated by an intramuscular injection of azaperone (5 mg/kg) (Stresnil, Janssen Pharmaceutica, Belgium). Anaesthesia was induced by an intravenous injection of pentobarbital (10 mg/kg), followed by intubation of pigs and maintaining anaesthesia by halothane inhalation (2% in oxygen) via a semi-closed circuit. Immediately after anaesthesia was obtained, a laparotomy was performed. A 40-cm segment of jejunum was excised 30 cm distal to the ligament of Treitz. The tissue was, by blunt dissection, stripped of its serosa and smooth muscle layers, including the myenteric plexus, thus leaving the submucosa/mucosa intact. The stripped segment was opened along the mesenteric border and placed in oxygenated HCO_3^-–Ringer solution containing (in mM): 145 Na^+, 6.3 K^+, 2.0 Ca^{2+}, 1.0 Mg^{2+}, 128 Cl^-, 25 HCO_3^-, 0.32 $H_2PO_4^-$, 1.0 SO_4^{2-} and 16.5 D-glucose. Pigs were euthanised by an intracardiac injection of pentobarbital (75 mg/kg). The experiments in the present paper comply with the recommendations approved by the Danish Council of Animal Experiments.

By the use of a pair of scissors tissue sheets were carefully prepared and mounted in a voltage clamp set-up. The Ussing chambers had an opening area of 1 cm^2, surrounded by a rubber ring on the mucosal side to minimise edge damage. Both sides of the tissue were bathed with 7 ml oxygenated (5% CO_2 in O_2) Ringer solution maintained at pH 7.4. The glass reservoirs of the air-lifts and the Ussing chambers were siliconised (SurfaSil, Pierce Chemical, USA) and kept at 39.1°C (porcine core temperature) by water-jackets connected to a water bath.

2.2. Electrical measurements

Tissues were short-circuited to a potential difference of 0 mV maintained during the entire experiment. The potential difference across each tissue was measured by two Ag–AgCl electrodes (Clark Electromedical Instruments, UK) in 3 M KCl, each connected to a half chamber by a 3% agar bridge. Current was passed through the tissue by Ag–AgCl electrodes. The electrodes were connected to a multichannel computer-controlled voltage clamp. The clamp apparatus has been described earlier (Clauss et al., 1988). Tissue resistance was determined by computer imposed bipolar voltage command steps (3 mV, 300 ms duration). The measurements were automatically corrected for solution resistance. The potential difference was calculated from tissue resistance and short-circuit current. The computer software provided corresponding values for potential difference, tissue resistance and short-circuit current every 20 s.

2.3. Data evaluation

The baseline short-circuit current was allowed to stabilise for approximately 45 min. Drugs and peptides were added to the serosal bathing medium, except for theophylline, which was added on both sides. Peptidase inhibitors and receptor antagonists were added to the serosal side 15 min prior to subsequent peptide addition, unless stated otherwise. Measurements of the peak changes in short-circuit current (i.e., difference between peak response and baseline short-circuit current) were used as a measure for effects of drugs and peptides.

Preliminary experiments showed that substance P and neurokinin A produced transient increases in short-circuit current. This confirmed earlier observations in pig jejunum (Parsons et al., 1992). To investigate the possibility that the transient response to substance P and neurokinin A was due to their rapid degradation by peptidases, the effect of peptidase inhibitors was investigated. The two peptides are substrates for neutral endopeptidase 24.11, whereas only neurokinin A is a substrate for aminopeptidases A and N (Nau et al., 1985, 1986). The peptidase inhibitors, phosphoramidon (10 μM) and bestatin (100 μM) were used, inhibiting neutral endopeptidase 24.11 and aminopeptidases A and N, respectively. As a phosphoramidon-insensitive component of endopeptidase 24.11 seems to exist, the effect a synthetic inhibitor of neutral endopeptidase 24.11, thiorphan (10 μM), was also investigated (Nau et al., 1986). Peptidase inhibitors did not alter basal short-circuit current (data not shown). None of the inhibitors changed significantly, neither the transient character (traces not shown), nor the peak response to 0.1 μM substance P (control, 27.2 ± 2.7 μA cm^{-2}, $n = 6$; phosphoramidon, 21.8 ± 2.9 μA cm^{-2}, $n = 6$; bestatin, 22.0 ± 3.1 μA cm^{-2}, $n = 5$; thiorphan, 28.9 ± 4.3 μA cm^{-2}, $n = 7$) or 0.1 μM neurokinin A (control, 19.5 ± 1.6 μA cm^{-2}, $n = 8$; phosphoramidon, 17.4 ± 1.9 μA cm^{-2}, $n = 5$;

