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Neurotransmitters involved in the fast inhibitory junction potentials in mouse distal colon

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Abstract

We investigated, in murine colon circular muscle, the role of adenosine 5′-triphosphate (ATP) and pituitary adenylate cyclase activating peptide (PACAP) as inhibitory neurotransmitters of the fast component of nerve-evoked inhibitory junction potential (fast IJP). Fast IJP was antagonised by apamin or suramin, abolished by desensitisation with the P2Y receptor agonist, adenosine 5′-*O*-2-thiodiphosphate (ADPβS), unaffected by desensitisation with P2X receptor agonist, α,β-methylene ATP (α,β-meATP), and reduced by PACAP-(6–38), a PACAP receptor antagonist. ATP induced membrane hyperpolarization resistant to tetrodotoxin, N^{ω}-nitro-L-arginine methyl ester (L-NAME) or PACAP-(6–38), but antagonised by apamin, suramin, P2X and P2Y receptor desensitisation. PACAP-(1–27) caused membrane hyperpolarization antagonised by PACAP-(6–38), apamin and P2Y receptor desensitisation, reduced by tetrodotoxin, but not affected by L-NAME and by P2X receptor desensitisation. Therefore, in murine colon circular muscle, an ATP-like endogenous P2Y purinoceptor ligand is the final nonadrenergic, noncholinergic (NANC) inhibitory mediator involved in the generation of fast IJP. A neuromodulator role of PACAP in the inhibitory pathway is supposed.

Keywords: Colon, murine; Fast inhibitory junction potential; Apamin; ATP; PACAP (pituitary adenylate cyclase activating peptide); Purinoceptor

1. Introduction

Nonadrenergic, noncholinergic (NANC) nerves play an important role in the inhibition of gastrointestinal smooth muscle, being involved in many important physiological reflexes. It has been reported that electrical field stimulation of enteric nerves under NANC conditions elicits a transient hyperpolarization of gastrointestinal smooth muscle cells (inhibitory junction potential, IJP) accompanied by smooth muscle relaxation (see Kuryama et al., 1998 for review). The shape of the NANC IJP and the neurotransmitter(s) involved has been extensively studied in various animal species. Different agents have been proposed as cotransmitters in the inhibitory NANC innervation of the gut, including nitric oxide (NO), adenosine 5′-triphosphate (ATP) or related purines and peptides (see Kuryama et al., 1998 for review). However, the relative biological role of each cotransmitter in different regions and different species has yet to be determined.

Mice are becoming increasingly important subjects for investigating gastrointestinal motility because of the availability of mutants and the advent of gene-targeting technology. Our laboratories are currently interested in studying electrical and mechanical differences in gastrointestinal motor function between normal and *mdx* mice, animals lacking dystrophin from their sarcolemma. However, since there are still few investigations, a full description of the pharmacology of neurotransmission in the mouse gastrointestinal tract under a normal condition, prerequisite to exploit and apply the new technologies, is not yet available.

We have previously reported (Serio et al., 2001) that circular muscle of mouse colon showed spontaneously occurring inhibitory junction potentials and that NANC inhibitory nerve activation induced inhibitory responses (IJPs) as originally described by Okasora and Okamoto (1986) and Wood et al. (1986). These are characterised, as are those in other preparations (Stark et al., 1993; Kishi et al., 1996; Xue et al., 1999), by an initial fast hyperpolarization (fast IJP) followed by a slower, longer-lasting hyperpolarization of smaller

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amplitude, nitrergic in nature (Shuttleworth et al., 1997; Spencer et al., 1998; Serio et al., 2001). So far, we do not have any information about the transmitters involved in the fast component of IJPs and in the spontaneous occurring IJPs. However, in this mouse preparation, both spontaneous and evoked IJPs seem to be dependent, at least in part, on the opening of small conductance Ca^{2+}-dependent K^+ channels sensitive to apamin (Spencer et al., 1998; Powell et al., 2001). In some species, ATP was reported to mediate the apamin-sensitive NANC fast IJP (Crist et al., 1992; Maggi and Giuliani, 1993; Zagorodnyuk and Maggi, 1994; Xue et al., 1999). Moreover, Satoh et al. (1999) have reported that nitric oxide and pituitary adenylate cyclase activating peptide (PACAP) mediate the relaxation of longitudinal muscle in the distal colon of ICR mice.

