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Mechanisms underlying the inhibitory effects induced by pituitary adenylate cyclase-activating peptide in mouse ileum

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Abstract

The aim of this study was to investigate the signal transduction mechanisms underlying the inhibitory effect induced by pituitary adenylate cyclase activating peptide (PACAP-27) on the spontaneous contractile activity of longitudinal muscle of mouse ileum. Mechanical activity of ileal segments was recorded isometrically in vitro. PACAP-27 produced apamin-sensitive reduction of the amplitude of the spontaneous contractions. 9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ 22,536), adenylate cyclase inhibitor, or genistein and tyrphostin 25, tyrosine kinase inhibitors, had negligible effects on PACAP-27-induced inhibition. PACAP-27 effects were significantly inhibited by U-73122, phopholipase C (PLC) inhibitor, by 2-aminoethoxy-diphenylborate (2-APB), permeable blocker of inositol 1,4,5-triphosphate (IP_3) receptors and by depletion of Ca^{2+} stores with cyclopiazonic acid or thapsigargin. Ryanodine did not reduce PACAP-27-inhibitory responses.

We suggest that, in mouse ileum, the inhibitory responses to PACAP-27 involve stimulation of PLC, increased production of IP_3 and localised Ca^{2+} release from intracellular stores, which could provide the opening of apamin-sensitive Ca^{2+}-dependent K^+ channels.
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Keywords: PACAP-27 (pituitary adenylate cyclase activating peptide); Phospholipase C; Inositol 1,4,5-triphosphate; Mouse ileum; Muscular inhibition

1. Introduction

Pituitary adenylate cyclase activating peptide (PACAP), a member of the glucagon/vasoactive intestinal peptide (VIP)/secretin family of peptides, is widely distributed in the gastrointestinal tract and it is considered a potential neurotransmitter involved in the neurally evoked relaxation (see Läuffer et al., 1999, for review). Two molecular forms of PACAP exist, PACAP-38 and the C-terminally truncated peptide PACAP-27. The relative potencies of these two form of PACAP differ not only between species, but also between various region of the gut. For example, in rat ileum PACAP-27 is more potent of PACAP-38 in inducing relaxatory responses, whilst in rat and in mouse colon they are equipotent (Ekblad and Sundler, 1997; Ekblad et al., 2000). Three G-protein-coupled receptor subtypes of the VIP/PACAP family have been cloned and pharmacologically characterised (Arimura and Shioda, 1995). $VPAC_1$ and $VPAC_2$ receptors display high affinity for both VIP and PACAP, whereas PAC_1 receptor is almost exclusively stimulated by PACAP-38 or PACAP-27. (Läuffer et al., 1999). In addition, pharmacological studies indicated in rat ileum the presence of PACAP-27 preferring receptors which may represent a subtype of the PAC_1 receptors (Ekblad and Sundler, 1997; Ekblad et al., 2000). In some animal preparations, PACAP, acting on PACAP preferring receptors, induces hyperpolarization and relaxation of intestinal muscle that, are coupled to apamin-sensitive changes in the membrane potential (Schworer, 1992; McConalogue et al., 1995b; Kishi et al., 1996; Imoto et al., 1998; Ekblad et al., 2000). In particular, in the mouse, PACAP has been shown to induce inhibitory effects in colon and ileum (Satoh et al., 1999; Mukai et al., 2002; Serio et al., 2003; Zizzo et al., 2004) through PACAP-27 preferring receptors and opening of apamin-sensitive K^+-channels (Serio et al., 2003; Zizzo et al., 2004).

