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# Electrophysiological evidence for different release mechanism of ATP and NO as inhibitory NANC transmitters in guinea-pig colon

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**1** The effect of the $P_2$-purinoceptor antagonist, suramin, the specific N-type voltage-dependent calcium channel blocker, ω-conotoxin GVIA (ω-CgTx) and the δ-opioid receptor agonist [D-Pen$^2$,D-Pen$^5$] enkephalin (DPDPE) on the apamin-sensitive and apamin-resistant inhibitory junction potentials (i.j.ps) produced by electrical field stimulation (EFS) were investigated by means of a sucrose-gap technique in the circular muscle of the guinea-pig colon.

**2** After incubation of muscle strips in either atropine (1 μM), guanethidine (3 μM) and N$^G$-nitro-L-arginine (L-NOARG, 30 μM) or atropine, guanethidine and apamin (0.3 μM), the addition of the $NK_1$ receptor antagonist, SR 140,333 (1 μM) abolished the non-adrenergic, non-cholinergic (NANC) excitatory junction potential (e.j.p.) and unmasked a pure apamin-sensitive i.j.p. (in the presence of L-NOARG) or a pure apamin-resistant i.j.p. (in the presence of apamin). Both types of i.j.p. were abolished by tetrodotoxin.

**3** Suramin (30–300 μM) concentration-dependently inhibited the apamin-sensitive i.j.p., while the apamin-resistant i.j.p. was not significantly affected by suramin (up to 300 μM). L-NOARG (30 μM) markedly reduced the apamin-resistant i.j.p.

**4** The δ-opioid receptor agonist, DPDPE (0.03–3 μM) concentration-dependently reduced the apamin-sensitive i.j.p., while leaving the apamin-resistant i.j.p. unaffected. Naloxone (1 μM) prevented the i.j.p. inhibition evoked by DPDPE (0.3 μM).

**5** ω-CgTx (0.3 μM) markedly reduced the apamin-sensitive but not the apamin-resistant i.j.p. The application of DPDPE (3 μM), after development of a steady state inhibitory effect by ω-CgTx, evoked further inhibition of the apamin-sensitive i.j.p., similar to the effect produced by DPDPE alone. The L-type calcium channel blocker, nifedipine (1 μM) did not significantly affect either the apamin-sensitive or the apamin-resistant i.j.ps.

**6** These findings support the purinergic origin of the fast, apamin-sensitive i.j.p. produced by EFS in the circular muscle of the guinea-pig colon and strongly suggest that the apamin-sensitive and the apamin-resistant components of the evoked i.j.p. utilize different mechanisms for the secretion of the NANC transmitters, ATP and NO, respectively.

**Keywords:** Non-adrenergic, non-cholinergic transmission; apamin; inhibitory junction potentials; suramin; nitric oxide (NO); ω-conotoxin; δ-opioid receptor agonist; [D-Pen$^2$,D-Pen$^5$] enkephalin

## Introduction

At present, it appears established that more than one transmitter is responsible for non-adrenergic, non-cholinergic (NANC) inhibition in the gut. There is considerable evidence that adenosine triphosphate (ATP; Burnstock, 1981; Vladimirova & Shuba, 1984; Zagorodnyuk & Shuba, 1986; Crist *et al.*, 1992), vasoactive intestinal polypeptide (VIP; Grider & Rivier, 1990; Crist *et al.*, 1992) and nitric oxide (NO, Boeckxstaens *et al.*, 1990; for review see Sanders & Ward, 1992) are involved as transmitters in NANC inhibitory neural responses to the intestinal smooth muscle from different animal species and man. Previously, we have shown that at least three inhibitory mechanisms are involved in the NANC inhibitory junction potential (i.j.p.) and relaxation in the circular muscle of the guinea-pig colon: (i) apamin-sensitive, (ii) N$^G$-nitro-L-arginine (L-NOARG)-sensitive and (iii) apamin- and L-NOARG-resistant (Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993). For the apamin-sensitive phase of i.j.p., ATP was suggested as a likely mediator in the guinea-pig and human colon (Vladimirova & Shuba, 1984; Zagorodnyuk & Shuba, 1986; Zagorodnyuk *et al.*, 1989). The L-NOARG-sensitive phase of i.j.p. and relaxation of the smooth muscle of the guinea-pig colon implies a possible role of NO or NO-generating substance(s) as inhibitory transmitter (Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993).