bestatin, 21.8 ± 2.9 μA cm^{-2}, $n = 8$; thiorphan, 18.4 ± 2.8 μA cm^{-2}, $n = 5$). Further experiments were done to ascertain that there was no significant degradation of exogenously added tachykinins. Substance P or neurokinin A were added to the serosal bathing media (each at 0.1 μM). Samples of both mucosal and serosal bathing media (2 ml each) were collected in polyethylene tubes (Minisorb®, Nunc, Roskilde, Denmark). The samples were kept at −20 C° until assay for substance P- and neurokinin A-immunoreactivity. The concentration of both peptides in the serosal media were measured, as described earlier (Schmidt et al., 1991). At 1, 6, 11 and 21 min after peptide addition no significant degradation was found (the concentration of substance P was 0.09 ± 0.02, 0.10 ± 0.03, 0.08 ± 0.01 and 0.10 ± 0.01 μM, respectively, and the concentration of neurokinin A was 0.11 ± 0.04, 0.07 ± 0.01, 0.10 ± 0.02 and 0.07 ± 0.01 μM, respectively, $n = 4$ in four pigs). Thus, peptidase inhibitors were not used in the subsequent investigations.

Preliminary experiments revealed rapid desensitisation upon cumulative addition of substance P and neurokinin A (data not shown), also confirming earlier observations in pig jejunum (Parsons et al., 1992). Accordingly, a non-cumulative concentration–response protocol was employed. Agonist effects were characterised by EC_{50}, E_{max} (maximal short-circuit current response), and n_H (Hill coefficient). These parameter values were obtained by Hill's equation ($\Delta SCC = E_{max} \cdot C^{n_H}/(C^{n_H} + EC_{50})$, where SCC is the short-circuit current and C is the concentration of the agonist), which was fitted non-linearly to data. Dose-ratios were calculated for each concentration of an antagonist producing dextral shift of the agonist concentration–response curve, without a depression of the maximal response and further analysed by a Schild regression. A slope of the Schild regression not significantly different from unity allows an estimate of pK_B (the negative logarithm of the antagonist equilibrium constant) (Kenakin, 1993).

2.4. Statistics

Results are expressed as means ± S.E.M. All reported n values represent the number of tissues. Unpaired Student's *t*-tests were used both to determine the significant difference between means and non-linear curve fitting estimates, respectively. In experiments with three or more means, data were subjected to one-way analysis of variance, followed by Bonferroni's test. The slope of the Schild regression was tested against unity by a *t*-test. A probability value (P) of 0.05 or less was considered statistically significant.

2.5. Compounds

Substance P, neurokinin A, the selective NK_1 receptor agonist [Sar^{9}Met(O$_2$)^{11}]substance P, the selective NK_2 receptor agonist [β-Ala^8]neurokinin A-(4–10), the selective NK_3 receptor agonist, senktide and the peptide NK_2 receptor antagonist GR 94,800 (PhCO–Ala–Ala–DTrp–Phe–DPro–Pro–NleNH_2) (McElroy et al., 1992) were purchased from Peninsula Laboratories Europe (St. Helens, Merseyside, UK).

The non-peptide NK_1 receptor antagonist, CP 99,994 ((2*S*,3*S*)-3-(2-methoxybenzyl)amino-2-phenylpiperidine) (McLean et al., 1993), and CP 100,263, the (2*R*,3*R*) enantiomer of CP 99,994, were kindly provided by Dr. S.B. Kadin, Pfizer (Groton, CT, USA).

The non-peptide NK_1 receptor antagonist, RP 67,580 ((3α *R*,7α *R*)-7,7-diphenyl-2-[1-imino-2(2-methoxyphenyl)ethyl] perhydroisoindol-4-one) (Garret et al., 1991), was a generous gift from Dr. C. Garret, Rhône Poulenc (Vitry, France).