There is a relative paucity of information available regarding the biochemical pathways by which PACAP signal is transduced at the intracellular level. PACAP\VIP receptors seem to act almost exclusively via the adenylate cyclase system, whereas PAC_1 appears to stimulate both adenylate cyclase and

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phospholipase C (PLC), the latter likely initiating the inositol phospate cascade (Christophe, 1993). Moreover, in rat colon, PACAP has been reported to activate tyrosine kinase, upstream event for activation of apamin-sensitive K^+-channels (Takeuchi et al., 1999). Anyway, how the second messenger pathways regulate apamin-sensitive K^+-channels has not been clarified yet. It has been suggested that the open probabilities of Ca^{2+}-dependent conductances in the plasma membrane may be dependent upon localized Ca^{2+} release events (Ca^{2+} sparks) from ryanodine-sensitive Ca^{2+} stores on the sarcoplasmic reticulum (SR), first observed in vascular smooth muscle cells (Nelson et al., 1995), and that contractile and relaxant agents would regulate smooth muscle function by modulating the amplitude and frequency of the Ca^{2+} sparks (Jaggar et al., 2000; Sanders, 2001). Moreover, Ca^{2+} release mediated by inositol 1,4,5-trisphosphate (IP_3) receptor-operated channels (Ca^{2+} puffs) could be also involved in regulating membrane ionic conductances, namely small conductance apamin-sensitive Ca^{2+}-dependent K^+ channels, as suggested by Bayguinov et al. (2000) in mouse colonic myocytes by stimulation of P2Y receptors.

Because, we have previously shown that in the mouse ileum longitudinal smooth muscle, PACAP-27 induces inhibitory effects through opening of apamin-sensitive K^+-channels (Zizzo et al., 2004), the aim of the present study was to investigate the intracellular second messenger systems activated by PACAP-27 and whether localized Ca^{2+} release from intracellular stores can be involved in the coupling between PACAP-27 receptor stimulation and opening of Ca^{2+}-dependent K^+ channels.

2. Materials and methods

Experiments, authorised by the Ministero della Sanità (Rome, Italy), were performed on adult male mice (C57BL/10SnJ, Charles River, Calco LC, Italia), killed by cervical dislocation. The abdomen was immediately opened and a segment of ileum (6–7 cm proximal to the ileocecal junction) was removed and placed in Krebs solution consisting of (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1. The contents of the excised segments were gently flushed out with Krebs solution. Segments (20 mm in length) were suspended in 10 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs solution maintained to 37 °C.

2.1. Recording of mechanical activity

The distal end of each segment was tied to an organ holder and the proximal end was secured with a silk thread to an isometric force transducer (FORT 10, Ugo Basile, Biological Research Apparatus, Comerio VA, Italy). Mechanical activity was digitized on a A/D converter, visualized, recorded and analysed on a personal computer using the PowerLab/400 system (Ugo Basile. Italy). Because we have demonstrated the existence in mouse ileum of an interplay between PACAP-27 and nitric oxide (NO) (Zizzo et al., 2004), experiments were carried out in the presence of L-NAME to exclude the component of the transduction mechanism due to NO.

Longitudinal preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min. Rhythmic spontaneous contractions of varying amplitude developed in all preparations.

2.2. Experimental protocol

After the equilibration time, concentration–dependent curve for PACAP-27 was constructed by non-cumulative addition of the peptide before and after the different drugs used. PACAP-27 was applied for approximately 2 min at 20 min interval, with at least three changes of Krebs solution between concentrations. All the antagonists were allowed to maintain contact with the tissue for at least 30 min before repeating the curve of the agonist. The interval between the two curves was at least 1 h. Each preparation was tested with a single antagonist. Time control experiments showed that a second curve to the agonist was reproducible. Concentrations of drugs used were determined from literature.