The aims of this study were two fold: first, we investigated the effect of suramin, a selective $P_2$-purinoceptor antagonist (Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990) on the apamin-sensitive and apamin-resistant i.j.p. in order to verify the 'purinergic hypothesis' for the apamin-sensitive part of NANC inhibitory innervation of the circular muscle of guinea-pig colon. Second, we aimed to assess whether two agents known to interfere with transmitter release at autonomic neuroeffector junctions, the peptide blocker of N-type calcium channels ω-conotoxin (ω-CgTx, Miller, 1987; Maggi *et al.*, 1988) and the δ-opioid receptor-selective agonist [D-Pen$^2$,D-Pen$^5$] enkephalin (DPDPE, Kosterlitz, 1982; Hoyle *et al.*, 1990a) might exert a differential inhibition on the apamin-sensitive and apamin-resistant i.j.ps in the circular muscle of the guinea-pig colon.

## Methods

A single sucrose-gap, modified as described by Artemenko *et al.* (1982) and Hoyle (1987) was used to investigate simultaneously changes in the membrane potential and contractile activity (load 1–2 mN) of the smooth muscle of guinea-pig

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proximal colon to electrical field stimulation (EFS). The sucrose-gap method was the same as described in detail previously (Zagorodnyuk *et al.*, 1993). Briefly, circular muscle strips approximately 0.5–0.8 mm wide and 10 mm long were superfused with oxygenated (95% $O_2$ and 5% $CO_2$) Krebs solution (35 ± 0.5°C) at a rate of 1 ml $min^{-1}$. The composition of Krebs solution (mM) was as follows: NaCl 119, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5 and glucose 11.

Junction potentials were evoked by submaximal electrical field stimulation (EFS, 10 Hz for 0.5 s, i.e. six stimuli) with 40 V stimulus strength and 0.2 ms pulse width.

When studying the effect of suramin, ω-CgTx and DPDPE, the smooth muscle strips were incubated for at least 2 h in Krebs solution containing either atropine (1 μM), guanethidine (3 μM), L-NOARG (30 μM) and SR 140,333 (1 μM), or atropine (1 μM), guanethidine (3 μM), apamin (0.3 μM) and SR 140,333 (1 μM), respectively, for studying their effect on the apamin-sensitive or the apamin-resistant i.j.p., respectively. The tachykinin $NK_1$ receptor antagonist, SR 140,333 (Emonds-Alt *et al.*, 1993) was present in most experiments to abolish the NANC excitatory junction potential (e.j.p.) which interfered with NANC i.j.p. (see results).

For both types of i.j.ps (apamin-sensitive and apamin-resistant), the following parameters were evaluated: latency, amplitude, duration, time to peak ($t_1$) and time of recovery from peak to baseline ($t_2$).

### *Statistical analysis*

All data in the text are mean ± standard error of the mean (s.e.mean). Statistical analysis was performed by means of Student's *t* test for paired or unpaired data, or by means of analysis of variance, when applicable. A $P$ level $<0.05$ was considered statistically significant.

### *Drugs*

Drugs used were: atropine HCl (Serva, Heidelberg, Germany); guanethidine sulphate (ICF); $N^G$-nitro-L-arginine (L-NOARG), apamin, [D-$Pen^2$,D-$Pen^5$] enkephalin (DPDPE) and naloxone (Sigma); ω-conotoxin GVIA (ω-CgTx, Peninsula). SR 140,333 or (S)-1-{2-[3-(3,4-dichlorophenyl)-1-3(3-isopropoxyphenylacetyl) piperidin-n-3-yl] ethyl)-4phenyl-1-azoniabicyclo[2,2,2]octane chloride was a kind gift from Dr X. Emonds-Alt, Sanofi Research, Montpellier, France. Suramin was a kind gift of Prof. M. Costa, Dept. of Human Physiology, Flinders University, Bedford Pk, SA, Australia.

