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Presynaptic modulation by VIP, secretin and isoproterenol of somatostatin release from enriched enteric synaptosomes: role of cAMP

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Abstract

The release of somatostatin-like immunoreactivity was studied in isolated enteric synaptosomes. A significant release of somatostatin-like immunoreactivity was observed in the presence of vasoactive intestinal polypeptide (VIP) (10^{-6} M: 53.0 ± 12.4 pg/mg, basal: 14.3 ± 1.7 pg/mg, $n = 5$, $P < 0.05$), secretin (10^{-6} M: 56.1 ± 3.8 pg/mg, basal: 25.8 ± 1.6 pg/mg, $n = 6$, $P < 0.01$) and isoproterenol (10^{-5} M: 54.0 ± 13.4 pg/mg, basal: 20.0 ± 3.4 pg/mg, $n = 8$, $P < 0.05$). Forskolin, an unspecific activator of the adenylate cyclase, caused a significant release of somatostatin-like immunoreactivity (10^{-6} M: 57.3 ± 13.2 pg/mg, basal: 30.0 ± 5.8 pg/mg, $n = 13$, $P < 0.01$) which was further augmented in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M) (77.0 ± 17.8 pg/mg, $n = 13$, $P < 0.01$). 3-Isobutyl-1-methylxanthine and N^6,2′-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate mimicked the effect of forskolin and VIP. The release of somatostatin was paralleled by an increase of cAMP immunoreactivity in the presence of VIP (10^{-6} M: 37.1 ± 9.4 pmol/mg, basal: 19.8 ± 4.2 pmol/mg, $n = 10$, $P < 0.05$), isoproterenol (10^{-5} M: 42.4 ± 9.8 pmol/mg, basal: 16.7 ± 2.4 pmol/mg, $n = 12$, $P < 0.01$) and forskolin (10^{-6} M: 47.1 ± 12.4 pmol/mg, basal: 19.8 ± 4.2 pmol/mg, $n = 10$, $P < 0.01$). The effect of nitric oxide (NO) which acts as an inhibitory neurotransmitter in the enteric nervous system was studied. NO is known to activate soluble guanylate cyclase to induce transmitter release. The NO-generating compound sodium nitroprusside and bromoguanosine-3′,5′-cyclic monophosphate (8-Br-cGMP) had no effect on the release of somatostatin-like immunoreactivity. These data demonstrate the stimulatory effect of VIP, secretin and isoproterenol on release of somatostatin-like immunoreactivity from enteric synaptosomes, which is presumably mediated by cAMP-dependent mechanisms. cGMP-dependent mechanisms seem to be of minor relevance.

Keywords: Synaptosome; VIP (vasoactive intestinal polypeptide); Secretin; Isoproterenol; cAMP; Nitric oxide (NO)

1. Introduction

Somatostatin modulates the secretion of other regulatory peptides in the pituitary gland, the endocrine pancreas and the gastrointestinal tract (Schusdziarra, 1992). The presence of somatostatin-containing neurons in the myenteric and submucous plexus of the enteric nervous system points to a potential role for this peptide in regulating gastrointestinal motility (Daniel et al., 1985). Somatostatin has been reported to hyperpolarize or depolarize myenteric neurons (Katayama and North, 1980) and has been suggested to be the non-adrenergic inhibitory post-synaptic slow potential mediator in submucosal neurons (Shen and Suprenant, 1993). Furthermore, somatostatin has been shown to induce intestinal migrating motor complexes (Thor et al., 1978) but also appears to inhibit intestinal motility due to suppression of the post-ganglionic cholinergic neuron (Guillemin, 1976). So far, it is not clear which mechanisms are responsible for the mode of somatostatin modulation of intestinal motor activity. Somatostatin secretion from the stomach and the pancreas is increased by agents that stimulate adenylate cyclase activity, including vasoactive intestinal polypeptide (VIP) (Chiba et al., 1980; Schusdziarra et al., 1986), norepinephrine (Hermansen et al., 1979), glucagon and secretin (Chiba et al., 1980), suggesting that cAMP is a second messenger for somatostatin release from endocrine D-cells. However, little information is available on the release mechanisms of *neuronal* somatostatin, as the somatostatin released from stomach and pancreas is predominantly of endocrine origin. In the enteric nervous system, somatostatin has been released from isolated ganglia of the myenteric plexus (Grider,