The aim of the present study was thus to identify the conductance changes and the transmitters involved in the fast component of the evoked IJPs and in the spontaneously occurring IJPs in the distal colon of the mouse C57BL/10, the wild-type strain for dystrophic mice. We investigated, in particular, the possibility of involvement of ATP and PACAP as inhibitory neurotransmitters.

2. Materials and methods

2.1. General

Experiments, authorised by the Ministero della Sanità (Rome, Italy), were performed on adult normal male mice (C57BL/10SnJ) killed by cervical dislocation. The abdomen was immediately opened and the distal colon was rapidly removed and placed in a dissecting dish filled with oxygenated Krebs solution. The colon was opened via a longitudinal incision made along the mesenteric border, remaining faecal material was washed off and the mucosal layer was gently removed. Longitudinally cut strips, 20-mm long, were dissected and pinned to the floor of a Sylgard-coated recording chamber with the circular muscle facing up. The preparation was continuously perfused at a constant rate (3.5 ml min^{-1}) with oxygenated (95% O_2 and 5% CO_2) and prewarmed (37 °C) Krebs solution. Muscle strips were allowed to equilibrate for 90–120 min before experiments were begun. Nifedipine (1 μM) was added to the perfusate to reduce muscular contractions, and atropine and guanethidine (1 μM each) were added to assess NANC conditions. In control experiments, two consecutive applications of ATP (1 mM) and PACAP (0.1 μM) were performed 20–25 min apart from each other. The concentrations were selected from preliminary experiments as consistently producing a comparable hyperpolarization when tested on the same muscle strips of mouse colon. All the antagonists were allowed to maintain contact with the tissue for 30 min before the effects of the agonists were tested. Each preparation was tested with a single antagonist, except when otherwise stated.

2.2. Intracellular electrical recording

Cells within the circular muscle layer were impaled with glass microelectrodes filled with 3 M KCl and having resistances of 50–90 MΩ. Successful impalement of circular muscle cells was indicated by a sharp negative deflection in membrane potential followed by the recording of spontaneously occurring inhibitory junction potentials and of responses to electrical field stimulation. Membrane potential was measured with a high-input impedance electrometer (WPI-intra 767, WPI, Sarasota, FL, USA), displayed on an oscilloscope (Tektronix 5113, Tektronix, Beaverton, OR, USA) and reproduced on chart paper (Grass Physiograph 79D, Grass Instruments, Quincy, MA, USA). Nerves in the muscle strips were stimulated by current pulses applied through two silver chloride plates, one on each side of the preparation and parallel to the long axis of the muscle strips. A Grass S88 electrical stimulator and a stimulus isolation units (SIU5) were used to deliver current pulses for electrical field stimulation. Single pulses (0.6-ms duration at variable stimulus strength) were used. To obtain reproducible responses, electrical field stimulation was performed at 5-min intervals. Time control experiments showed no decay in the amplitude of the responses to electrical field stimulation for several hours.

2.3. Statistical analysis

All data are given as means ± S.E. *n* in the results section refers to the number of animal preparations on which observations were made. Statistical analysis was performed by means of paired Student's *t*-test. A probability value of less than 0.05 was regarded as significant.

2.4. Drugs

Krebs solution had the following ionic composition (mM) NaCl 119, KCl 4.5, $MgSO_4$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5 and glucose 11.1. The following drugs were used: ATP, adenosine 5′-*O*-2-thiodiphosphate (ADPβS), α,β-methylene ATP (α,β-meATP), apamin, atropine sulphate, guanethidine monosulphate, nifedipidine, N^{ω}-nitro-L-arginine methyl ester (L-NAME), vasoactive intestinal peptide (VIP), tetrodotoxin (Sigma-Aldrich, St. Louis, USA), suramin (Sigma-RBI, Sigma-Aldrich), pituitary adenylate cyclase activating peptide-(1–27) amide (PACAP-(1–27)), pituitary adenylate cyclase activating peptide-(6–38) amide (PACAP-(6–38)) (Bachem, Bubendorf, Switzerland). A stock solution of nifedipine was prepared in ethanol and all the other drugs were dissolved in distilled water. The working solutions were prepared fresh on the day of the experiment by diluting the stock solutions in Krebs and were added to the perfusing solution. Lastly, experiments were conducted in a darkened laboratory because of the photosensitive nature of nifedipine.