## Results

### *General*

In the presence of atropine (1 μM) and guanethidine (3 μM), EFS (10 Hz, 40 V, 0.2 ms for 0.5 s) evoked a composite i.j.p. and relaxation of the circular muscle of the guinea-pig colon (Figure 1). In most experiments (e.g. Figure 1), the two components of the i.j.p. are not easily resolved from each other but, after addition of apamin or L-NOARG, the observed changes in latency, amplitude, duration, time to peak ($t_1$) and time of recovery to baseline ($t_2$) (Table 1; Zagorodnyuk *et al.*, 1993) clearly indicate that apamin (0.3 μM) blocked the first fast phase of i.j.p. while leaving the slow phase unaffected. On the contrary, L-NOARG (30 μM) inhibited the second, slow phase, of i.j.p. leaving the early, apamin-sensitive, phase unaffected (Figure 1). Under the present experimental conditions, apamin had little effect on the amplitude of the evoked NANC relaxation which was largely inhibited or suppressed by L-NOARG (Figure 1).

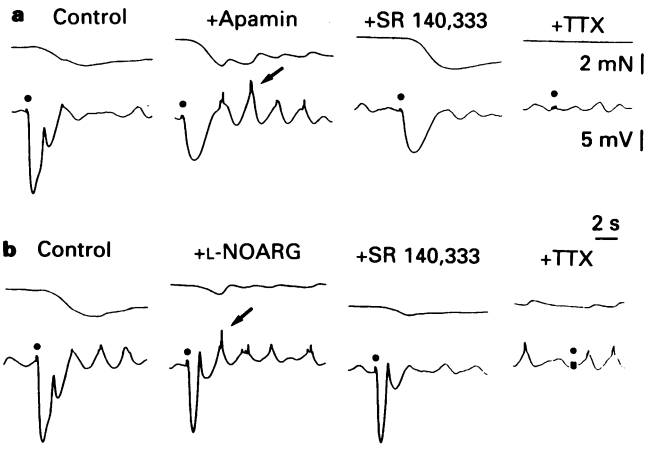


**Figure 1** Typical tracings showing the effects of apamin (0.3 μM), $N^G$-nitro-L-arginine (L-NOARG, 30 μM), SR 140,333 (1 μM) and tetrodotoxin (TTX, 0.3 μM) on the junction potentials and changes in tension evoked by electrical field stimulation (EFS, 10 Hz, 0.5 s, 40 V, 0.2 ms, applied at dots) in the circular muscle from guinea-pig proximal colon. The effect of apamin and L-NOARG is shown after 20 min of action; the effect of SR 140,333 (1 μM) applied in apamin- or L-NOARG-containing Krebs solution is shown at 40 min of action; TTX (0.3 μM for 10 min) blocked i.j.p.s evoked by EFS in apamin- or L-NOARG-containing Krebs solution. Upper tracing is mechanical activity, lower tracing membrane potential. Arrows indicate e.j.p.s.

In the presence of either apamin or L-NOARG a NANC e.j.p. followed by spikes was evident in the response to EFS (arrows in Figure 1) which interferred with the evaluation of drug action on the apamin-sensitive or apamin-resistant i.j.p. Since we showed previously that activation of tachykinin $NK_1$ receptors mediates the NANC e.j.p. in this preparation, we used the $NK_1$ receptor antagonist, SR 140,333 (Emonds-Alt *et al.*, 1993) to eliminate this component of the response to EFS. The addition of SR 140,333 (1 μM) to the Krebs solution containing apamin or L-NOARG abolished the NANC e.j.p. and enabled study of a pure NANC apamin-resistant or apamin-sensitive i.j.p. (Figure 1). The electrophysiological parameters of these i.j.ps are summarized in Table 1. Both the apamin-sensitive and apamin-resistant i.j.ps