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1989), a culture of canine jejunal submucosal plexus (Buchan et al., 1990) and cultured human submucosal ganglia (Accili et al., 1993). The presence of somatostatin in synaptosomal preparations of the brain (Bennett et al., 1979) and the enteric nervous system (Kurjak et al., 1995), together with evidence showing that somatostatin has direct actions on neuronal activity (Guillemin, 1976; Katayama and North, 1980), has prompted speculation that it may have an important regulatory function at synaptic level. In enriched synaptosomes from the enteric nervous system, we have demonstrated the release of somatostatin in response to depolarizing levels of potassium and veratridine and in the presence of cholinergic agents (Kurjak et al., 1995). To date, there is no information on the possible coupling between neuropeptide and cAMP formation as the stimulatory mechanism of somatostatin secretion in the enteric nervous system. Previous studies with lipophilic cAMP have demonstrated an increase in acetylcholine release from myenteric synaptosomes of the guinea-pig small intestine (Reese and Cooper, 1984), supporting the role of cAMP in the process of neurotransmitter secretion in the enteric nervous system. The feasibility of stimulation of adenylate cyclase and of cAMP in enzymatically dissociated myenteric ganglia is established. Forskolin and brain-gut peptides stimulate cAMP formation in these ganglia (Xia et al., 1991; Baidan et al., 1992). Preliminary experiments with enteric synaptosomes revealed subpopulations of vesicles which release neuropeptides (bombesin and VIP) following stimulation with cGMP and nitric oxide (NO)-related compounds (Kurjak et al., 1994b). As the release of NO exerts inhibitory control of intestinal motility (Christinck et al., 1991), it is of interest whether this substance has a modulatory action on the somatostatin-containing neuron. In the present study, experiments were designed to: (1) examine the effect of direct (forskolin) and receptor-mediated (VIP, secretin and isoproterenol) activation of adenylate cyclase on stimulation of release of somatostatin-like immunoreactivity from enriched enteric synaptosomes; (2) demonstrate that enteric synaptosomes are capable of accumulating cAMP and determine the effects of forskolin, isoproterenol and VIP on cAMP levels; and (3) examine the effect of NO and cGMP on the release of somatostatin-like immunoreactivity.

2. Materials and methods

2.1. Tissue handling and isolation of synaptosomes

Isolation of synaptosomes was performed as described previously (Kurjak et al., 1994a, Allescher et al., 1989). Briefly, male Wistar rats were killed by cervical dislocation and the small intestine was quickly removed and suspended in ice-cold isolation buffer (25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 10 mM $MgCl_2$, 8% w/v sucrose, pH 7.4). All further preparative steps were carried out at 0–4°C. The ileum was dissected, cleaned of mesenteric arcade and fat, and opened along the mesenteric attachment line. The mucosal layer was removed. For preparation of synaptosomes, the tissue was resuspended in isolation buffer, minced with scissors and homogenized with a Polytron PT20 homogenizer at approximately 1500 rpm setting for 15 s (3×5 s).

The tissue homogenate was centrifuged in two steps of $800 \times g$ for 10 min. The supernatant was collected (post-nuclear supernatant) and re-centrifuged at $3500 \times g$ for 10 min to obtain the crude synaptosomal fraction (P1). The supernatant after this spin was centrifuged at $120\,000 \times g$ for 60 min. The pellet from this spin (microsomal 1) was re-suspended and centrifuged again at $10\,000 \times g$ for 10 min. The resulting pellet and the supernatant were then further referred to as enriched synaptosomal fraction (P2) and microsomal fraction 2, respectively. The integrity of the synaptosomes has been demonstrated by electron microscopy (EM-10-CR) following fixation in 3% glutaraldehyde containing 4.5% sucrose, 1 mM CaCl2 in 75 mM cacodylate buffer (pH 7.4).

2.2. Enzyme and protein assays

Protein was measured by the folin phenol method of Lowry (Lowry et al., 1951). The specific activity of 5'-nucleotidase (EC 3.1.3.5) was determined by incubation of the membrane fraction (20–50 μg of protein) for 30 min at 37°C in 50 mM imidazole-buffer pH 7.0 containing 5 mM AMP-sodium salt and 5 mM $MgCl_2$ to a final volume of 1 ml. The reaction was stopped by adding 1 ml of ice-cold trichloroacetic acid. The precipitated protein was sedimented by centrifugation and the liberated phosphate was determined.

2.3. Peptide release

Peptide release studies were carried out as described previously (Kurjak et al., 1994a). Briefly, 1050 μl of Krebs-Ringer bicarbonate solution (NaCl 115.5 mM, $MgSO_4$ 1.16 mM, NaH_2PO_4 1.16 mM, glucose 11.1 mM, $NaHCO_3$ 21.9 mM, $CaCl_2$ 2.5 mM, KCl 4.16 mM, gassed with 95% O_2 and 5% CO_2) and 150 μl of drugs or Krebs-Ringer solution serving as blank were incubated in separate test tubes at 37°C using a gently shaking water bath. The reaction was started by adding 300 μl of synaptosomes (300 μg of protein) to each tube at timed intervals and they were incubated for 5 min. Preliminary experiments with an incubation of a given amount of exogenous somatostatin demonstrated that the loss of somatostatin-like immunoreactivity due to proteolytic breakdown is less than 5%, when the incubation period was reduced to 5 min, compared to a loss of somatostatin-like immunoreactivity of more than 10%, when the incubation period was 30 min.

2.4. Radioimmunoassay

Somatostatin-like immunoreactivity was determined as described elsewhere (Harris et al., 1978) with an antibody raised against [Tyr^1]somatostatin. Antiserum 80c for measurements of somatostatin-like immunoreactivity was generously provided by Dr. R.H. Unger (Dallas, TX, USA). [Tyr^1]Somatostatin and synthetic somatostatin as standard were generously supplied by Dr. Jean Rivier (Salk Institute, La Jolla, CA, USA). The assay probe (100 μl) was incubated with the tracer (2000–3000 cpm/100 μl) for 72 h at 4°C. Free label was separated from that bound by absorption to dextran-coated charcoal. The sensitivity of the assay was 12.5 pg/ml. The coefficient of variation within and between assays was 6 and 18%, respectively.