2.3. Drugs

The following drugs were used: 2-aminoethoxy-diphenylborate (2-APB), ciclopiazonic acid (CPA), forskolin, guanethidine monosulphate, Nω-nitro-L-arginine methyl ester (L-NAME), ryanodine, sodium nitroprusside, thapsigargin, 9-(Tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ 22,536) (Sigma-Aldrich, Inc., St. Louis, USA); genistein, tyrphostin 25, U-73122 (Calbiochem, Darmstadt, Germany); pituitary adenylate cyclase activating peptide-(1–27) amide (PACAP-27), (Bachem AG, Bubendorf, Switzerland). CPA, genistein, ryanodine, SQ 22,536, thapsigargin, tyrphostin 25, U-73122 were dissolved in dimethylsulfoxide (DMSO). 2-APB was dissolved in ethanol. 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. Drugs were added to the organ bath in volumes of less than 1.0% of the bathing solution. Control experiments using 1.0% DMSO or 1.0% ethanol alone showed that they have no effect on the spontaneous contractile activity nor on the concentration–response curve to PACAP-27.

2.4. Statistical analysis

All data are given as means ± S.E.M. "*n*" in the results section refers to the number of animal preparations on which observations were made. Inhibitory effects induced by PACAP-27 were calculated as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27. PACAP-27 responses in the absence or in the presence of the different antagonists were fitted to sigmoid curves (Prism 4.0, GraphPAD, San Diego, CA, USA), and EC_{50} values with 95% confidence limits (95% CL) were determined from these curves. Statistical analysis was

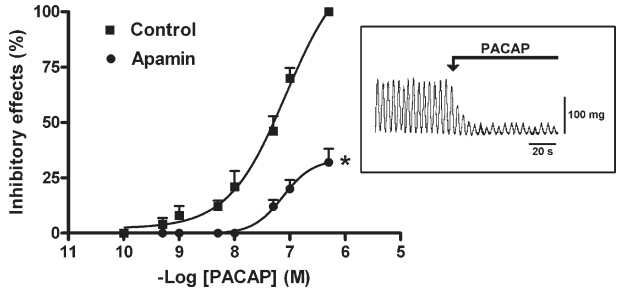

Fig. 1. Concentration–response curves for the inhibitory effects induced by PACAP-27 alone or in the presence of apamin (10^{-7} M, $n=4$). Data are reported as means±S.E.M. and are expressed as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27. *$P<0.05$ when the concentration–response curve was compared to that obtained in respective control condition. Insert: Representative recording showing the effect of PACAP-27 (5×10^{-7}M) on the longitudinal muscle of mouse ileum, in the presence of L-NAME (10^{-4}M).

performed by means of Student's *t* test. A probability value of less than 0.05 was regarded as significant.

3. Results

As previously reported, PACAP-27 in the presence of L-NAME, to exclude the component of the transduction mechanism due to NO, produced a concentration dependent reduction of the amplitude of spontaneous contractions (Zizzo et al., 2004). Due to the presence of L-NAME the dose–response curve to PACAP-27 showed a low sensitivity with an EC_{50} (95% CL) values of 70 (39–126) nM ($n=15$) (Fig. 1). PACAP-27-induced effects were significantly antagonized by apamin (10^{-7} M), a blocker of small and intermediate conductance Ca^{2+}-activated K^+ channels (Fig. 1).

At first we analysed whether the inhibitory effects of PACAP-27 were sensitive to the adenylate cyclase inhibitor SQ 22,536. Treatment with SQ 22,536 (10^{-4} M) did affect neither mechanical spontaneous activity nor the PACAP-27-induced effects at any concentration used (Table 1, Fig. 2). However, SQ 22,536 (10^{-4} M) was able in the same preparation to antagonized the inhibitory effects induced by forskolin (10^{-7} M), adenylyl cyclase activator (data not shown).

Table 1
EC_{50} values for PACAP-27-induced inhibitory effects before and after different pharmacological treatments

	EC_{50} (nM)	95% CL (nM)	n
Control	70	39–126	5
SQ 22,536	91	66–128	
Control	71	38–149	5
Tyrphostin 25	96	50–183	
Control	62	30–130	5
Genistein	80	34–1895	
Control	70	40–148	6
U-73122	3686 *	1847–7357	
Control	78	48–158	6
2-APB	2181 *	1350–7264	
Control	80	30–160	5
CPA	173 *	64–464	
Control	77	40–180	5
Thapsigargin	291 *	135–626	
Control	68	44–139	6
Ryanodine	75	45–127	

* $P<0.05$ when compared to the respective control.