**Table 1** Electrophysiological parameters of the apamin-sensitive and apamin-resistant inhibitory junction potential (i.j.p.) evoked by electrical field stimulation (10 Hz, 40 V, 0.2 ms pulse width for 0.5 s) in the circular muscle of the guinea-pig colon

| | *Latency* (ms) | *Amplitude* (mV) | *Duration* (ms) | $t_1$ (ms) | $t_2$ (ms) |
|---|---|---|---|---|---|
| Apamin-sensitive i.j.p. | 192 ± 3 | 11.8 ± 0.6 | 1161 ± 56 | 497 ± 6 | 664 ± 54 |
| Apamin-resistant i.j.p. | 292 ± 10 | 8.0 ± 0.7 | 2660 ± 201 | 1153 ± 53 | 1529 ± 166 |

Each value is mean ± s.e.mean of 26–27 experiments for the apamin-sensitive i.j.p. and 14–15 experiments for the apamin-resistant i.j.p.
All values of the apamin-sensitive i.j.p. are significantly different from the corresponding values of the apamin-resistant i.j.p., $P<0.05$.
$t_1$ = time to peak; $t_2$ = time of recovery to baseline.

were abolished by 10 min application of tetrodotoxin (TTX, 0.3 μM, $n = 4$) (Figure 1).

*Effects of suramin on the apamin-sensitive and apamin-resistant i.j.ps*

Application of suramin (30–300 μM) in L-NOARG- or apamin-containing Krebs solution produced a decrease in tone of the muscle strips with no significant changes in membrane potential. Suramin (30–300 μM) concentration-dependently inhibited the apamin-sensitive i.j.p. (Figure 2 and Figure 4). The inhibition developed slowly and 30–40 min superfusion with suramin was required to reach a steady state. The maximal effect of suramin (300 μM) was 67.7 ± 4.3% ($n = 3$) inhibition of the control response. The latency of the i.j.p. was slightly but significantly increased by suramin being 205 ± 3.4 ms ($n = 6$) and 247 ± 12 ms ($n = 6$, $P < 0.05$) before and after suramin (100–300 μM). The effects produced by the two concentrations of suramin on this parameter were comparable and data were pooled for statistical analysis.

Suramin, at the maximal concentration tested (300 μM) did not affect significantly the amplitude of the apamin-resistant i.j.p. during 40 min of application (Figure 2). At the same time, the apamin-resistant i.j.p. was inhibited by L-NOARG (30 μM) by 62 ± 3% ($n = 3$).

*Effects of DPDPE and ω-CgTx on the apamin-sensitive and apamin-resistant i.j.ps*

The δ-opioid receptor agonist, DPDPE (3 μM) did not produce a significant change in potential of the smooth muscle membrane. At the same time, DPDPE (0.03–3 μM) concentration-dependently inhibited the apamin-sensitive i.j.p. (Figure 3 and Figure 4). The maximum inhibitory effect (60 ± 5%, $n = 6$) was observed with 3 μM DPDPE at 3–7 min of application. The i.j.p. inhibition by DPDPE was not complete, leaving about 40% of the i.j.p. amplitude unaffected. Naloxone (1 μM) prevented the i.j.p. inhibition caused by DPDPE (0.3 μM): DPDPE produced 45 ± 0.9% and 9 ± 0.9% inhibition of i.j.p. before and after naloxone, respectively ($n = 4$, $P < 0.05$).

At the highest concentration tested (3 μM), DPDPE did not significantly affect the amplitude of the apamin-resistant i.j.p. (Figure 3).

The application of ω-CgTx (0.3 μM) in apamin- or L-NOARG-containing Krebs solution did not evoke any significant change in membrane potential of the circular smooth muscle. ω-CgTx (0.3 μM) inhibited the amplitude of the apamin-sensitive i.j.p. by 64.1 ± 3.9% ($n = 7$) (Figure 5). The inhibitory effect developed slowly and reached a steady state within 20 min. The application of DPDPE (3 μM) after development of a steady-state inhibitory effect of ω-CgTx,