The non-peptide NK_2 receptor antagonist, SR 48,968 ((*S*)-*N*-methyl-*N*-(4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl)bezamide) (Emonds-Alt et al., 1992), and the non-peptide NK_3 receptor antagonist, SR 142,801 ((*S*)-(*N*)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-*N*-methyacetamide) (Emonds-Alt et al., 1994), were kindly donated by Dr. X. Emonds-Alt, Sanofi Recherche (Montpellier, France).

The peptide NK_2 receptor antagonist, PD 147,714 ((2,3-diOMeZ)-(*S*)Trp(*S*)α MePheGlyNH_2), (Guard et al., 1993), was kindly provided by Dr. J. Hughes, Parke-Davis (Cambridge, England).

All other drugs and chemicals were purchased from Sigma (St. Louis, MO, USA).

3. Results

3.1. Basal electrical parameters

The basal electrical parameters of porcine jejunal tissue preparations from a series of five pigs averaged for potential difference −5.0 ± 0.2 mV, for short-circuit current 156.0 ± 7.5 μA cm^{-2} and for tissue resistance 34.2 ± 1.4 Ω cm^2 ($n = 60$). Baseline short-circuit current is mainly accounted for by Na^+ absorption (Skadhauge et al., 1986).

3.2. Effect of substance P and neurokinin A on baseline short-circuit current

Substance P and neurokinin A, added to the serosal side, produced transient increases in short-circuit current, which peaked after 2–3 min (traces not shown). The peak decayed to baseline within 15 min. Luminal addition of substance P and neurokinin A (each at 0.1 μM) did not change baseline short-circuit current.

Substance P produced a concentration-dependent increase in short-circuit current, demonstrating a double sigmoidal form with the first plateau reached at 0.1 μM (Fig. 1). A non-linear fitting was performed to data up to

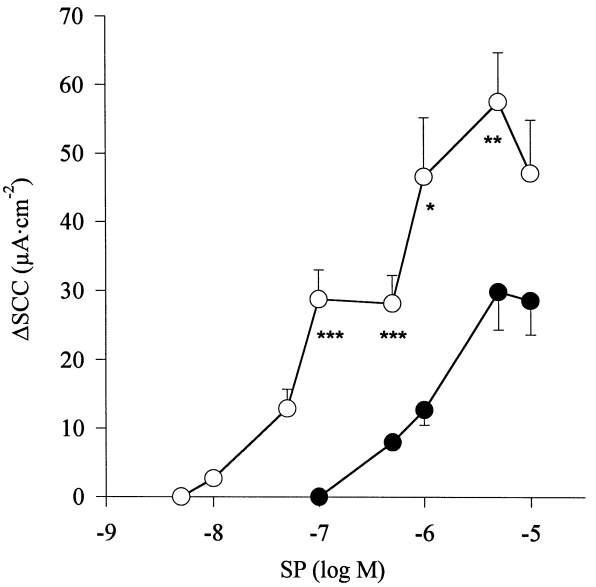

Fig. 1. Concentration–response curves showing peak changes in short-circuit current (SCC) (μA cm^{-2}) induced by substance P (SP) in porcine jejunum in the absence (○) and presence (●) of CP 99,994 (1 μM). Data points represent mean ± S.E.M. of 6–11 experiments in at least four pigs. $*\ P < 0.05$, $**\ P < 0.01$, $***\ P < 0.001$.