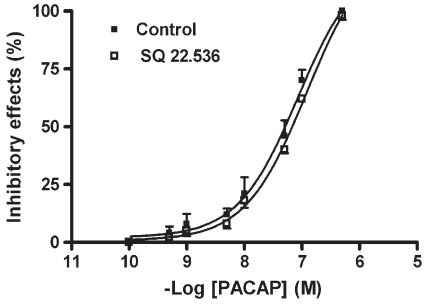

Fig. 2. Concentration–response curves for the inhibitory effects induced by PACAP-27 alone or in the presence of SQ 22,536 (10^{-4} M, $n=5$). Data are reported as means±S.E.M. and are expressed as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27.

Since it has been reported that, in rat colon, tyrosine kinase is involved in PACAP-induced relaxation (Takeuchi et al., 1999), we examined the effects of genistein and tyrphostin 25, two competitive tyrosine kinase inhibitors, on the response to PACAP-27. In our preparation, neither drug affected spontaneous activity. Moreover, neither genistein (10^{-5} M) nor tyrphostin 25 (10^{-5} M) antagonised PACAP-27 inhibitory effects (Table 1, Fig. 3).

To investigate about a possible role of the PLC/IP_3 signalling pathway, we carried out experiments using U-73122, inhibitor of the PLC, thus limiting the generation of IP_3, and 2-APB, membrane-permeant IP_3 receptor inhibitor. U-73122 (10^{-4}M) or 2-APB (3×10^{-5} M) decreased the spontaneous mechanical activity and significantly shifted to the right the concentration–response curve to PACAP-27 (Table 1, Fig. 4). Sodium nitroprusside which causes as well inhibition of mechanical activity was tested in the presence of U-73122 (10^{-4} M) or 2-APB (3×10^{-5} M). In these conditions the inhibitory response to sodium nitroprusside was not modified (data not shown).

The role of calcium ions released from intracellular stores in the inhibitory effects induced by PACAP-27, was further investigated pre-treating the intestinal segments with drugs known to interfere with calcium handling. In particular, we tested two known inhibitors of the sarcoplasmatic reticulum Ca^{2+}-ATPase pump, CPA (10^{-5} M) and thapsigargin (2×10^{-7} M), thus allowing internal calcium stores to deplete

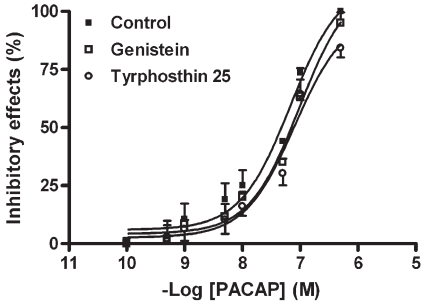

Fig. 3. Concentration–response curves for the inhibitory effects induced by PACAP-27 alone or in the presence of genistein (10^{-5}M, $n=5$) or tyrphostin 25 (10^{-5} M, $n=5$). Data are reported as means±S.E.M. and are expressed as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27.

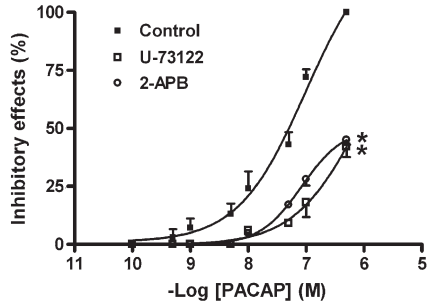

Fig. 4. Concentration–response curves for the inhibitory effects induced by PACAP-27 alone or in the presence of U-73122 (10^{-4} M, $n=6$), or 2-APB (3×10^{-5} M, $n=6$). Data are reported as means±S.E.M. and are expressed as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27. *$P<0.05$ when the concentration–response curves were compared to that obtained in respective control condition.