2.5. Determination of adenosine-3',5'-cyclic monophosphate

The experiments were carried out in Krebs-Ringer solution containing 10 mM 3-isobutyl-1-methylxanthine. The basal values for cAMP were determined without any stimulus in the incubation medium. For stimulation of adenylate cyclase forskolin (10^{-6}, 10^{-5} M), VIP (10^{-8} to 10^{-6} M) and isoproterenol (10^{-7} to 10^{-5} M) were added. The reaction was started by the addition of 300 μl of membranes (300 μg protein). After 5 min, the reaction was stopped by immediate heating and the synaptosomes were ruptured by addition of distilled water. The suspension was centrifuged using a Eppendorf centrifuge and the supernatant was frozen at −20°C until further determination by RIA. CAMP levels were measured using a commercially available radioimmunoassay (RIANEN [^{125}I]Assay System, Du Pont).

2.6. Drugs

The reagents were purchased from the indicated sources: Secretin, glucagon, isoproterenol, sodium nitroprusside, L-arginine and N^G-nitro-L-arginine (L-NNA) (Sigma, Munich, Germany), forskolin (Calbiochem, La Jolla, CA, USA), 3-isobutyl-1-methylxanthine (Serva), VIP (Peninsula, Heidelberg, Germany), N^6,2'-O-dibutyryl-3',5'-cyclic monophosphate (dbcAMP), 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP) (Biolog Life Science Institute, Bremen, Germany). Adequate controls were performed with the vehicles used for solubilizing each reagent.

2.7. Statistics

Data are given as means ± S.E.M., n indicates the number of independent observations in separate experiments from separate preparations. For each value of a given drug of a single preparation, the release study was carried out in duplicate. When statistical difference of two means was determined, a paired two-tailed Student's t-test was performed. For multiple comparisons, analysis of variance (ANOVA), followed by post-hoc test with Bonferroni-Holm procedure for multiple testings, was carried out to determine statistical differences. Values of $P < 0.05$ or less were considered significant.

3. Results

3.1. Effect of VIP on somatostatin release

VIP caused a substantial release of somatostatin-like immunoreactivity from the synaptosomes. VIP increased somatostatin levels from a baseline of 14.3 ± 1.7 to 53.0 ± 12.4 pg/mg at 10^{-6} M ($n = 5$, $P < 0.05$). 3-isobutyl-1-methylxanthine 10^{-4} M alone significantly stimulated release of somatostatin-like immunoreactivity when compared to baseline (33.3 ± 9.0 pg/mg, $n = 5$, $P < 0.05$). In the presence of both 3-isobutyl-1-methylxanthine 10^{-4} M and VIP 10^{-6} M, somatostatin release was only slightly increased (10^{-6} M + IBMX: 62.0 ± 13.4 pg/mg) compared to VIP alone, but significantly different from the effect of 3 − isobutyl-1-methylxanthine alone. Exposure of synaptosomes to VIP at concentrations of 10^{-7} M and 10^{-8} M did not cause any significant change in release of somatostatin-like immunoreactivity (VIP 10^{-7} M: 37.3 ± 11.2 pg/mg; VIP 10^{-8} M: 27.6 ± 8.7 pg/mg) (Fig. 1).

3.2. Effect of secretin and glucagon on somatostatin release

Secretin caused a significant stimulation of release of somatostatin-like immunoreactivity when compared to baseline (secretin 10^{-6} M: 56.1 ± 3.8 pg/mg, basal: 25.8 ± 1.6 pg/mg, $n = 6$, $P < 0.01$). In the presence of 3-isobutyl-1-methylxanthine 10^{-4} M, this effect was not further

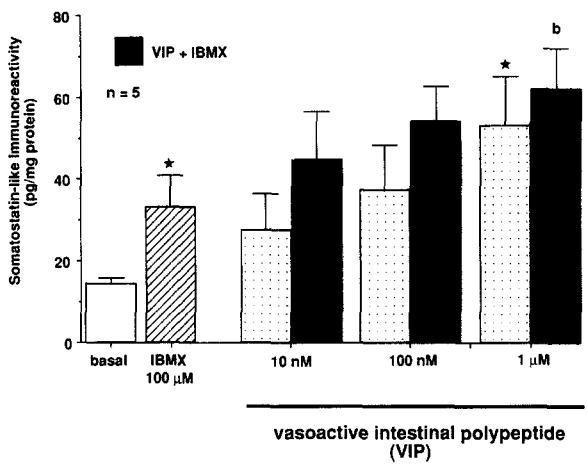

Fig. 1. Effect of VIP on the release of somatostatin-like immunoreactivity from enteric synaptosomes in the absence and presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M). Values are expressed as means ± S.E.M. of $n = 5$ independent experiments. * $P < 0.05$ relative to baseline, ^{b} $P < 0.05$ relative to IBMX. For ANOVA, $P < 0.01$, difference among group means was significant.