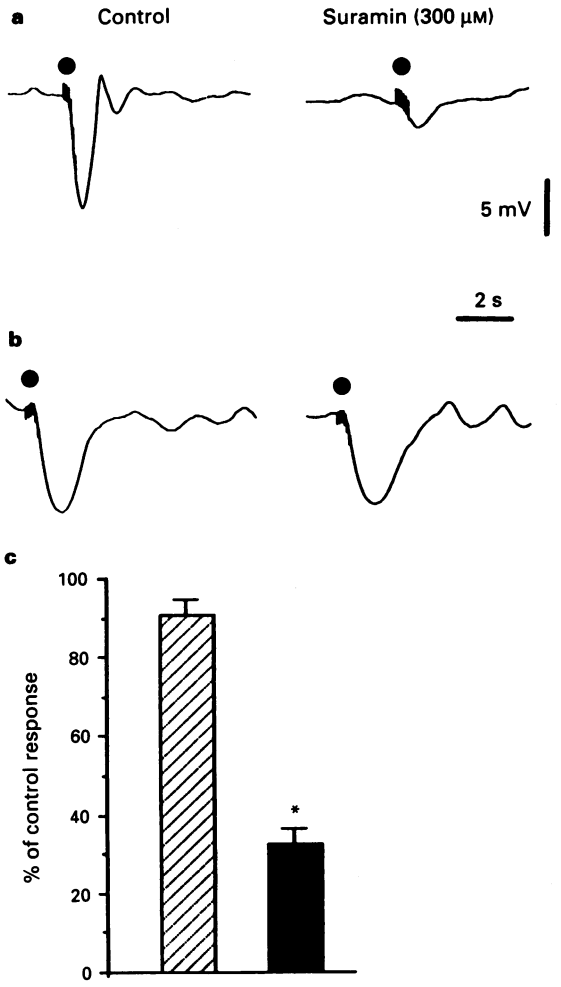


**Figure 2** Effect of suramin (300 μM for 40 min) on the apamin-sensitive (a) and apamin-resistant (b) i.j.ps evoked by electrical field stimulation in the circular muscle of the guinea-pig colon; (c) shows the effect of suramin (300 μM) on the amplitude of the apamin-sensitive i.j.p. (solid column) and apamin-resistant i.j.ps (hatched column). Each value is mean ± s.e.mean of three experiments. *Significantly different from control values: $P < 0.05$.

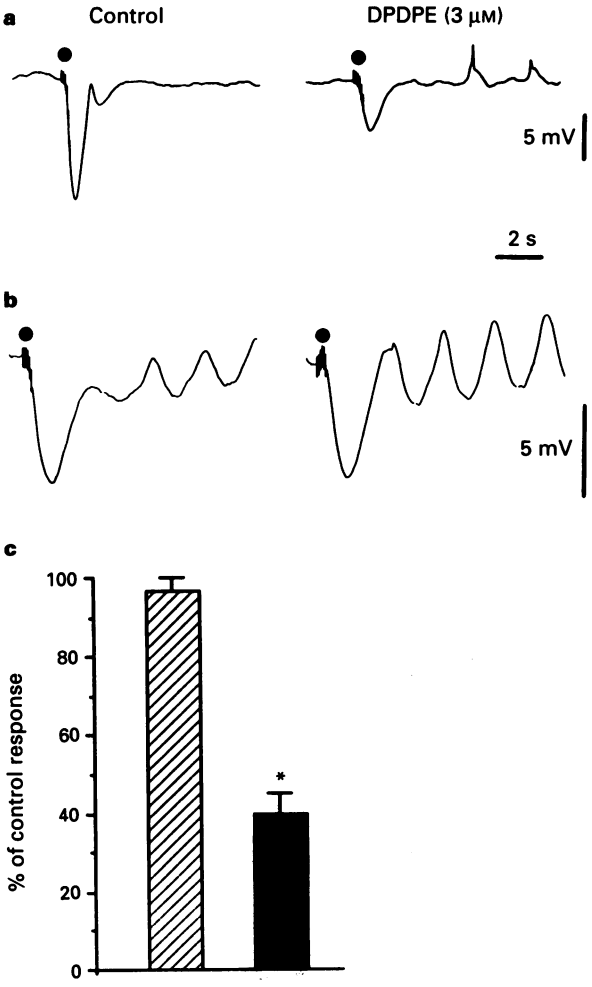


**Figure 3** Effect of [D-Pen$^2$,D-Pen$^5$]enkephalin (DPDPE, 3 μM for 7 min) on the apamin-sensitive (a) and apamin-resistant (b) i.j.ps evoked by EFS in the circular muscle of the guinea-pig colon; (c) shows the effect of DPDPE (3 μM) on the amplitude of the apamin-sensitive i.j.p. (solid column) and apamin-resistant i.j.ps (hatched column). Each value is mean ± s.e.mean of 4–6 experiments. *Significantly different from control values; $P < 0.05$.