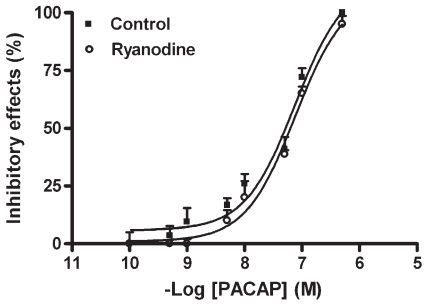

Fig. 6. Concentration–response curves showing the inhibitory effects induced by PACAP-27 alone or in the presence of ryanodine (10^{-5} M, $n=6$). Data are reported as means±S.E.M. and are expressed as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27.

progressively. These drugs transitorily increased the basal tone and the amplitude of spontaneous contractions. PACAP-27-inhibitory effects were decreased by CPA and by thapsigargin (Table 1, Fig. 5).

To test whether ryanodine-sensitive Ca^{2+} stores are also involved in the inhibitory effects induced by PACAP-27 we tested the effects of ryanodine, an inhibitor of Ca^{2+} release from the SR. Ryanodine (10^{-5} M) induced a reduction of the amplitude of spontaneous contractions but did not modify the effects of PACAP-27 (Table 1, Fig. 6). Ryanodine at this concentration was indeed able to antagonize sodium nitroprusside-induced inhibitory effects (Zizzo et al., in press).

4. Discussion

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 in several segments of the gastrointestinal tract from different animal species (Kishi et al., 1996; Imoto et al., 1998; Chakder and Rattan, 1998; Plujà et al., 2000; Serio et al., 2003; Zizzo et al., 2004). Some reports indicate that PACAP induces hyperpolarization and relaxation of intestinal muscle that, are coupled to the apamin-sensitive changes in the membrane potential (Schworer, 1992; McConalogue et al., 1995b; Kishi et al., 1996; Imoto et al., 1998). Other reports suggest that the mechanism mediating PACAP relaxation is not strictly coupled to the apamin-sensitive changes in the membrane potential (Zagorodnyuk et al., 1996; Plujà et al., 2000). In mouse ileum, we have reported that, exogenous PACAP induces inhibition of intestinal muscle through smooth muscle PAC_1 receptors, not activated by VIP, and coupled to apamin sensitive Ca^{2+}-dependent K^+ channels, and indirectly through the stimulation of NO production (Zizzo et al., 2004). The mechanism by which PACAP activates apamin-sensitive K^+ channels is still unknown. Takeuchi et al. (1999) reported that in rat colon PACAP activates tyrosine kinase, upstream event for activation of apamin-sensitive K^+-channels. Moreover, there are some reports that PACAP activates adenylate cyclase and increases the intracellular cyclic AMP content in intestinal muscle (Murthy and Maklouf, 1994; McConalogue et al., 1995a; Murthy et al., 1997).

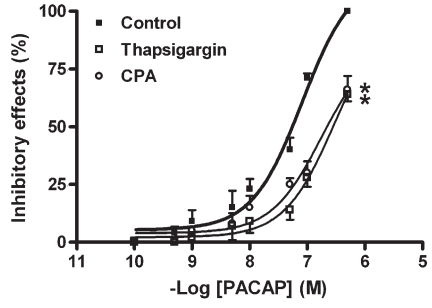

Fig. 5. Concentration–response curves for the inhibitory effects induced by PACAP-27 alone or in the presence of CPA (10^{-5} M, $n=5$), or thapsigargin (2×10^{-7}M, $n=5$). Data are reported as means±S.E.M. and are expressed as a percentage of the maximal effect induced by 5×10^{-7} M PACAP-27. *$P<0.05$ when the concentration–response curves were compared to that obtained in respective control condition.