3. Results

3.1. General

Circular muscle cells of mouse distal colon had a mean resting potential (RPM) of -53.2 ± 1.4 mV ($n=23$). Electrical activity consisted of tetrodotoxin-sensitive spontaneous hyperpolarizations (IJPs) of variable frequency and amplitude. Electrical field stimulation with single pulses elicited NANC IJPs in all preparations. No excitatory junction potentials were observed. NANC IJPs, which lasted 6.6 ± 1.4 s ($n=24$), consisted of a "fast" hyperpolarization followed by a "slow" hyperpolarization (slow IJP). The actual amplitude of the fast IJP at 80 V was 15.5 ± 1.2 mV ($n=24$) and the response recovered to $74.6 \pm 3.8\%$ ($n=24$) of control RMP in about 1 s following the stimulus. On the contrary, spontaneous occurring IJPs consisted of only a fast developing hyperpolarization (Fig. 1).

3.2. Pharmacology of spontaneous ad evoked IJPS

Apamin (0.1 μM) induced a muscular depolarization of about 10 mV (RPM = -49.7 ± 2 mV in the control and -36.3 ± 2.4 mV after apamin, respectively, $P<0.05$, $n=5$) and markedly reduced the amplitude and the occurrence of the spontaneous IJPs (maximal amplitude from about 6 to about 1 mV and frequency from about 0.3 to 0.06 s^{-1} in the presence of 0.1 μM apamin). Moreover, apamin significantly antagonised the fast IJP as shown in Fig. 2. This effect was dose-dependent because increasing the concentration of apamin 10-fold (1 μM) produced a further reduction of the amplitude of fast IJP. However, at high stimulus strength, a residual IJP was recorded (Fig. 2). Spontaneously occurring IJPs were virtually abolished by 1 μM apamin. Apamin hardly affected the amplitude of the slow IJP. L-NAME at the dose of 100 μM, which we have previously shown to abolish the slow IJP (Serio et al., 2001), had no effect on the apamin-insensitive fast IJP. Electrical field stimulation at 100 V evoked a fast IJP with amplitude of 3.6 ± 0.7 mV in the presence of apamin and of 3.4 ± 0.6 mV in the presence of both apamin and L-NAME ($n=3$, $P>0.05$).

Suramin (100 μM), a non-specific P2 purinoceptor antagonist, did not modify membrane potential and slightly reduced the fast component of the evoked IJPs (at 80 V fast IJP amplitude was 17.7 ± 1.3 mV in Krebs solution compared to 12.0 ± 1.7 V in the presence of suramin, $n=5$, $P<0.05$, Fig. 3), without affecting the slow component (4.8 ± 0.6 mV in Krebs solution compared to 4.9 ± 0.8 mV in the presence of suramin, $n=5$, $P>0.05$, Fig. 3). The effect of suramin was reversible on washout. P2X purinoceptor desensitisation, induced by perfusion with α,β-meATP (1 μM for 30 min), did not modify either the fast IJP (at 80 V fast IJP amplitude was 17.7 ± 1.4 mV in Krebs solution compared to 17.5 ± 1.4 mV in the presence of α,β-meATP, $n=5$, $P>0.05$, Fig. 3) or the slow IJP evoked by electrical field stimulation (4.5 ± 0.9 mV in Krebs solution compared to 4.4 ± 0.6 mV in the presence of α,β-meATP, $n=5$, $P>0.05$, Fig. 3). A transient hyperpolarization lasting about 10 min occurred on addition of α,β-meATP to the bath after which the membrane potential repolarized back to the baseline value. P2Y purinoceptor desensitisation, induced by perfusion with ADPβS at the dose of 10 μM (for 20 min), markedly antagonised the amplitude of fast IJP (at 80 V fast IJP amplitude was 18.2 ± 2.2 mV in Krebs solution compared to 6 ± 1.0 mV in the presence of ADPβS, $n=5$, $P<0.05$, Fig. 3), but it did not affect the slow IJP (4.9 ± 0.6 mV in Krebs solution compared to 4.9 ± 0.9 mV in the presence of ADPβS, $n=5$, $P>0.05$, Fig. 3). A transient hyperpolarization occurred on addition of ADPβS to the bath after which the membrane potential repolarized back to the baseline value. This transient hyperpolarization was not blocked by tetrodotoxin, but