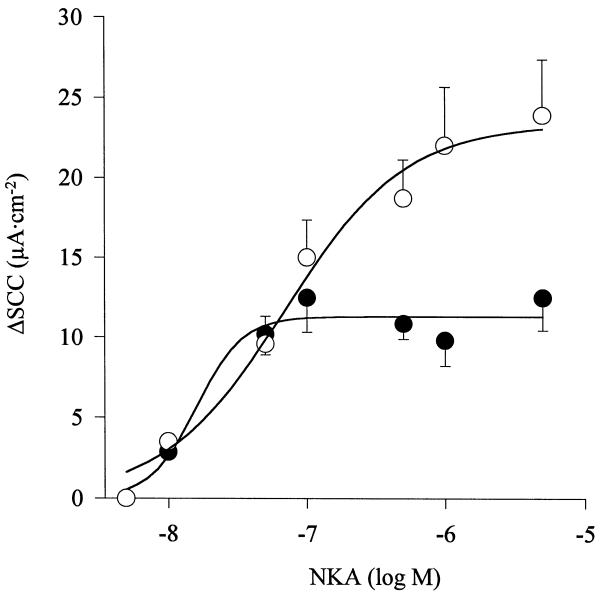

Fig. 2. Concentration–response curves showing peak changes in short-circuit current (SCC) (μA cm^{-2}) induced by neurokinin A (NKA) in porcine jejunum in the absence (○) and presence (●) of CP 99,994 (1 μM). Data points represent mean ± S.E.M. of 5–10 experiments in at least five pigs.

0.5 μM (figure not shown). Thus, the reported agonist effects of substance P relate to the curve at substance P concentrations below 1 μM (Table 1). neurokinin A also caused a concentration-dependent increase in short-circuit current (Fig. 2). As only part of the substance P concentration response curve could be analysed, a more detailed comparison between substance P and neurokinin A agonist effects was not performed. However, the maximal increase in short-circuit current obtained by substance P (Fig. 1) was more than two fold larger than the value for neurokinin A (Fig. 2).

3.3. Effect of selective tachykinin receptor agonists

The selective NK_1 receptor agonist, [Sar^{9}Met-(O_2)^{11}]substance P, added to the serosal side, caused a change in basal short-circuit current (trace not shown), similar to that for substance P and neurokinin A. The response was concentration dependent (Fig. 3). Luminal addition of [Sar^{9}Met(O_2)^{11}]substance P (0.5 μM) did not elicit any change in short-circuit current.

Table 1
EC_{50}, E_{max} and Hill coefficient (n_H) values for substance P, neurokinin A and [Sar^{9}Met(O_2)^{11}]substance P induced peak changes in short-circuit current (μA cm^{-2}) in porcine jejunum

	EC_{50} (nM)	E_{max} (μA cm^{-2})	n_H
Substance P	66.27 ± 11.14	32.02 ± 2.14	1.38 ± 0.23
Neurokinin A	68.87 ± 16.23	23.36 ± 1.39	0.99 ± 0.20
[Sar^{9}Met(O_2)^{11}] substance P	235.0 ± 53.90	26.63 ± 1.84	0.95 ± 0.13

Serosal addition of the selective NK_2 receptor agonist, [β-Ala^8]neurokinin A-(4–10), did not elicit any change in basal short-circuit current until reaching μM concentra-

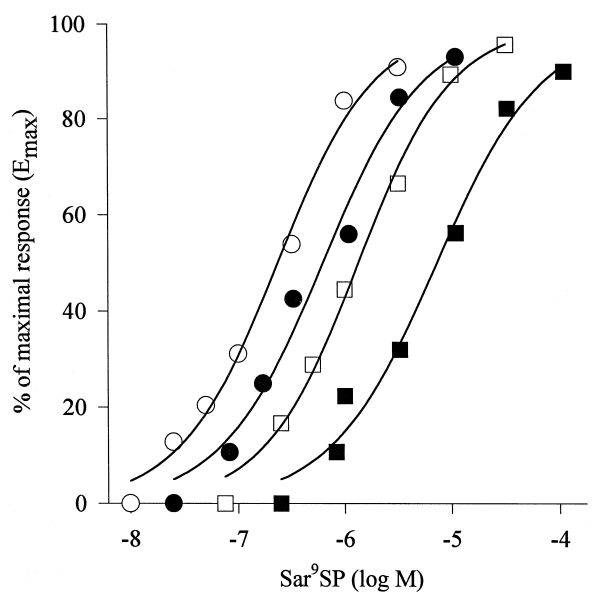

Fig. 3. Concentration–response curves showing peak changes in short-circuit current (μA cm^{-2}) induced by [Sar^{9}Met(O_2)^{11}]substance P (Sar^{9}SP) in porcine jejunum in the absence (○) and presence of increasing concentrations of CP 99,994. Antagonist concentrations used were 0.1 μM (●), 0.3 μM (□) and 1 μM (■). For each curve, data points are expressed as a percentage of the E_{max} value. A typical representative of the [Sar^{9}Met(O_2)^{11}]substance P control curve is shown. Data points represent mean of 5–7 experiments in at least five pigs. S.E.M. values are not shown for clarity of the figure. No S.E.M. value exceeded 20% of the respective mean value.

tions. [β-Ala^8]neurokinin A-(4–10) at 1 and 3 μM gave increases in short-circuit current of 3.8 ± 0.4 μA cm^{-2} ($n = 6$) and 4.2 ± 0.4 μA cm^{-2} ($n = 5$) in four pigs.

