Mechanisms for Muscarinic Inhibition of Somatostatin Release From Canine Fundic D Cells

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We undertook the present studies to explore the mechanisms by which carbachol inhibits the release of somatostatin-like immunoreactivity (SLI) from D cells. D cells were isolated from canine fundic mucosa by collagenase/EDTA dispersion followed by counterflow elutriation. Carbachol inhibited the release of SLI induced by forskolin, dibutyryl 3':5' cyclic adenosine monophosphate (cAMP), pentagastrin (PG), and 12-0-tetradecanoyl-phorbol-13-acetate in a fashion that could be prevented by pertussis toxin (PT) pretreatment of the D cells. Pertussis toxin also prevented the carbachol-induced inhibition of forskolin-stimulated cAMP generation and PG-stimulated $[Ca^{2+}]i$ mobilization. These data indicate that pertussin toxin sensitive inhibitory guanine nucleotide binding proteins mediate many of carbachol's inhibitory actions on D cells.
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MUSCARINIC cholinergic agonists inhibit somatostatin release from gastric D cells.[1] Since the inhibitory action of muscarinic agonists in other systems is associated with decreased cellular 3':5' cyclic adenosine monophosphate (cAMP) production that is preventable by pertussis toxin (PT) pretreatment, muscarinic inhibition is thought to be mediated, at least in part, by inhibition of adenylate cyclase via the action of an inhibitory guanine nucleotide binding protein (Gi).[2-4] However, we have observed in D cells that carbachol also inhibits somatostatin release induced by various agents that do not affect adenylate cyclase,[5,6] suggesting the existence of mechanisms for muscarinic inhibition other than via inhibition of cAMP production. We undertook these studies to explore the potential mechanisms for this inhibitory action of muscarinic agents.

MATERIALS AND METHODS

Cell Isolation and Culture

Mucosal cells were dispersed from stripped canine fundus by sequential exposure to collagenase (0.35 mg/mL) and EDTA (1 mmol/L), and somatostatin containing D cells were enriched by centrifugal elutriation as described previously.[1,5-7] After 40 hours of culture on a bed of collagen in Ham's F12-Dulbecco's modified Eagle's medium (HF12/DMEM) (50:50, vol/vol) (Irvine Scientific, Santa Ana, CA) containing 10% dog serum, the cells (4×10^6/well) were incubated in Earle's balanced salt solution (EBSS) containing various test agents for 2 hours. Somatostatin-like immunoreactivity (SLI) that was released into the media was measured by specific radioimmunoassay.[8] Some experiments were performed after the cells had been preincubated with PT (200 ng/mL) for 4 hours.

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Production of cAMP

For measurement of cAMP production, the incubations were terminated after only 15 minutes by adding chilled trichloroacetic acid to achieve a final concentration of 5%. Following ether extraction, cAMP content was measured by competitive protein binding assay.[9]

Protein Kinase C Activity

After 40 hours of culture, D cells were dispersed with trypsin (0.05%) and EDTA (0.02%) and allowed to stabilize for 4 hours in HF12/DMEM. Subsequently, the cells (10^7) were incubated in 3 mL of EBSS with various test agents for 5 minutes at 37°C, and the reactions were stopped by adding 4 mL of ice cold EBSS. After washing, the cells were suspended in 20 mmol/L Tris/HCl, 0.25 mol/L sucrose, 10 mmol/L EGTA, and 2 mmol/L EDTA, pH 7.5, vortex-sonicated, and centrifuged at $10,000 \times g$ for 30 minutes at 2°C; the supernatant was used as the particulate or membrane fraction. These fractions were applied to a DE52 column (Sigma, St Louis, MO) and protein kinase C was then eluted with 20 mmol/L Tris/HCl containing 1 mmol/L EDTA, 1 mmol/L EGTA, 50 mmol/L 2-mercaptoethanol, and 100 mmol/L NaCl as described previously.[10,11] Fractions were assayed by measuring their ability to catalyze the phosphorylation of histone H3S using (α-^{32}P) adenosine triphosphate (ATP) as a substrate at 0.5 mmol/L $CaCl_2$ in the presence or absence of 50 μg/mL phosphatidylserine and 1 μg/mL diolein. The difference in incorporation of radioactivity was used to calculate phosphatidylserine and diolein-dependent histone kinase activity.