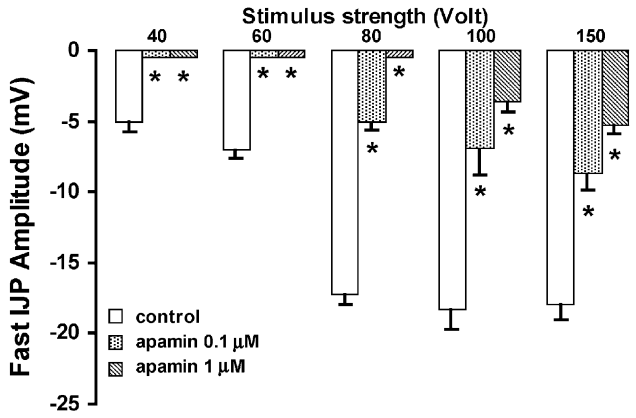

Fig. 2. Histogram showing the effects of apamin on the amplitude of the "fast component" of the IJP evoked by single pulses at different stimulus strengths. Data are expressed as means ± S.E. ($n=4$). *$P<0.05$ when compared to the respective value obtained in the control. Note that apamin dose dependently and significantly antagonized the fast IJP.

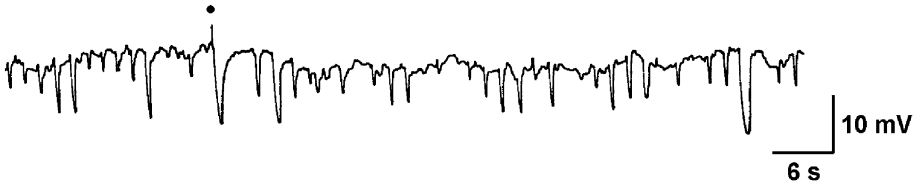

Fig. 1. Intracellular microelectrode recording from the circular muscle cells of murine distal colon. Electrical activity consisted of irregularly occurring spontaneous hyperpolarization. A single pulse (0.6 ms, 60 V) (see dot) evoked a NANC IJP.

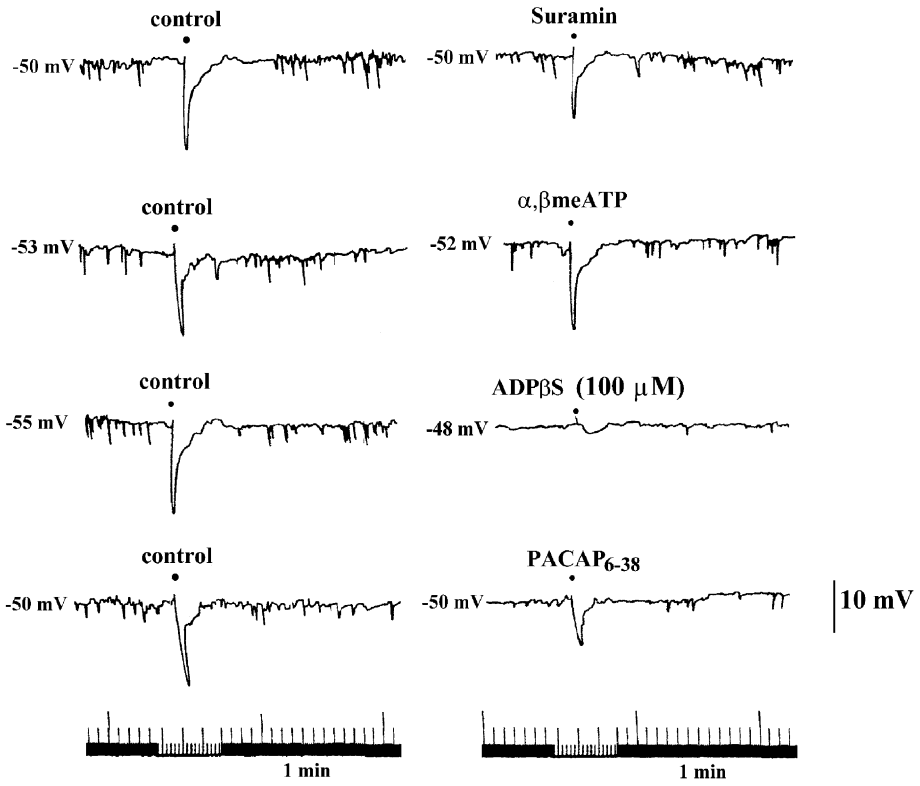

Fig. 3. Original recordings showing the effects of suramin (100 μM), α,β-meATP (1 μM for 30 min), ADPβS (100 μM for 20 min) and PACAP-(6–38) (3 μM) on the amplitude of the "fast component" of the IJP evoked by electrical field stimulation (delivered at ●, single pulses at 80 V). Note that the time scale is increased from 25 to 50 mm/min during the electrical field stimulation.