Senktide (1 μM), a selective NK_3 receptor agonist, did not elicit any change in short-circuit current ($n = 6$) in three pigs.

3.4. Effect of NK_1 antagonists

Preliminary experiments had shown that in porcine jejunum, CP 99,994 seemed to be more potent than RP 67,580 in inhibiting substance P-mediated responses. To analyse this in further detail, tissue preparations preincubated with either RP 67,580 (1 μM) or CP 99,994 (1 μM), were stimulated with substance P (0.1 μM). RP 67,580 did not show any agonist effects, and further did not alter the substance P response, whereas CP 99,994 totally abolished it (control, 11.8 ± 1.2 μA cm^{-2}, $n = 12$; RP 67,580, 14.1 ± 1.2 μA cm^{-2}, $n = 6$; CP 99,994, 0 μA cm^{-2}, $n = 6$). Thus, CP 99,994 was chosen to characterise the involvement of NK_1 receptors in tachykinin-mediated responses.

CP 99,994 (1 μM), added to the serosal side, had no effect on baseline short-circuit current, but caused a dextral shift of the first part of the substance P concentration–response curve (Fig. 1). CP 99,994 (1 μM) totally abolished part of the neurokinin A response, but in contrast to substance P, in concentrations of 0.5 μM and above. The antagonist did not change the response to concentrations of neurokinin A below 0.5 μM (Fig. 2).

Experiments were done to ascertain that the inhibition of substance P and neurokinin A responses by CP 99,994 was not due to a non-specific depression of jejunal ion transport. Tissue preparations, pretreated with CP 99,994 (1 μM), were stimulated with either the muscarinic agonist, carbachol (1 μM), causing a calcium-mediated secretion (Chandan et al., 1991), or the phosphodiesterase inhibitor, theophylline (0.5 mM), causing a cAMP-mediated secretion (McEwan et al., 1990). CP 99,994 did not significantly change the response to carbachol (control, 41.0 ± 5.2 μA cm^{-2}, $n = 7$; CP 99,994, 45.2 ± 6.4 μA cm^{-2}, $n = 5$) or theophylline (control, 58.7 ± 4.2 μA cm^{-2}, $n = 6$; CP 99,994, 54.6 ± 7.3 μA cm^{-2}, $n = 6$).

The NK_1 receptor affinity resides in the (2*S*,3*S*) enantiomer (CP 99,994), whereas CP 99,994 and its (2*R*,3*R*) enantiomer, CP 100,263, have almost identical affinity for verapamil sensitive calcium channels (McLean et al., 1993). CP 100,263 at 1 μM, did not alter the response to 0.1 μM substance P (control, 10.7 ± 1.5 μA cm^{-2}, $n = 7$; CP 100,263, 9.8 ± 0.7 μA cm^{-2}, $n = 6$), a response, which was totally abolished by CP 99,994 at 1 μM (Fig. 1).

Experiments were done in an attempt to determine the affinity of CP 99,994 for porcine jejunal NK_1 receptors. As substance P seemed to cause a complex activation of neurokinin receptors in porcine jejunum, the inhibition of [Sar^{9}Met(O$_2$)^{11}]substance P responses were examined. CP 99,994, used at 0.1, 0.3 and 1 μM, produced parallel concentration-dependent dextral shifts of the [Sar^{9}Met(O$_2$)^{11}]substance P concentration-response curve, without a depression of the maximal response (Fig. 3). The slope of the Schild regression was 1.59, which was significantly different from unity (Fig. 4). Thus, we were unable to estimate the pK_B value for CP 99,994.