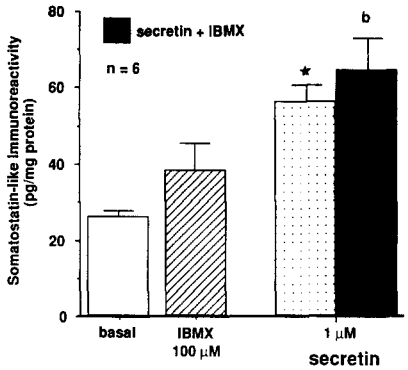

Fig. 2. Effect of secretin on the release of somatostatin-like immunoreactivity from enteric synaptosomes in the absence and presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M). Values are expressed as means ± S.E.M. of $n = 6$ independent experiments. * $P < 0.05$ relative to baseline, [b] < 0.05 relative to IBMX. For ANOVA, $P < 0.01$, difference among group means was significant.

augmented (64.2 ± 8.4 pg/mg, $n = 6$, $P < 0.05$). Secretin 10^{-8} M caused a smaller release (50.0 ± 13.4 pg/mg) which did not reach statistical significance (Fig. 2).

In contrast to secretin, glucagon failed to induce a significant change in release of somatostatin-like immunoreactivity (basal: 26.7 ± 2.0 pg/mg, glucagon 10^{-8} M: 26.5 ± 3.7 pg/mg, glucagon 10^{-6} M: 27.1 ± 9.6 pg/mg, $n = 5$). There was no significant change in the presence of 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M) (glucagon 10^{-8} M + IBMX: 19.3 ± 6.9 pg/mg; glucagon 10^{-6} M + IBMX: 39.3 ± 14.8 pg/mg, $n = 5$).

3.3. Effect of isoproterenol on somatostatin release

The β-adrenoceptor agonist isoproterenol showed a significant stimulatory effect on the release of somatostatin-like immunoreactivity at both 10^{-6} M and 10^{-5} M (basal: 20.0 ± 3.4 pg/mg, 10^{-6} M: 44.3 ± 10.1 pg/mg, 10^{-5} M: 54.0 ± 13.4 pg/mg, $n = 8$, $P < 0.05$). In the presence of 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M), this effect was increased (isoproterenol 10^{-6} M + IBMX: 66.3 ± 8.8 pg/mg, isoproterenol 10^{-5} M + IBMX: 64.1 ± 9.0 pg/mg) but only at the higher concentration significantly different compared to isoproterenol alone. 3-isobutyl-1-methylxanthine alone also stimulated somatostatin release (47.3 ± 5.2 pg/mg, $n = 7$, $P < 0.01$) significantly. The combined effect of isoproterenol and IBMX was significantly different from the effect of IBMX alone. At concentrations of 10^{-7} M and 10^{-8} M isoproterenol had no apparent effect on release of somatostatin-like immunoreactivity (10^{-8} M: 36.6 ± 6.9 pg/mg, 10^{-7} M: 30.0 ± 5.9 pg/mg) (Fig. 3).

3.4. Effect of forskolin on somatostatin release

Forskolin, an unspecific activator of the adenylate cyclase, caused a significant release of somatostatin-like immunoreactivity when compared to baseline (basal: 33.9 ± 6.7 pg/mg protein; 10^{-6} M: 57.3 ± 13.2 pg/mg, $n =$

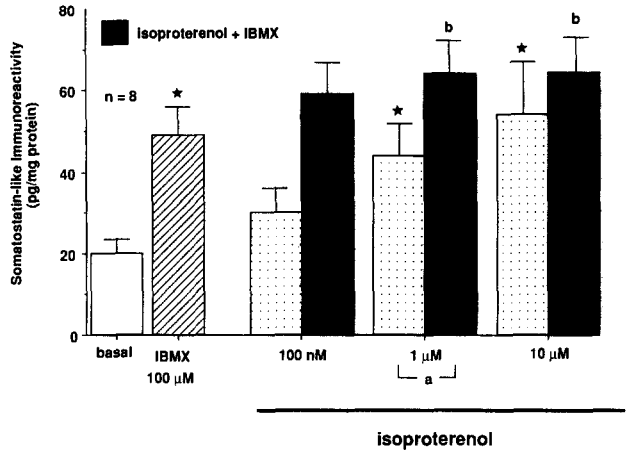

Fig. 3. Effect of isoproterenol on the release of somatostatin-like immunoreactivity from enteric synaptosomes in the absence and presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M). Values are expressed as means ± S.E.M. of $n = 8$ independent experiments. * $P < 0.05$ relative to baseline, [b] $P < 0.05$ relative to IBMX. For ANOVA, $P < 0.005$, difference among group means was significant.

13, $P < 0.01$; 10^{-5} M: 67.5 ± 17.6 pg/mg, $n = 13$, $P < 0.01$) (Fig. 4). In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M), this stimulatory effect of forskolin was further augmented (forskolin 10^{-6} M + IBMX 10^{-4} M: 77.0 ± 17.8 pg/mg, $n = 13$, $P < 0.01$; forskolin 10^{-5} M + IBMX 10^{-4} M: 101.0 ± 19.7 pg/mg, $n = 13$, $P < 0.01$). The phosphodiesterase inhibitor alone also increased release of somatostatin-like immunoreactivity to 46.0 ± 12.2 pg/mg, without reaching statistical significance (Fig. 4).