was antagonized by apamin (12.5 ± 1.4 in the control compared to 1.8 ± 0.8, $n=3$, $P<00.5$), suggesting that it was due to a postjunctional effect of ADPβS. On increasing the desensitizing concentration of ADPβS to 100 μM, a depolarization of about 9 mV of the resting membrane potential was observed (RPM = − 52.7 ± 2.1 in the control and − 43.3 ± 1.2 after 100 μM ADPβS, respectively, $P<0.05$, $n=5$). Moreover, the fast component of the evoked IJPs was abolished at all stimulus strengths used (at 80 V fast IJP amplitude was 17.5 ± 2 mV in Krebs solution compared to 0 ± 0 mV in the presence of ADPβS, $n=5$, $P<0.05$, Fig. 3). Indeed, the slow component was not affected (4.2 ± 0.6 mV in Krebs solution compared to 3.8 ± 0.8 mV in the presence of ADPβS, $n=5$, $P>0.05$, Fig. 3). One hour after washout of ADPβS, the fast IJP had recovered to 89% of the control response.

The PACAP receptor antagonist, PACAP-(6–38) (3 μM), did not have any effect on the resting membrane potential. However, the amplitude of fast IJP was significantly reduced by PACAP-(6–38) (at 80 V fast IJP amplitude was 18.7 ± 1.8 mV in Krebs solution compared to 10.2 ± 1.2 mV in the presence of PACAP-(6–38), $n=5$, $P<0.05$, Fig. 3), while the slow IJP was not modified (4.3 ± 0.7 mV in Krebs solution compared to 3.9 ± 0.8 mV in the presence of PACAP-(6–38), $n=5$, $P>0.05$, Fig. 3).

Lastly, the spontaneously occurring IJPs showed the same sensitivity to the drugs as did the evoked fast IJP (Fig. 3).

3.3. Effects of ATP and PACAP on the resting membrane potential

Perfusion of ATP (1 mM, $n=31$) induced membrane hyperpolarization of 12.6 ± 0.6 mV (Fig. 4). This hyperpolarization was resistant to tetrodotoxin (1 μM) and L-NAME (100 μM). Apamin (1 μM) significantly antagonised ATP-induced hyperpolarization. Suramin (100 μM), P2X receptor desensitisation induced by perfusion with α,β-meATP (1 μM for 30 min) and P2Y receptor desensitisation induced by perfusion with ADPβS (100 μM for 20 min), significantly reduced ATP-induced hyperpolarization. The combined exposure to either suramin and α,β-meATP or suramin and ADPβS or α,β-meATP and ADPβS had additive effects on the ATP response. In particular, the combined exposure to α,β-meATP and ADPβS was the most effective, reducing by more than 90% the ATP-induced hyperpolarization (from 13.1 ± 0.5 mV in the control to 1.2 ± 0.3 mV in the presence of both α,β-meATP and ADPβS, $n=3$, $P<0.05$). Lastly, ATP-induced effects were insensitive to 3 μM PACAP-(6–38), a PACAP receptor antagonist. The effects of the various drugs on ATP-evoked hyperpolarization are summarised in Fig. 4.

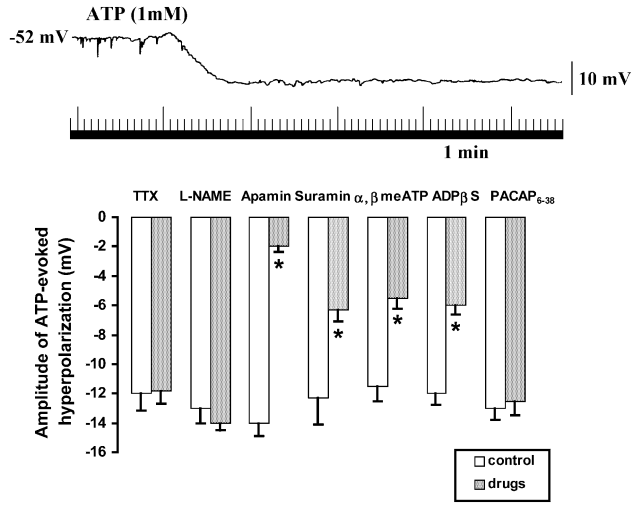

Fig. 4. Top: intracellular microelectrode recording showing the effects of ATP (1 mM) on the resting potential under control condition. Bottom: summary of effects of tetrodotoxin (1 μM, $n=3$), L-NAME (100 μM, $n=3$), apamin (1 μM, $n=5$), suramin (100 μM, $n=5$), α,β-meATP (1 μM, $n=5$), ADPβS (100 μM, $n=5$) and PACAP-(6–38) (3 μM, $n=5$) on hyperpolarization evoked by ATP (1 mM). Data are expressed as means ± S.E. *$P<0.05$ when compared to the respective value obtained in the control.