evoked a further inhibition of the apamin-sensitive i.j.p. by 65 ± 11% ($n = 4$), quantitatively similar to the inhibitory effect produced by DPDPE (3 μM) in normal Krebs solution. DPDPE (3 μM) or ω-CgTx (0.3 μM) alone did not change the latency of the apamin-sensitive i.j.p. but, when the two drugs were applied together, the latency of the i.j.p. was about twice (465 ± 54 ms, $n = 4$) that observed in controls (220 ± 20 ms, $n = 4$).

The application of ω-CgTx (0.3 μM for 30 min) did not affect significantly the amplitude of the apamin-resistant i.j.p. (Figure 5).

Nifedipine (1 μM for 30 min) did not significantly affect the amplitude of the apamin-sensitive or apamin-resistant i.j.ps which, in the presence of nifedipine, averaged 93 ± 1.3% and 103 ± 1.2% of the control response, respectively ($n = 3$ each, $P > 0.05$).

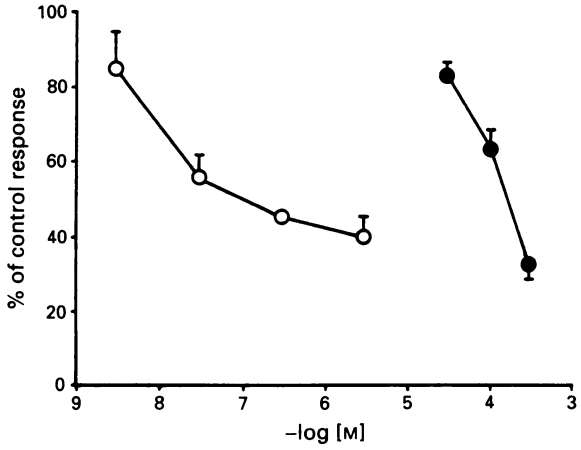


**Figure 4** Concentration-dependent inhibition by [D-Pen$^2$,D-Pen$^5$] enkephalin (DPDPE, ○) and suramin (●) of the apamin-sensitive i.j.p. evoked by electrical field stimulation in the circular muscle of the guinea-pig colon. Each value is mean ± s.e.mean of 3–6 experiments.