3.5. Effect of N^6,2′-O-dibutyryl-cAMP (dbcAMP) on somatostatin release

The stable and membrane permeable analogue of cAMP, N_6,2′-O-dibutyryl-3′,5′-cyclic monophosphate (dbcAMP),

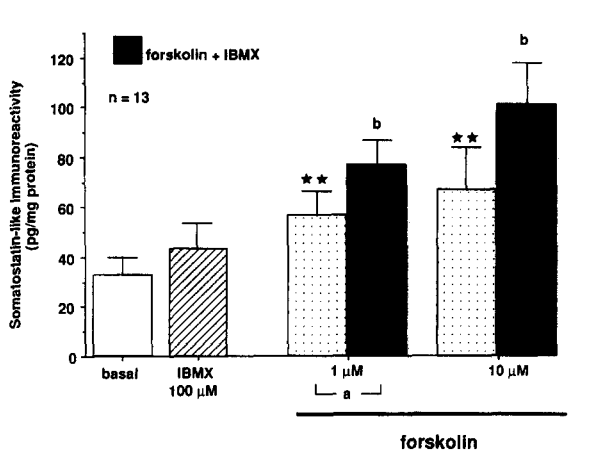

Fig. 4. Effect of forskolin on the release of somatostatin-like immunoreactivity from enteric synaptosomes in the absence and presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M). Values are expressed as means ± S.E.M. of $n = 13$ independent experiments. ** $P < 0.01$ relative to baseline, [b] $P < 0.05$ relative to IBMX. For ANOVA, $P < 0.05$, difference among group means was significant.

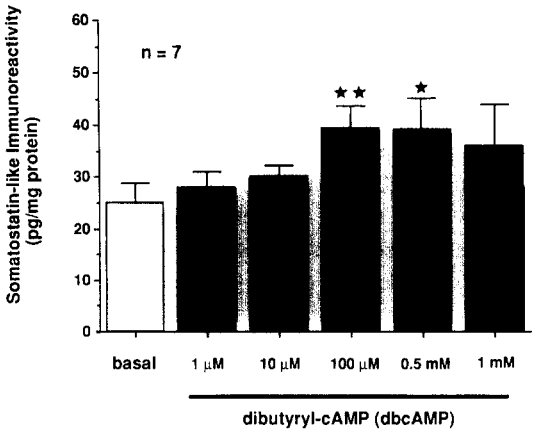

Fig. 5. Effect of N^6,2′-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate (dbcAMP) on the release of somatostatin-like immunoreactivity from enteric synaptosomes. Values are expressed as means ± S.E.M. of $n = 5$ independent experiments. ** $P < 0.01$, * $P < 0.05$ relative to baseline. For ANOVA, $P < 0.05$, difference among group means was significant.

had a slight but significant stimulatory effect on the release of somatostatin-like immunoreactivity when compared to baseline (basal: 27.0 ± 2.0 pg/mg, dbcAMP 10^{-4} M: 39.4 ± 4.4 pg/mg, $n = 7$, $P < 0.01$), higher concentrations had no additional effect on release of somatostatin-like immunoreactivity (dbcAMP 5×10^{-3} M: 39.2 ± 10.4 pg/mg, $n = 7$, $P < 0.05$). Lower concentrations of 10^{-5} to 10^{-6} M dbcAMP had no significant effect on release of somatostatin-like immunoreactivity (Fig. 5).

3.6. Effect of NO-related substances on somatostatin release

The NO agonist sodium nitroprusside (10^{-7} to 10^{-4} M) had no significant stimulatory effect on release of somatostatin-like immunoreactivity (basal: 18.7 ± 2.1 pg/mg, 10^{-7} M: 21.2 ± 3.5 pg/mg, 10^{-6} M: 23.7 ± 3.3 pg/mg, 10^{-5} M: 23.6 ± 1.9 pg/mg, 10^{-4} M: 26.4 ± 5.2 pg/mg, $n = 10$). When the effect of sodium nitroprusside was tested in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX 10^{-4} M), there was a small increase of somatostatin release, which was, however, not different from the increase in somatostatin release obtained with 3-isobutyl-1-methylxanthine alone (basal 18.7 ± 2 pg/mg, IBMX 10^{-4} M: 24.3 ± 3.2 pg/mg, IBMX + sodium nitroprusside 10^{-7} M: 23 ± 3.9 pg/mg, IBMX + sodium nitroprusside 10^{-6} M: 28.6 ± 3.3