Perfusion of PACAP-(1–27) (0.1 μM, $n=31$) caused hyperpolarization of the membrane potential of 16.1 ± 1.6 mV (Fig. 5). This inhibitory effect was partially reduced by tetrodotoxin (1 μM), but was not affected by L-NAME (100 μM). Apamin (0.1 μM) significantly antagonised PACAP-(1–27)-induced hyperpolarization. In the presence of 1 μM apamin, a small depolarization in response to PACAP-(1–27) was detected. PACAP-induced hyperpolarization was also significantly antagonised by the PACAP receptor antagonist, PACAP-(6–38) (3 μM). In order to check the specificity of PACAP-(6–38) as PACAP receptor antagonist, we tested the effects of PACAP-(6–38) on the response to VIP. VIP (0.1 μM) induced membrane hyperpolarization, which was not modified by PACAP-(6–38) (3 μM) (8.7 ± 1.8 mV in the control and 9.1 ± 1.4 mV after PACAP-(6–38), $n=3$, $P>0.05$). A depolarization in response to PACAP has been observed in the presence of suramin (100 μM). Desensitisation of P2X purinoceptors induced by perfusion with α,β-meATP (1 μM for 30 min) did not modify PACAP-(1–27)-induced hyperpolarization. However, desensitisation of P2Y purinoceptors induced by perfusion with ADPβS (100 μM for 20 min) reduced the amplitude of PACAP-induced hyperpolarization. In some

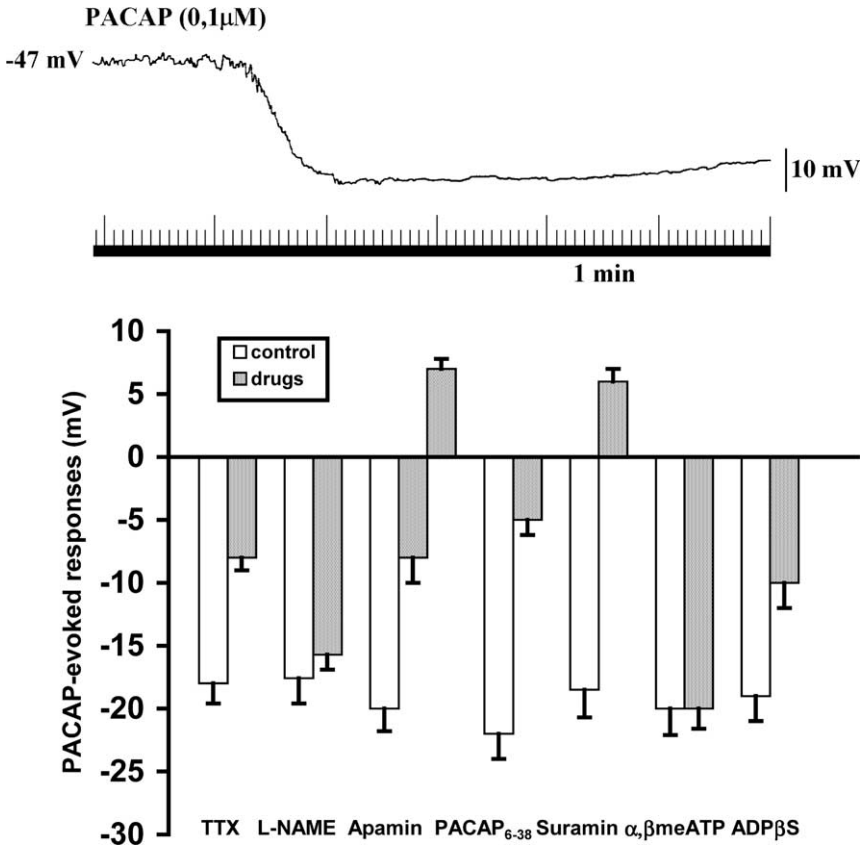

Fig. 5. Top: intracellular microelectrode recording showing the effects of PACAP-(1–27) (0.1 μM) on the resting potential under control conditions. Bottom: summary of effects of tetrodotoxin (1 μM, $n=3$), L-NAME (100 μM, $n=3$), apamin (0.1–1 μM, $n=5$), suramin (100 μM, $n=6$), α,β-meATP (1 μM, $n=5$), ADPβS (100 μM, $n=5$) and PACAP-(6–38) (3 μM, $n=5$) on responses evoked by PACAP-(1–27) (0.1 μM). Data are expressed as means ± S.E. *$P<0.05$ when compared to the respective value obtained in the control.

preparations, after P2Y receptors desensitization, PACAP induced a biphasic response consisting of an early depolarization followed by a low amplitude hyperpolarization (data not shown). The effects of the various drugs on PACAP-evoked hyperpolarization are summarised in Fig. 5.