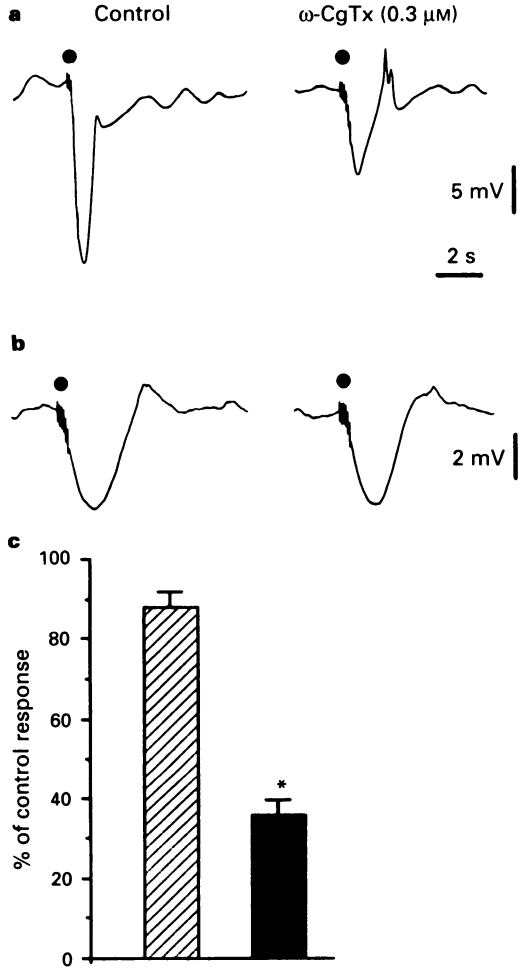


**Figure 5** Effect of ω-conotoxin (ω-CgTx, 0.3 μM for 20 min) on the apamin-sensitive (a) and apamin-resistant (b) i.j.ps evoked by electrical field stimulation in the circular muscle of the guinea-pig colon; (c) shows the effect of ω-CgTx (0.3 μM) on the amplitude of the apamin-sensitive i.j.p. (solid column) and apamin-resistant i.j.ps (hatched column). Each value is mean ± s.e.mean of 3–7 experiments. *Significantly different from control values; $P < 0.05$.

## Discussion

### *ATP and NO as mediators of the NANC i.j.p.*

In agreement with the findings of a previous study using other antagonists (Zagorodnyuk *et al.*, 1993), we found that the potent tachykinin $NK_1$ receptor antagonist, SR 140,333 (Emonds-Alt *et al.*, 1993) eliminates the NANC e.j.p. produced by EFS in the circular muscle of the guinea-pig colon. After blockade of tachykinin $NK_1$ receptors, a pure i.j.p. can be evoked in this preparation: the response is biphasic and two further components can be discerned by the use of apamin which blocks the fast phase of i.j.p., and of L-NOARG which inhibits the second component.

Following desensitization of $P_2$ purinoceptors by means of stable ATP analogues (Zagorodnyuk & Shuba, 1986; Crist *et al.*, 1992) or by using the purinoceptor antagonists, reactive blue 2 or suramin (Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990b; Crist *et al.*, 1992; Ohno *et al.*, 1993), the proposal has been advanced that ATP is responsible for the fast, apamin-sensitive i.j.p. and part of NANC relaxation in various regions of the guinea-pig intestine (stomach, ileum and taenia coli). This proposal is further supported by the present findings showing that suramin selectively inhibited the fast, apamin-sensitive i.j.p. while leaving unaffected the apamin-resistant response.

It is important to note that, in the present experimental conditions, the apamin-sensitive i.j.p. does not appear to contribute significantly to the evoked relaxation which was almost abolished by L-NOARG instead. Since the aim of this study was to investigate a differential pharmacological modulation of the two components of i.j.p., and L-NOARG alone almost abolished the evoked NANC relaxation, we did not systematically investigate the effect of drugs on this parameter.

The slow i.j.p. evoked by EFS in the presence of apamin was inhibited by the NO-synthase inhibitor, L-NOARG, implying a role of NO, a NO-like, or a NO-generating transmitter(s) in its genesis (cf. Maggi & Giuliani, 1993; Zagorodnyuk *et al.*, 1993). NO-synthase has been shown to be expressed in nerves (Bredt *et al.*, 1990), including neuronal elements of the myenteric plexus of the guinea-pig ileum, where NO synthase-like immunoreactivity co-localizes with vasoactive intestinal polypeptide (VIP) (Costa *et al.*, 1992). Although the application of NO-generating agents, like sodium nitroprusside, produces hyperpolarization and relaxation of the circular muscle of the guinea-pig colon (data not shown), it is as yet uncertain whether the L-NOARG-sensitive i.j.p. and relaxation is produced by NO generated within nerves diffusing to produce its effect in smooth muscle cells. In fact, L-NOARG has been reported to inhibit partially the action of VIP in some intestinal preparations (guinea-pig stomach and ileum, rat stomach) (Li & Rand, 1990; Grider *et al.*, 1992; He & Goyal, 1993). Thus the hypothesis was advanced that VIP released from nerves may induce NO generation in smooth muscle as a contributing and amplifying mechanism to the VIP-induced relaxation (Grider *et al.*, 1992). On the other hand, the action of VIP is unaffected by L-NOARG in certain intestinal preparations (guinea-pig taenia coli, dog

intestine etc.) (Boeckstaens *et al.*, 1990; Sanders & Ward, 1992; Grider *et al.*, 1992) and it is likely that, in the latter, both VIP and NO are released from nerves to produce NANC relaxation via independent mechanisms. It is possible that the circular muscle of the guinea-pig colon belongs to this second group of preparations since preliminary experiments have shown that L-NOARG (30–100 μM) barely affects the relaxation produced by exogenous VIP at this level (Maggi & Zagorodnyuk, unpublished data).