Results from our experiments indicate that, in mouse ileum, PACAP-27 induces inhibition of the spontaneous contractions via opening of apamin-sensitive K^+ channels and via PLC/IP_3 signalling pathway. In fact, in our preparation, in the presence of L-NAME to exclude the component of the transduction mechanism due to NO, PACAP-27-induced inhibition of mechanical activity was strongly antagonised by apamin. Moreover, it was not affected by the adenylate cyclase inhibitor SQ-22536, or by genistein and tyrphostin 25, two competitive tyrosine kinase inhibitors, whilst it was significantly antagonized by U-73122, known inhibitor of the PLC. In support of this idea, we found that the membrane-permeant IP_3 receptor antagonist, 2-APB, markedly antagonized PACAP-27-induced effects. Activation of PLC, but not of adenylate cyclase, has been reported to be involved in activation of p38 MAP kinase by PACAP in PC12 cells (Sakai et al., 2002). The possibility that the reduction of the PACAP-27 effects observed in the presence of U-73122 or 2-APB occurs as a consequence of the decrease of the mechanical activity can be ruled out since sodium nitroprusside was able in the same circumstances to still inhibit muscular activity, and, in addition, PACAP-27 effects were not affected when the phasic contractions were decreased by ryanodine. Indeed, the observation that U-73122 or 2-APB interfere with the spontaneous mechanical activity confirms that the release of Ca^{2+} from IP_3-sensitive intracellular stores plays a critical role in the genesis of spontaneous activity. Regulation of

the slow waves amplitude and frequency by IP_3-sensitive release has been reported in the murine small intestine (Malysz et al., 2001). Moreover, absence of slow waves activity has been reported also in the stomach of mice which lack the IP_3 receptors (Suzuki et al., 2000). Our data suggested that Ca^{2+} release from SR mediated by IP_3 receptor-operated channels is also required for PACAP-27-induced inhibition of the muscular activity.

The confirmation that intracellular Ca^{2+} stores play a role in the inhibitory effects induced by PACAP-27 is suggested by the experiments using CPA and thapsigargin which interfere with calcium handling by blocking SR Ca^{2+}-ATPase pump. Both inhibitors antagonized PACAP-27-induced relaxation suggesting that functional SR Ca^{2+}-ATPase pump is an essential requirement for PACAP-27-induced effects. PACAP-27 receptor activation would induce muscular inhibition regulating the IP_3-dependent transient Ca^{2+} release from intracellular stores near the plasma membrane. Activation of apamin-sensitive K^+ channels by localized Ca^{2+} release events has been reported in isolated myocytes of murine colon following P2Y receptor stimulation via a mechanism involving PLC and IP_3 receptors (Bayguinov et al., 2000).

Lastly, we investigated the possibility that localized Ca^{2+} release events (Ca^{2+} sparks) from ryanodine-sensitive Ca^{2+} stores on the SR can be also involved in the PACAP-27 inhibitory effects. In fact, ryanodine sensitive mechanisms have been also proposed to be responsible for PACAP-induced Ca^{2+} release (Tanaka et al., 1998; Dehaven and Cuevas, 2004). Moreover, Boittin et al. (1998) provided evidence that, in rat portal vein myocytes, IP_3-dependent Ca^{2+} release is amplified by ryanodine receptors, and this would facilitate the development of Ca^{2+} waves. In our experiments inhibition of Ca^{2+} release from ryanodine stores had no influence on PACAP-27-induced inhibitory effects, thus not supporting a role for ryanodine-sensitive Ca^{2+} release mechanism in response to PACAP-27 receptor activation. Ryanodine reduced the amplitude of the spontaneous activity. Calcium release from these stores is reported to be linked to the regulation of membrane potentials in murine small intestine (Malysz et al., 2001) and this could account for the effects on the spontaneous contraction amplitude.

In conclusion, in mouse ileum, we suggest that smooth muscle PAC_1 receptor activation involves PLC/IP_3 signalling pathway. IP_3-dependent transient Ca^{2+} release from intracellular stores near the plasma membrane would increase the opening probability of apamin-sensitive K^+ channels and so, leading to muscular inhibition.

Acknowledgements

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