4. Discussion

This study suggests that an ATP-like endogenous P2Y purinoceptor ligand, with a smaller contribution of PACAP, is the neurotransmitter that mediates the spontaneously occurring IJPs and the "fast component" of the IJP in the circular muscle cells from murine distal colon. Activation of P2Y receptors induces opening of apamin-sensitive Ca^{2+}-dependent K^+ channels and thereby causes hyperpolarization of the cell membrane.

IJPs in different animal preparations (He and Goyal, 1993; Keef et al., 1993; Serio et al., 1996; Ohno et al., 1996; Imoto et al., 1998), including mouse colon (Shuttleworth et al., 1997; Spencer et al., 1998; Serio et al., 2001), are characterised by apamin-sensitive and insensitive components. While the apamin-insensitive component is attributed to an action of NO and VIP, ATP has been proposed as the neurotransmitter responsible for the apamin-sensitive component (see Kuryama et al., 1998 for review). However, some concerns with the role of ATP as mediator of the apamin-sensitive component of IJP have been raised. In some preparations, ATP does not play any role in the apamin-sensitive IJP (Ohno et al., 1996; Serio et al., 1992; 1996; Ivancheva et al., 2000) and, recently, a VIP-related peptide, PACAP, has been reported to produce apamin-sensitive effects in some intestinal tracts (Schworer et al., 1992; Kishi et al., 1996; Imoto et al., 1998; Plujà et al., 2000).

Our results have shown that (i) the purinoceptor block by the P2-purinoceptor antagonist, suramin, and, especially, desensitisation of P2Y-purinoceptors inhibited the fast IJP evoked by electrical field stimulation; (ii) exogenous ATP induced muscular hyperpolarization, antagonised by suramin and by both P2X and P2Y receptor desensitisation; (iii) apamin, a blocker of subtypes of small and intermediate conductance Ca^{2+}-activated K^+ channels, inhibited a large part of the fast IJP evoked by electrical field stimulation and ATP-induced hyperpolarization. Therefore, in circular muscle from murine colon, an ATP-like endogenous P2Y purinoceptor ligand acting through apamin-sensitive Ca^{2+}-activated K^+ channels is responsible for fast IJP.

Patch-clamp studies have shown that murine colon myocytes express K^+ channels with properties consistent with small conductance K^+ channels present in other gastrointestinal muscle and these channels may mediate membrane hyperpolarization evoked by ATP, because P2-purinoceptor agonists increased their open probability (Koh et al., 1997). Our results indicate that apamin-sensitive small conductance Ca^{2+}-activated K^+ channels are involved in the inhibitory responses to NANC nerve stimulation in mouse distal colon. However, it was possible to evoke a residual fast IJP after apamin. This can be explained by the fact that more than a single class of channels involved in the generation of fast IJP or apamin-sensitive and -insensitive isoforms of small conductance Ca^{2+}-activated K^+ channels contribute to the fast IJP. Apamin-sensitive and -insensitive isoforms of small conductance Ca^{2+}-activated K^+ channels have been identified in mammalian brain (Kohler et al., 1996).

Under our experimental conditions, desensitisation of P2Y receptors by prolonged application of ADPβS, a putative selective P2Y receptor agonist, abolished fast IJP and antagonised the effects of exogenous ATP. These data suggest that the fast IJP and the effect of ATP on membrane voltage in mouse colon are mediated by ADPβS-sensitive P2Y receptors. These receptors also turned out to be partially sensitive to suramin. Involvement of such receptors in the fast IJP has also been reported in human jejunum (Xue et al., 1999). P2X receptors activated by α,β-meATP seem not to be involved in the generation of fast IJP in our preparation, as suggested for example in the guinea-pig colon (Zagorodnyuk et al., 1996), because their desensitisation by prolonged application of α,β-meATP failed to affect the fast IJP. However, the desensitisation of P2X receptors antagonised the effects of exogenous ATP, indicating that such receptors are present in the distal colon of the mouse. The observation that ATP-hyperpolarization is resistant to L-NAME and that desensitisation of P2Y or P2X receptors leaves the slow component of the evoked IJPs ruled out a possible interplay between ATP and NO, as suggested for instance for the canine terminal ileum (Boeckxstaens et al., 1991).