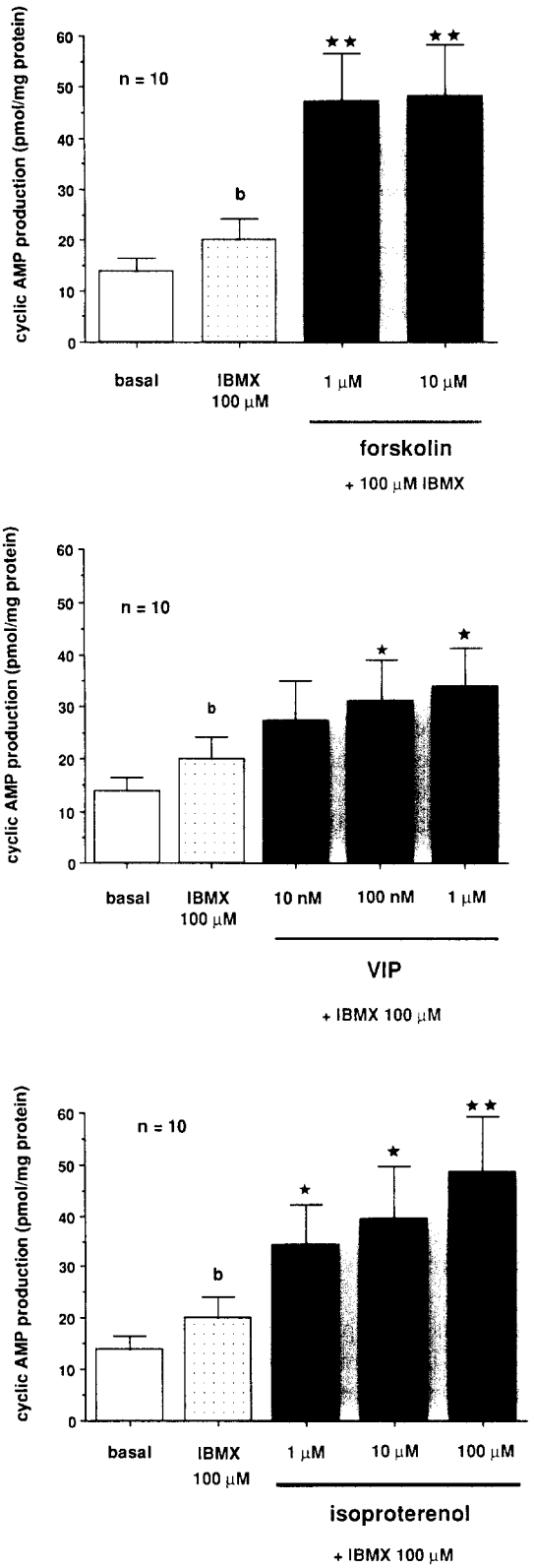

Fig. 6. (Top panel) Effect of forskolin on the generation of cAMP in enriched enteric synaptosomes. Values are expressed as means ± S.E.M. of $n = 10$ independent experiments. ** $P < 0.01$ relative to IBMX, ^{b} $P < 0.01$ relative to basal. For ANOVA, $P < 0.005$, difference among group means was significant. (Middle panel) Effect of VIP on the generation of cAMP in enriched enteric synaptosomes. Values are expressed as means ± S.E.M. of $n = 10$ independent experiments. * $P < 0.05$ relative to IBMX, ^{b} $P < 0.01$ relative to baseline. For ANOVA, $P < 0.01$, difference among group means was significant. (Bottom panel) Effect of isoproterenol on the generation of cAMP in enriched enteric synaptosomes. Values are expressed as means ± S.E.M. of $n = 10$ independent experiments. ** $P < 0.01$, * $P < 0.05$ relative to IBMX, ^{b} $P < 0.01$ relative to basal. For ANOVA, $P < 0.005$, difference among group means was significant.

pg/mg, IBMX + sodium nitroprusside 10^{-5} M: 28.1 ± 5.0 pg/mg, IBMX + sodium nitroprusside 10^{-4} M: 24.4 ± 3.1 pg/mg). Neither blockade of endogenous NO synthesis by the NO synthase inhibitor N^G-nitro-L-arginine (L-NNA) (basal: 18.7 ± 2.1 pg/mg, 5×10^{-4} M: 22.6 ± 3.8 pg/mg, $n = 10$), nor the substrate for NO synthesis L-arginine (10^{-3} M: 20.3 ± 1.6 pg/mg, $n = 10$), nor the combination of L-arginine and L-NNA (31.7 ± 9.9 pg/mg, $n = 10$) had any significant effect on the release of somatostatin-like immunoreactivity.

3.7. Effect of 8-bromoguanosine-3',5'-cyclic monophosphate on somatostatin release

The membrane permeable analogue of cGMP, 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP) (10^{-7} to 10^{-4} M), had no significant effect on the somatostatin release from synaptosomes (basal: 30.1 ± 2.3 pg/mg; 10^{-7} M: 29.2 ± 1.4 pg/mg, 10^{-6} M: 27.1 ± 2.7 pg/mg, 10^{-5} M: 27.8 ± 2.7 pg/mg, 10^{-4} M: 23.0 ± 10.6 pg/mg, $n = 10$).

3.8. Effect of VIP, isoproterenol and forskolin on cAMP levels

To demonstrate the stimulation of the adenylate cyclase, cAMP levels were measured in the presence of 3-isobutyl-1-methylxanthine and an unspecific activator (forskolin) and receptor-mediated activators (VIP and isoproterenol) of adenylate cyclase. cAMP accumulation was significantly increased in the presence of forskolin (10^{-6} M: 47.1 ± 12.4 pmol/mg, $n = 10$, $P < 0.01$; 10^{-5} M: 48.1 ± 10.5 pmol/mg, $n = 10$, $P < 0.05$) (Fig. 6, top panel) and VIP (VIP 10^{-7} M: 34.0 ± 8.2 pmol/mg; VIP 10^{-6} M: 37.1 ± 9.4 pg/mg, $n = 10$, $P < 0.05$) compared to baseline 19.8 ± 4.2 pmol/mg (Fig. 6, middle panel). VIP at lower concentration (10^{-8} M) had no stimulatory effect on cAMP accumulation. Isoproterenol also significantly stimulated cAMP immunoreactivity when compared to baseline (basal: 16.7 ± 2.4 pg/mg; isoproterenol 10^{-6} M: 37.6 ± 8.2 pmol/mg, $n = 12$, $P < 0.05$; isoproterenol 10^{-5} M: 42.4 ± 9.8 pmol/mg, $n = 12$, $P < 0.05$; isoproterenol 10^{-4} M: 48.4 ± 12.2 pg/mg, $n = 12$, $P < 0.01$) (Fig. 6, bottom panel).

