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Bombesin-Stimulated Acetylcholine Release from Myenteric Plexus Neurons[1]

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The ability of bombesin to stimulate acetylcholine release from guinea pig myenteric plexus neurons was studied using a primary neuronal culture system. Bombesin caused dose-dependent increases in [^{3}H]-acetylcholine (ACh) release from guinea pig myenteric plexus neurons. ACh release in response to 0.5 m*M* bombesin (160 ± 12% of control) was blocked by exposure to a calcium-free medium (116 ± 13%) by nifedipine (101 ± 11%) and by omega conotoxin (107 ± 10%). Bombesin-stimulated ACh release was inhibited the protein kinase C inhibitor, H7, but was not affected by inhibitors of the cAMP signaling pathway. Interactions with inhibitory neuropeptides was implied by sensitivity of bombesin-stimulated ACh release to neuropeptide Y and galanin. The findings suggest that bombesin activation of protein kinase C in myenteric neurons results in increased acetylcholine release. © 1993 Academic Press, Inc.

INTRODUCTION

Bombesin is an important peptide neurotransmitter in the enteric nervous system. Bombesin-positive nerve fibers have been demonstrated immunocytochemically in myenteric ganglia of the guinea pig ileum and taenia coli, and electrophysiologic studies have revealed bombesin receptors on myenteric neurons [4, 17, 36]. *In vitro,* bombesin stimulates ileal contractility, with effects exerted by both myogenic and neural mechanisms [15, 20, 23]. Pharmacologic study of ileal longitudinal muscle strips, prepared with attached myenteric plexus, suggests that the neural actions of bombesin are mediated by a predominantly cholinergic mechanism [34]. While bombesin has been demonstrated to release acetylcholine from the guinea pig antrum [19], direct evidence that bombesin releases acetylcholine from myenteric plexus neurons has been lacking.

Recently, primary cultures of guinea pig myenteric plexus neurons have been used in investigations of the enteric nervous system. The preparation is useful because it retains a number of differentiated structural and functional characteristics observed *in situ* [1, 2, 12]. Specifically, cultured myenteric neurons have been used to investigate control of cholinergic transmission and have been demonstrated to release acetylcholine (ACh) in response to low concentrations of peptidergic neurotransmitters [24, 25]. In the current study we sought to: (1) examine the release of [^{3}H]ACh from myenteric neurons in primary culture stimulated by bombesin; (2) study the signal transduction pathways involved in bombesin-stimulated ACh release; and (3) observe interactions of bombesin with other inhibitory myenteric neuropeptides.

MATERIALS AND METHODS

Materials. The following materials were purchased: hemicholinium, collagenase, physostigmine, penicillin, streptomycin (Sigma Chemical, St. Louis, MO); bombesin, somatostatin, galanin, neuropeptide Y (NYP) poly-L-lysine (Peninsula Labs, Belmont CA); medium 199 (GIBCO, Grand Island, NY). Neonatal Duncan–Hartley guinea pigs were purchased from Simonsen Labs (Gilroy, CA). Tritiated choline was obtained from New England Nuclear (Boston, MA). R_p-cAMPS was purchased from BioLab (La Jolla, CA).

Methods. Acetylcholine release was examined using myenteric plexus neurons in primary culture. Plexus explants were obtained for culture by a previously published method [18, 31]. The taenia coli from 2-day-old male Duncan–Hartley guinea pigs was dissected aseptically, divided into segments, and placed in Hanks' balanced salt solution (HBSS) containing collagenase (1 mg/ml). Muscle strips were incubated for 12 h at 4°C and then for 30 to 60 min at 37°C. Tissue fragments were then transferred to fresh HBSS without collagenase and loosely adherent smooth muscle fibers were removed from neuronal plexuses using a dissecting microscope.

The tissues of each animal were divided into 12 aggregates, and the explants were placed into culture wells that had been previously coated with poly-L-lysine (10 μg/ml). Nerves were exposed to culture medium con-

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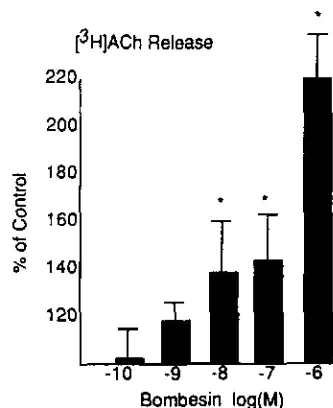

FIG. 1. Tritiated ACh release in response to graded concentrations of bombesin. Data expressed as means ± SEM; $n = 8$ for each group. $^*P < 0.05$ vs control incubation.