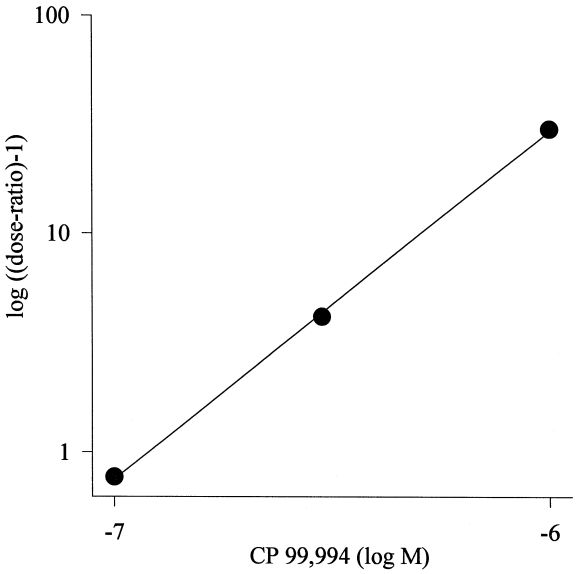

Fig. 4. Schild regression for antagonism of [Sar^{9}Met(O$_2$)^{11}]substance P induced responses in porcine jejunum by CP 99,994. The data points represent a value for each antagonist concentration tested.

3.5. Effect of NK_2 receptor antagonists

The non-peptide antagonist SR 48,968 (0.1 μM), added to the serosal side, had no effect on baseline short-circuit current and did not significantly alter the response to neurokinin A (Fig. 5). Additional experiments were performed to determine if NK_2 receptors were involved in the neurokinin A response. A discrepancy has been observed for SR 48,968 to reach equilibrium binding at NK_2 receptors in functional studies (Emonds-Alt et al., 1992; Maggi et al., 1993). Therefore, neurokinin A (1 μM) was added after tissue preparations were pretreated with SR 48,968 (0.1 μM) for either the usual 15 min or for 30 min. In the same experimental protocol, the effect of two other peptide NK_2 receptor antagonists, GR 94,800 (1 μM) and PD 147,714 (1 μM), were tested. None of these altered the response to neurokinin A (control, 13.6 ± 2.3 μA cm^{-2}, $n = 9$; SR 48,968 (incubation time 15 min), 17.0 ± 1.9 μA cm^{-2}, $n = 9$; SR 48,968 (incubation time 30 min), 14.0 ± 1.7 μA cm^{-2}, $n = 8$; PD 147,714, 16.3 ± 3.1 μA cm^{-2}, $n = 7$; GR 94,800, 12.1 ± 3.1 μA cm^{-2}, $n = 8$. Furthermore, GR 94,800 (1 μM) did not change the response to neurokinin A (0.1 μM) (control, 11.2 ± 1.1 μA cm^{-2}, $n = 6$; GR 94,800, 11.9 ± 1.5 μA cm^{-2}, $n = 7$).

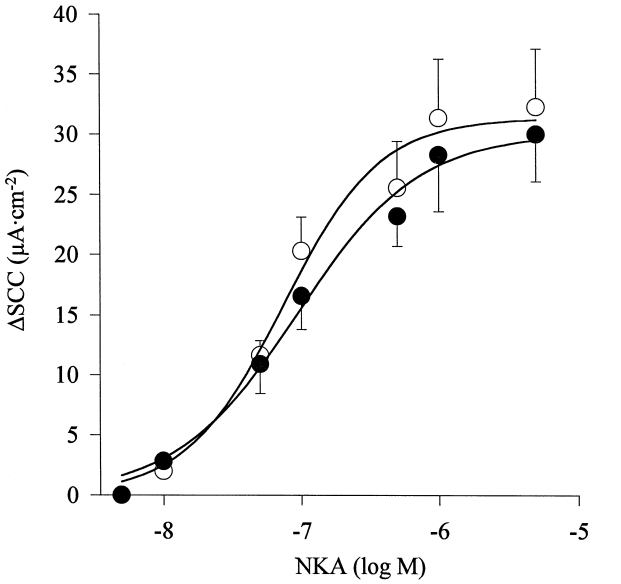

Fig. 5. Concentration–response curves showing peak changes in short-circuit current (SCC) (μA cm^{-2}) induced by neurokinin A (NKA) in porcine jejunum in the absence (○) and presence (●) of SR 48,968 (0.1 μM). Data points represent mean ± S.E.M. of 5–13 experiments in at least four pigs.