4. Discussion

The results of the present study clearly demonstrate that somatostatin-like immunoreactivity can be released from enriched enteric synaptosomes by VIP, secretin and isoproterenol, but not by glucagon. Furthermore, forskolin, addition of the stable cAMP analogue N^6,2'-*O*-dibutyryladenosine-3',5'-cyclic monophosphate or even 3-isobutyl-1-methylxanthine evoke the release of somatostatin-like immunoreactivity.

In the gastrointestinal tract, somatostatin-like immunoreactivity is released from the pancreas and the stomach following stimulation with VIP (Ipp et al., 1978; Chiba et al., 1980; Saffouri et al., 1984; Schusdziarra et al., 1986), isoproterenol (Hermansen et al., 1979), glucagon (Chiba et al., 1980) and secretin (Chiba et al., 1980; Ipp et al., 1977). However, tissue extraction studies revealed that somatostatin released from the gastrointestinal tract is predominantly of endocrine origin (Penman et al., 1983). The mechanisms of somatostatin release from neuronal elements have been studied in isolated enteric ganglia (Grider, 1989), cultured submucous ganglia (Accili et al., 1993) and cultured enteric submucosal neurons (Buchan et al., 1990). As VIP represents a potential candidate for the non-adrenergic non-cholinergic inhibitory transmitter in the enteric nervous system and both, somatostatin and VIP are released during propulsive complexes in isolated ileum (Schmidt et al., 1993), it is of considerable interest whether there is functional connection between somatostatin- and VIP-containing neurones in the gut. In preparations of isolated ganglia of the myenteric plexus, VIP abolished the increase of somatostatin release induced by dimethylphenylpiperazinium. It was speculated that this is due to a regulatory feedback control of somatostatin by VIP (Grider, 1989). This result is not in contrast to the stimulatory effect of VIP on release of somatostatin-like immunoreactivity demonstrated in our study, as in synaptosomes the neuronal circuitry is disrupted. Isolated ganglia leave the synaptic connection intact which predisposes to a modulatory action of interneurons. In the central nervous system, VIP has been shown to increase cAMP production and to release somatostatin from rat diencephalic cells (Pares-Herbute et al., 1989; Tapia-Arancibia et al., 1988) and rat cerebral cortical cells (Tapia-Arancibia et al., 1988). Recently, VIP receptors have been demonstrated on enriched enteric synaptosomes from the myenteric plexus, submucosal plexus and deep muscular plexus of the canine small intestine using receptor binding studies. VIP receptor distribution in the small intestine suggested the predominant localization of VIP receptors on presynaptic nerve membranes (Mao et al., 1991). From synaptosomal preparations of the superior cervical ganglion (Takahashi et al., 1992) and myenteric plexus (Yau et al., 1985), there is good experimental evidence for a neuromodulatory role of VIP at the presynaptic site of myenteric neurons. Evidence for a functional connection between somatostatin-containing neurons and VIP-ergic neurons in the enteric nervous system is the supported by the observation that secretin, a hormon which shares structure homologies with VIP, significantly increased somatostatin release in our preparation. The fact that glucagon failed to stimulate release of somatostatin could be due to a low affinity of glucagon at the two binding sites which have been demonstrated to be present on enteric synaptosomes. In contrast, secretin revealed a similar order of affinity as VIP at both receptors (Mao et al., 1991).

Other neuromodulators known to act at the presynaptic site to produce inhibition of both electrical and mechanical activity of gastrointestinal smooth muscle are β-adrenoceptor agonists. In our preparation, isoproterenol, a β-adrenoceptor agonist, evoked somatostatin release from enteric synaptosomes, suggesting the presence of a β-adrenoceptor at the presynaptic site. Norepinephrine, which partially acts as β-adrenoceptor agonist had no effect on release of somatostatin from cultured cortical cells at concentrations from 100 pM to 10 μM (Robbins et al., 1982), however, there are also reports that high concentrations of norepinephrine were stimulatory in cortical synaptosomes (Bennett et al., 1979) and in hypothalamic preparations (Negro-Vilar et al., 1978). Taken together the stimulatory effect of VIP, isoproterenol and secretin on somatostatin release could contribute to their inhibitory effect on gastrointestinal motility reported from previous studies.

NO is present in neurons of the enteric nervous system (Costa et al., 1992) and is suggested to be a non-adrenergic non-cholinergic transmitter, mediating smooth muscle relaxation by activation of cGMP-dependent protein kinase (Christinck et al., 1991). Furthermore, the mode of somatostatin activation of intestinal motor activity seems to depend more on inhibition of NO than of VIP output (Vergara et al., 1995). Thus, it was interesting whether NO exerts a modulatory action on somatostatin release from synaptic nerve terminals. L-Arginine, the precursor of NO, has been shown to be a potent stimulus of somatostatin release from the ileum (Hermansen, 1985), whereas it showed no effect on somatostatin release in the central nervous system (Robbins and Landon, 1983). We have demonstrated that neither the NO-generating compound sodium nitroprusside nor 8-bromoguanosine-3′,5′-cyclic monophosphate did influence the release of somatostatin-like immunoreactivity. Thus, it is suggested that release of somatostatin from enteric neurons presumably is not stimulated by NO involving cGMP-dependent mechanisms.