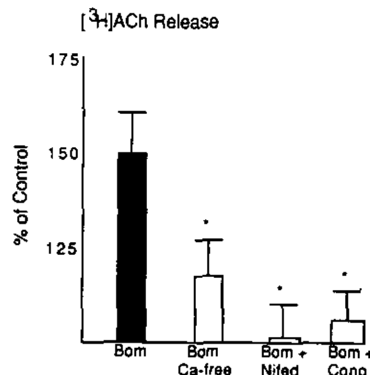

FIG. 2. [^{3}H]ACh release stimulated by bombesin (5×10^{-7} *M*) in the presence of control medium (Bom), calcium-free medium (1 m*M* EGTA), or control medium containing nifedipine (Nifed; 10^{-5} *M*) or omega conotoxin (Cono; 5×10^{-6} *M*). For each group, n = 6 or greater. $^*P < 0.05$ vs bombesin alone.

taining 10% fetal calf serum in addition to medium 199 (GIBCO), non-essential amino acids (0.1 m*M*), sodium pyruvate (1 m*M*), Hepes buffer (1 m*M*), L-glutamine (2 m*M*), and glucose (5 mg/ml). Initial culture medium contained penicillin/streptomycin (100 U/ml); antibiotics were omitted after the second day. Culture medium was changed every second day.

Studies examining ACh release were performed with plexuses after 5 to 7 days in culture. All plexuses were firmly adherent and surrounded with an extensive outgrowth zone of neurite processes. ACh release was examined using [^{3}H]ACh as a marker. Plexuses were exposed to [^{3}H]choline (0.2 μM, sp act 80 Ci/mmol) in oxygenated Krebs–Ringer buffer containing 50 μM physostigmine for 60 min at 37°C. The labeled tissues were washed twice with buffer and allowed to recover for 30 min before exposure to agonists. Each plexus was exposed to only one agonist.

Total ^{3}H release was measured and used to indicate transmitter release. The release medium contained 118 m*M* NaCl, 4.7 m*M* KCl, 1 m*M* NaH_2PO_4, 0.5 m*M* $MgCl_2$, 2.5 m*M* $CaCl_2$, 25 m*M* $NaHCO_3$, 11 m*M* glucose, 50 μM physostigmine, and 10 μM hemicholinium. Tissues were exposed to agonists in release medium for 15 min. The medium was then aspirated and added to scintillation fluid. The remaining tissue was solubilized, added to scintillation fluid, and counted separately. Fractional release of ACh was calculated by expressing the radioactivity in the release medium as a percentage of the total radioactivity present in the tissue at the time of the experiment. Percentage release of [^{3}H]ACh over basal induced by various agonists was calculated by dividing the fractional release caused by the agonist by the basal release. Greater than 90% of released ^{3}H was in the form of ACh, as determined by separation on an ion exchange column.

Statistical analysis. Results are expressed as group means ± SEM. Sample size was taken as the number of animals from which cultures were derived. Two-way analysis of variance was used for examination of the data, and when significant differences were noted ($P <$ 0.05), post hoc testing was performed to ascertain differences between groups.

RESULTS

Bombesin caused dose-dependent increases in [^{3}H]-ACh release from myenteric plexus neurons in primary culture (Fig. 1). Significant increases were noted at concentrations of 10^{-8} *M* and greater. The ability of bombesin to release ACh was dependent upon extracellular calcium, as bombesin-stimulated (5×10^{-7} *M*) [^{3}H]ACh release was inhibited by exposure to the peptide in a calcium-free medium (1 m*M* EGTA) (Fig. 2). Bombesin-stimulated [^{3}H]ACh release was abolished by coincubation with the calcium channel blocker nifedipine (10^{-5} *M*) and significantly inhibited by omega conotoxin coincubation (5×10^{-6} *M*).

Bombesin-stimulated (5×10^{-7} *M*) [^{3}H]ACh release was not affected by coincubation with dideoxyadenosine (DDA) or R_p-cAMPS (Fig. 3). DDA is a soluble cogener

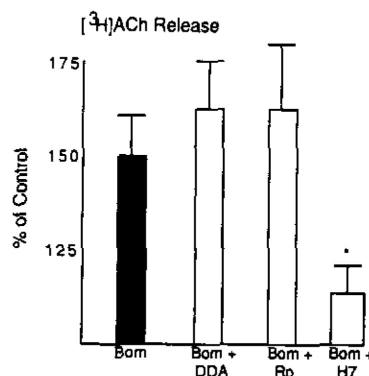

FIG. 3. [^{3}H]ACh release stimulated by bombesin (5×10^{-7} *M*) in the presence of control release medium (Bom), or medium containing dideoxyadenosine (DDA; 10^{-4} *M*), R_p-cAMP (10^{-4} *M*), or H7 (10^{-5} *M*). n = 8. $^*P < 0.05$ vs bombesin alone.