3.6. Effect of an NK_3 receptor antagonist

The double sigmoidal form of the substance P concentration–response curve indicated that at least two receptors were involved in the response (Fig. 1). As CP 99,994 almost totally abolished the first part of the curve, an NK_1 receptor might be involved. To investigate if an NK_3 receptor was involved in the second part, we examined if the substance P-CP 99,994 curve could be shifted further to the right by adding SR 142,801 (0.1 μM), a selective NK_3 receptor antagonist. The non-peptide antagonist was added 60 min prior to substance P, as a time-dependency of this length for SR 142,801 to reach equilibrium at NK_3 receptors in guinea-pig ileum has been demonstrated (Patacchini et al., 1995). The presence of SR 142,801 had no significant effect on the position of the substance P-CP 99,994 curve (figure not shown).

4. Discussion

In the present study, substance P and neurokinin A have been shown to alter electrolyte transport in vitro, in porcine jejunum, as observed in other studies in the small intestine of several species, including pig (Walling et al., 1977; Kachur et al., 1982; Chang et al., 1986; Brown et al., 1992). The substance P-induced change in short-circuit current in porcine jejunum has previously been shown to be partly due to an increase in an electrogenic Na^+–HCO_3^- cotransport mechanism and Cl^- secretion (Brown et al., 1992). In all species substance P and neurokinin A cause a typical transient increase in short-circuit current, which was also seen in the present study. We did not find any evidence for peptide degradation being responsible for this response. The transient shape might simply reflect desensitisation caused by several mechanisms, e.g., phosphorylation and internalisation of receptors. Indeed, it has recently been shown, that substance P-induced internalisation of the NK_1 receptor in endothelial and cultured cells may be involved in desensitisation (Bowden et al., 1994).

4.1. NK_1 receptor

In the present study, substance P caused a concentration-dependent increase in short-circuit current. The concentration–response curve had a double sigmoidal form, indicating that at least two receptors are involved in the response. The first plateau was reached at 0.1 μM, similar to what has previously been observed in porcine jejunum (Parsons et al., 1992). The selective NK_1 receptor antagonist, CP 99,994, caused a dextral shift of the first part of the curve (Fig. 1), indicating that part of the substance P response might be mediated through an NK_1 receptor. At higher concentrations substance P seemed to have affinity for another tachykinin receptor. The presence of NK_1 receptors in porcine jejunum are further substantiated by the concentration-dependent response of the NK_1 receptor agonist, [Sar^{9}Met(O$_2$)^{11}]substance P (Fig. 3).

Neurokinin A seemed to be a partial agonist in the porcine jejunum, as the maximal increase in short-circuit current obtained by neurokinin A was almost two fold less than the value for substance P. The concentration response curve of neurokinin A at concentrations below 0.5 μM was not altered by CP 99,994, whereas the antagonist completely inhibited the neurokinin A responses at 0.5 μM and higher concentrations (Fig. 2). Thus, neurokinin A seems to activate two receptors in porcine jejunum, one of these being an NK_1 receptor.

Caution in the interpretation of results concerning CP 99,994 is needed, since the compound in μM concentrations is shown to have affinity for the phenylalkylamine Ca channel binding site (Lombet and Spedding, 1994). However, the inactive enantiomer of CP 99,994 (2*S*,3*S*), CP 100,263 (2*R*,3*R*) did not change the response to substance P, giving indirect evidence that CP 99,994 might actually block NK_1 receptors in porcine jejunum. Furthermore, we did not observe any non-specific effects of CP 99,994, as shown by the integrity of the secretory response to theophylline and carbachol.

CP 99,994 produced parallel concentration-dependent dextral shifts of the [Sar^{9}Met(O$_2$)^{11}]substance P concentration–response curve. The slope of the Schild regression was significantly different from unity. Thus, a non-equilibrium steady state situation might be present, thereby precluding us from estimating the affinity of CP 99,994 for porcine jejunal NK_1 receptors (Kenakin, 1993). Non-equi-

librium could be due to an inadequate time for equilibration between the antagonist and its receptor. Other reasons, such as a saturable removal mechanism of CP 99,994, cannot not be definitively excluded. However, in the present study, a non-equilibrium steady state does not exclude a competitive antagonism exerted by CP 99,994, as indicated by the parallel dextral shift of the [$Sar^9Met(O_2)^{11}$]substance P concentration–response curve, without a concomitant change in the maximal response (Fig. 3) (Kenakin, 1993).