Since β-adrenoceptor agonists and VIP appear to inhibit gastrointestinal muscles by a mechanism involving cAMP (Smith et al., 1993) and both N^6,2′-*O*-dibutyryl-3′,5′-cyclic monophosphate (dbcAMP) and 3-isobutyl-1-methylxanthine mimicked the stimulatory effects of VIP and isoproterenol on somatostatin release in our preparation there was good evidence for a receptor coupling to adenylate cyclase in enteric neurons. Measurements of cAMP levels showed that VIP and isoproterenol produce significant increases in the levels of cAMP in enteric synaptosomes. Additionally, forskolin, a direct activator of adenylate cyclase, evoked a significant release of somatostatin and was demonstrated to stimulate cAMP accumulation in the synaptosomes. The amount of basal cAMP production and the stimulatory effect of forskolin reported in our study was lower than the previously reported cAMP production and the stimulatory effect on isolated myenteric ganglia (Baidan et al., 1992; Xia et al., 1991) or cultured cerebral cortical cells (Tapia-Arancibia et al., 1988). However, the cAMP levels are in good agreement with other cAMP measurements in isolated enriched canine enteric synaptosomes (Barnett et al., 1990). In this preparation, 10^{-5} M forskolin led to a 3-fold increase of cAMP levels and VIP caused an increase of cAMP levels with a threshold at 10^{-8} to 10^{-7} M. Thus, there is clear evidence that enteric synaptosomes are capable of accumulating cAMP. Taken together with the stimulatory effect of VIP on somatostatin release in our study, these data suggest that VIP might be coupled to a cAMP-generating system as a second messenger in the enteric nervous system. There is good experimental evidence for the involvement of cAMP as a second messenger in the neuronal signal transduction of the enteric nervous system, i.e., of slow synaptic potentials (Palmer et al., 1986). Elevation of intraneuronal cAMP by intracellular injection of cAMP or treatment with membrane permeable cAMP, phosphodiesterase inhibitors or forskolin mimicked a slow excitatory post-synaptic potential in myenteric neurons (Nemeth et al., 1986; Palmer et al., 1986; Zafirov et al., 1993). The induction of slow excitatory post-synaptic potentials almost exclusively occurred in AH/Type 2 cells and combined immunohistochemical-electrophysiological studies demonstrated that these cells contain most of the enteric somatostatin (Daniel et al., 1985). Furthermore, forskolin and cAMP were shown to increase transmitter release, i.e., acetylcholine or neurotensin, from isolated myenteric ganglia (Yau et al., 1987) or enteric cultured neurons (Barber et al., 1989).

In contrast, despite its stimulatory effect on cAMP levels, forskolin had no significant effect on somatostatin release from cultured cortical cells (Tapia-Arancibia et al., 1988) or cultured neurons of the canine ileum (Buchan et al., 1990). Therefore, it was speculated that cAMP increase alone is not sufficient to stimulate somatostatin release and VIP regulates somatostatin release by a cAMP-independent mechanism. The 'cross-activation' phenomenon between second messenger substances could be a possible explanation for this observation. Forskolin (1 and 10 μM) was shown to activate the cGMP-dependent protein kinase in pig coronary arteries (Jiang et al., 1992). This subsequently could alter neurotransmitter release, e.g. by stimulating the release of an inhibitory interneuron from cultured neurons. This could also explain the findings in our experiments, where the combined effect of VIP and 3-isobutyl-1-methylxanthine was not significantly different from that obtained with VIP alone. From previous studies, there is evidence for the involvement of protein kinase C in the release process of somatostatin from enteric nerves (Baidan et al., 1992; Accili et al., 1993). The mechanisms responsible for the observation that higher concentrations of N^6,2′-*O*-dibutyryladenosine-3′,5′-cyclic monophosphate did not further stimulate somatostatin release from our enteric synaptosomes also remain to be clarified. Whether this is due to a 'cross-activation' phenomenon or a negative feedback control mediated by the released somatostatin

modifying intracellular mechanisms as demonstrated in cultured diencephalic cells (Pares-Herbute et al., 1989) could not be answered in our experiments. To what extent, another transmembrane-signalling system independent of cAMP contributes to the effect of VIP, secretin and isoproterenol on somatostatin release from enteric synaptosomes remains to be determined.

In summary, this study demonstrates that somatostatin immunoreactive material can be released from enriched enteric synaptosomes by VIP, isoproterenol or secretin presumably involving a cAMP-dependent mechanism. The results confirm the potential of intrinsic enteric somatostatinergic neurons to respond to endogenous neurotransmitters and further establishes somatostatin as an enteric neuromodulator. The observed presynaptic modulatory action of VIP, secretin and isoproterenol could contribute to their inhibitory effect on gastrointestinal motility observed in vivo and in vitro.

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