PACAP is a neurotransmitter that might be involved in inhibitory actions in the gastrointestinal tract (see Shuttleworth and Keef, 1995 for review). The inhibitory effect of PACAP has been described for several segments of gastrointestinal tract from different animal species (Kishi et al., 1996; Imoto et al., 1998; Rattan and Chakder, 1997; Plujà et al., 2000) and can be due to activation of apamin-sensitive K^+ channels (Schworer et al., 1992; Kishi et al., 1996; Imoto et al., 1998; Plujà et al., 2000). In particular, in mouse colon, PACAP has been proposed as a neurotransmitter mediating NANC relaxation of longitudinal muscle (Satoh et al., 1999). Moreover, the presence of neurons immunoreactive to PACAP has been shown in mouse intestine (Sundler et al., 1992). Data from our experiments suggest that PACAP may be involved in the NANC inhibitory pathway. In fact, the PACAP-induced muscular hyperpolarization of membranes was due to the opening of apamin-sensitive small conductance Ca^{2+}-activated K^+ channels as the hyperpolarization of the fast IJP and PACAP-(6–38), a selective PACAP receptor antagonist, reduced the amplitude of the fast IJP to about 60% of the control value. Our finding of a lack of effect of PACAP-(6–38) on VIP-induced hyperpolarization indicates the presence in distal colon of PACAP-specific receptors, as reported for mice and other animal preparations (Ekblad and Sundler, 1997; Ekblad et

al., 2000). Part of the PACAP-evoked hyperpolarization was tetrodotoxin-sensitive and reduced by P2Y purinergic desensitisation. Therefore, PACAP inhibitory effects depend partially on neural action potential and on ATP acting on P2Y receptors. Activation of PACAP receptors at the myenteric neurons, leading to the release of neurotransmitters, has been reported for other animal preparations (Rattan and Chakder, 1997; Fox-Threlkeld et al., 1999; Ekblad et al., 2000). So, we suggest that PACAP can be involved in the inhibitory pathway as a neuromodulator, enhancing the release of ATP from inhibitory neurons and/or acting at the postjunctional level on apamin-sensitive Ca^{2+}-dependent K^+ channels, and thereby enhancing the response of the muscle to the primary neurotransmitter. The observation that the spontaneous IJPs were also sensitive to a PACAP receptor antagonist suggests that PACAP tonically plays such a permissive role. PACAP-induced hyperpolarization was resistant to L-NAME, so an involvement of the NO pathway in the PACAP-induced hyperpolarization in mouse colon appears unlikely. Moreover, PACAP-(6–38), a PACAP receptor antagonist, reduced only the fast IJP, excluding the possibility that PACAP induces NO production as suggested by Murthy et al. (1997) and Ekblad et al. (2000). Furthermore, the observation that, in the presence of apamin, suramin and, occasionally, in the presence of P2Y purinergic desensitisation, a depolarization was unmasked, which was likely due to the removal of the inhibitory effects, might suggest the existence in mouse colon of multiple PACAP receptors, as shown for other preparations (Zagorodnyuk et al., 1996). However, further studies are needed to examine this effect.

The observation that apamin and P2Y-receptor desensitisation by ADPβS caused membrane depolarization indicates that ATP and apamin-sensitive channels are also involved in maintenance of the tonic inhibition of the membrane potential, as suggested by Spencer et al. (1998).

Lastly, the observation that spontaneous IJPs showed the same sensitivity of the "fast component" of the evoked IJPs to the drugs used suggests that both are generated by the same population of inhibitory neurons. However, as described by Spencer et al. (1998) and Powell et al. (2001), apamin abolished the spontaneous IJPs, while the evoked fast IJP shows an apamin-resistant component. These authors have proposed that transmural stimulation can activate an additional population of nerves, which are not involved in the generation of spontaneous IJPs, with the evocation of the apamin-resistant component of fast IJP. However, the differences between both types of IJPs could be in the amount of neurotransmitter released and in the subsequent intensity of the evoked response.

In conclusion, the results of the present study indicate that, in the circular muscle of murine distal colon, an ATP-like endogenous P2Y purinoceptor ligand is the final NANC inhibitory mediator involved in the generation of fast IJPs. A role of PACAP as neuromodulator in the inhibitory pathway is also supposed.

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