4.2. NK_2 receptor

Neurokinin A is the preferred natural ligand for the NK_2 receptor. In this study, the selective non-peptide NK_2 receptor antagonist, SR 48,968, did not significantly alter the neurokinin A concentration–response curve (Fig. 5). A concentration of 0.1 μM of the antagonist was used. Higher concentrations of SR 48,968 were precluded, due to reported antagonism at NK_1 receptors and non-specific effects, unrelated to blockade of tachykinin receptors, in μM concentrations (Martin et al., 1993; Lombet and Spedding, 1994; Maggi et al., 1994; Wang et al., 1994). To further clarify the neurokinin A response, the effect of two high affinity peptide NK_2 receptor antagonists, GR 94,800 and PD 147,714, were investigated (McElroy et al., 1992; Guard et al., 1993). Both compounds, used in μM concentrations, did not cause any significant change of the neurokinin A-induced response.

The selective NK_2 receptor agonist, [β-Ala^8]neurokinin A-(4–10), caused an increase in short-circuit current, but only in μM concentrations. Compared with the neurokinin A concentration-response curves, a 100-fold higher concentration was needed to elicit a response. This discrepancy of agonist effects might reflect that NK_2 receptors do not mediate the agonist responses. Indeed, it has recently been reported that NK_2 selective receptor agonists, in μM concentrations, stimulate other tachykinin receptors (Patacchini and Maggi, 1995). Summarising the results of NK_2 receptor agonist and antagonist experiments, relatively strong evidence is provided that NK_2 receptors are not functionally present in porcine jejunum. Our results are in accordance with similar studies in canine colon (Crowther et al., 1994), whereas an NK_2 receptor seems to be functionally present in rat colon (Cox et al., 1993).

An incubation time of 120 min was needed for the full effect of SR 48,968 on NK_2 receptors in the original functional study describing the antagonist (Emonds-Alt et al., 1992). However, in another thorough investigation of this issue, the affinity of SR 48,968 on NK_2 receptors was not altered when comparing an incubation time of 15 and 120 min (Maggi et al., 1993). In porcine jejunum, we did not observe any effect on neurokinin A-mediated responses by increasing the incubation time from 15 to 30 min. An explanation for these discrepancies has been addressed (Maggi et al., 1993). The use of an almost 100-fold higher concentration of SR 48,968 (present study and Maggi et al., 1993), as compared with the original paper (Emonds-Alt et al., 1992), might be able to induce a faster equilibrium at NK_2 receptors.

4.3. NK_3 receptor

As discussed above, substance P might activate two receptors. Since NK_2 receptors did not seem to be functionally present in the porcine jejunum, we investigated the possibility that an NK_3 receptor could mediate tachykinin responses. The non-peptide NK_3 receptor antagonist, SR 142,801, did not shift the substance P-CP 99,994 concentration–response curve further to the right, indicating that NK_3 receptors do not seem to be involved in the substance P response. In these experiments the concentration of the agonist was relatively high compared with the antagonist and could thus conceal an effect of the antagonist. Meanwhile, we desisted from the use of SR 142,801 in concentrations higher than 0.1 μM due to reported non-specific effects in μM concentrations (Emonds-Alt et al., 1994). Results from studies using SR 142,801 should be evaluated with caution, as the compound exhibits highly species dependent variations in its affinity for NK_3 receptors. (Emonds-Alt et al., 1994; Patacchini et al., 1995). However, the selective NK_3 receptor agonist, senktide, did not change short-circuit current in porcine jejunum, further indicating that NK_3 receptors are not functionally present.

In conclusion, by the use of a wide array of agonists and antagonists, the present functional results indicate, that substance P and neurokinin A mediate ion transport in porcine jejunum through NK_1 receptors. We do not find any strong evidence for the involvement of NK_2 and NK_3 receptors. However, tachykinins seem to activate another receptor. Two active conformers of the NK_1 receptor, which binds substance P with different affinity, has recently been proposed (Maggi and Schwartz, 1997). The structural or cell-biological basis for these two putative conformers of the NK_1 receptor needs to be clarified.

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