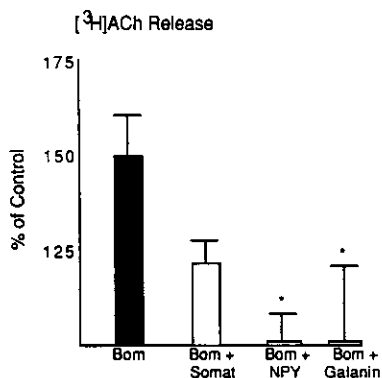

FIG. 4. Effects of inhibitory neuropeptides somatostatin, neuropeptide Y (NPY), and galanin (each 10^{-8} *M*) on [^{3}H]ACh release stimulated by bombesin (0.5 m*M*). *$P < 0.05$ vs bombesin alone; $n = 8$.

of cyclic AMP that binds to adenylate cyclase and inhibits formation of cAMP [32]. R_p-cAMPS is an diastereomer of cAMP that acts as an inhibitor of cAMP-dependent protein kinases [3, 29, 30]. In contrast, bombesin-stimulated ACh release was nearly abolished when plexuses were exposed to H7, an inhibitor of protein kinase C activity [14, 16, 27].

Bombesin stimulation of ACh release from myenteric neurons was sensitive to low concentrations of inhibitory neuropeptides (Fig. 4). Coincubation of neuropeptide Y or galanin (each 10^{-8} *M*) abolished release of [^{3}H]-ACh stimulated by 0.5 μM bombesin. Coincubation with somatostatin (10^{-8} *M*) did not achieve statistical significance.

DISCUSSION

The current studies demonstrate that bombesin is a potent stimulant for acetylcholine release from myenteric plexus neurons. Cholinergic stimulation was dose-dependent and significant effects were noted at peptide concentrations approximating 10 n*M*. Similar concentrations have been noted by other investigators to stimulate contractile responses in guinea pig ileal longitudinal smooth muscle [34] and to release ACh from guinea pig antrum [19]. However, because the current *in vitro* preparation is extrinsically denervated, alterations in responsiveness to agonist are possible and have not been excluded. Stimulation of acetylcholine release required the presence of extracellular calcium and could be inhibited by nifedipine or omega conotoxin. The latter findings imply that bombesin effects may be mediated by L-type calcium channels [35]. Guo *et al.* have reported that nifedipine blocks bombesin-stimulated release of gastrin and somatostatin from the perfused rat stomach [11]. Nifedipine has also been shown to block bombesin activation of L-type voltage-dependent Ca^{2+} channels in rat pancreatic acinar cells [9]. The stimulatory actions of bombesin were also inhibited by coincubation with galanin or NPY, indicating potential interactions with inhibitory neurotransmitters present within the myenteric plexus. Galanin and NPY have been demonstrated previously to suppress ACh release from myenteric neurons stimulated by vasoactive intestinal polypeptide, cholecystokinin octapeptide, or calcitonin gene-related peptide [26].

The ability of bombesin to stimulate ACh release was not affected by DDA or R_p-cAMPS coincubation. Taken together, these findings indicate that the cAMP second messenger system is not involved in transduction of cholinergic stimulation by bombesin. DDA has been reported to inhibit accumulation of intracellular cAMP by binding to adenylate cyclase [32]. R_p-cAMPS is a cAMP-dependent protein kinase antagonist which competes with cAMP on the regulatory subunit without causing dissociation of the holoenzyme [3, 29, 30].

In contrast, bombesin stimulation of ACh release was blocked by H7, an inhibitor of protein kinase C. Bombesin has previously been demonstrated to activate protein kinase C in a number of cell lines. In pancreatic HIT cells, membrane-associated protein kinase C activity is increased with exposure to bombesin, and bombesin incubation is associated with a burst in insulin secretion [28, 33]. In Swiss 3T3 cells, glucose transport and phosphorylation of intracellular protein substrates is stimulated by bombesin via protein kinase C activation [5, 7]. To our knowledge, the current study is the first demonstration that bombesin causes protein kinase C activation in myenteric neurons.

The effect of protein kinase C activation in neurons is not well understood and may depend both upon the tissue examined and the state of membrane polarization at the time of study. Gross and Macdonald have reported phorbol ester activation of protein kinase C causes decreases in peak calcium currents in cultured sensory neurons, when examined near resting membrane potential [10]. During membrane hyperpolarization, inhibitory effects were not observed. Protein kinase C activators have also been reported to inhibit Ca^{2+} currents in dorsal root ganglia and in hippocampal neurons [6, 8]. In contrast, in cultured sympathetic neurons, net accumulation of $^{45}Ca^{2+}$ during electrical stimulation was increased in the presence of active phorbol esters and was associated with a marked increase in norepinephrine release [21]. Protein kinase C activation has been reported to enhance depolarization-stimulated release of norepinephrine from PC12 cells [22] and K^+-stimulated release of norepinephrine and acetylcholine from guinea pig enteric neurons [13]. The current study suggests that bombesin-stimulated activation of protein kinase C in myenteric neurons has, as its net effect, increased acetylcholine release.

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