The relatively short latency (about 200 ms) of the slow, L-NOARG-sensitive i.j.p. also points to a non-peptidergic type of transmission since longer latencies have been shown (Zagorodnyuk *et al.*, 1993; Santicioli & Maggi, unpublished data) to be required for peptidergic neuromuscular transmission. We will therefore discuss the effect of ω-CgTx and DPDPE on the apamin-sensitive and apamin-resistant i.j.p. on the assumption that these two junctional events are mediated by ATP and NO, respectively.

### *Differential modulation of ATP and NO release in guinea-pig colon*

The present findings demonstrate that the peptide blocker of N-type calcium channels, ω-CgTx and the selective δ-opioid receptor agonist, DPDPE, both exert a selective inhibitory action on the ATP-mediated, apamin-sensitive i.j.p., without affecting the NO-mediated, apamin-resistant response to EFS.

ω-CgTx has been repeatedly shown to produce a prejunctional blockade of transmitter release at various neuroeffector autonomic junctions in mammals, without any evidence for significant postjunctional effects (Maggi *et al.*, 1988; De Luca *et al.*, 1990; Boeckstaens *et al.*, 1993). The inhibition by ω-CgTx of the ATP-mediated i.j.p. was partial and this may indicate that, in addition to N-type calcium channels, other types of voltage-sensitive calcium channels are involved in ATP release in this preparation. Marino *et al.* (1993) found that both ω-CgTx-sensitive and nifedipine-sensitive calcium channels mediate acetylcholine release in guinea-pig colon; however, nifedipine had no effect on the ATP- or NO-mediated i.j.p., suggesting that L-type calcium channels do not play a role in NANC inhibitory transmission at this level.

Like ω-CgTx, DPDPE produced a selective inhibition of the ATP-mediated i.j.p. For both ω-CgTx and DPDPE, the failure to affect the NO-mediated i.j.p. indicates that their inhibitory effect on the ATP-mediated i.j.p. is not due to nonspecific postjunctional effects on membrane excitability. Furthermore, both agents inhibited the ATP-mediated i.j.p. without affecting the resting membrane potential. The action of DPDPE observed here is in line with the results of previous studies showing a prejunctional modulatory influence of opioid receptor agonists on NANC inhibitory transmission in the human colon (Hoyle *et al.*, 1990a), in which the evoked i.j.p. is mainly ATP-mediated (Zagorodnyuk & Shuba, 1986).

Although opioid receptor agonists are known to reduce N-type calcium currents in neurones (Gross & McDonald, 1987; Akins & McCleskey, 1993) the inhibitory effect of DPDPE on the ATP-mediated i.j.p. (amplitude and latency of i.j.p.) was additive to the inhibitory effect of ω-CgTx, i.e. it occurs independently of the availability of N-type channels to sustain the evoked transmitter release. Since opioid receptor agonists are also known to enhance resting and voltage-gated K currents in neurones (North *et al.*, 1987), we speculate that a similar action may underlie the action of DPDPE on the evoked ATP release in guinea-pig colon. In fact, an effect on K currents could inhibit transmitter secretion independently from the type of voltage-sensitive calcium channels used to sustain transmitter secretion.

In conclusion, the present findings lend support to the purinergic origin of the fast, apamin-sensitive i.j.p. produced by EFS in the circular muscle of the guinea-pig colon and demonstrate that the apamin-sensitive and the apamin-resistant components of the evoked i.j.p. utilize different mechanisms for secretion of the NANC transmitters, ATP and NO, respectively. Whether or not this may indicate that different subsets of enteric neurones mediate the two components of the overall response cannot be decided from the present findings.

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