$[Ca^{2+}]$ Measurement

D cells that were dispersed after 40 hours of culture were suspended in modified Krebs-Ringer bicarbonate buffer (5 mmol/L glucose, 118 mmol/L NaCl, 3.0 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.2 mmol/L NaH_2PO_4, 1.5 mmol/L $CaCl_2$, and 23 mmol/L $NaHCO_3$, pH 7.4) at a density of 2.5×10^6 cells/mL. The cells were loaded with Fura 2-AM (10 μmol/L) (Molecular Probes, Eugene, OR) at 37°C for 30 minutes. The loaded cells were rinsed twice and resuspended at 10^7 cells/mL in buffer then placed in a square quartz cuvette. Intracellular Fura 2 fluorescence (excitation 340 nm and 380 nm, emission 500 nm) was measured, and the ratio, obtained by dividing the fluorescence values at 340 nm (I_{340}) by those at 380 nm (I_{380}), was recorded with a CAF-1000 Ca^{2+} analyzer (Jasco Co, Tokyo, Japan) at 37°C under oxygenated conditions. Autofluorescence was negligible. $[Ca^{2+}]$ was calculated using the following equation, as described by Grynkiewicz et al[12]: $[Ca^{2+}]i = Kd \times (R - Rmin)/(Rmax - R) \times Sf/Sb$ (R = the ratio of fluorescence intensity 340 nm to 380 nm; Rmax = I_{340}/I_{380} at a saturated Ca^{2+} after addition of 50 μmol/L digitonin;

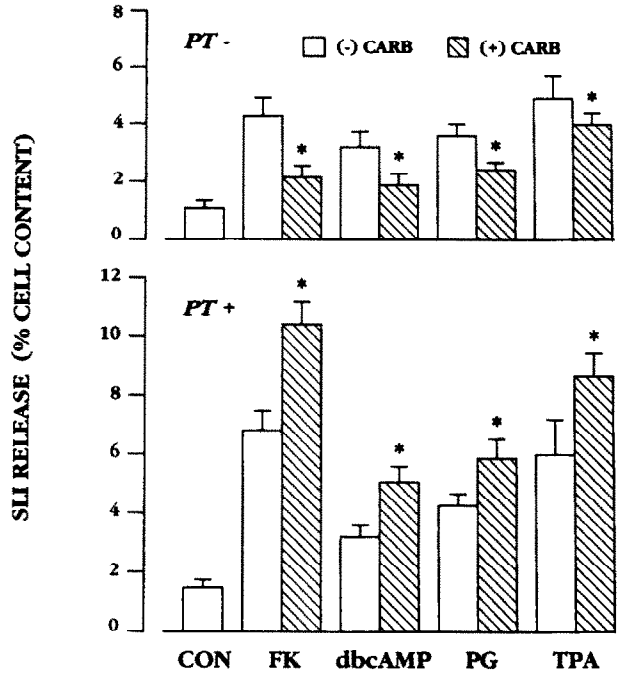

Fig 1. Effects of PT (200 ng/mL) pretreatment on inhibition of forskolin (FK, 10^{-4} mol/L), dibutyryl cAMP (dbcAMP, 10^{-4} mol/L), TPA (10^{-7} mol/L) and PG (10^{-7} mol/L)-induced SLI release by carbachol (CARB, 10^{-4} mol/L). Data are presented as means ± SE from four separate dog preparations. CON, Control. * $P < .05$ versus respective treatment group.

Rmin = I_{340}/I_{380} at virtually zero $[Ca^{2+}]i$ after addition of 10 mmol/L EGTA and 0.3 mol/L NaOH to cells made permeable with digitonin; K_d = the dissociation constant for Ca^{2+} binding to Fura 2; Sf = I_{380} at zero $[Ca^{2+}]i$; Sb = I_{380} at saturated $[Ca^{2+}]i$.

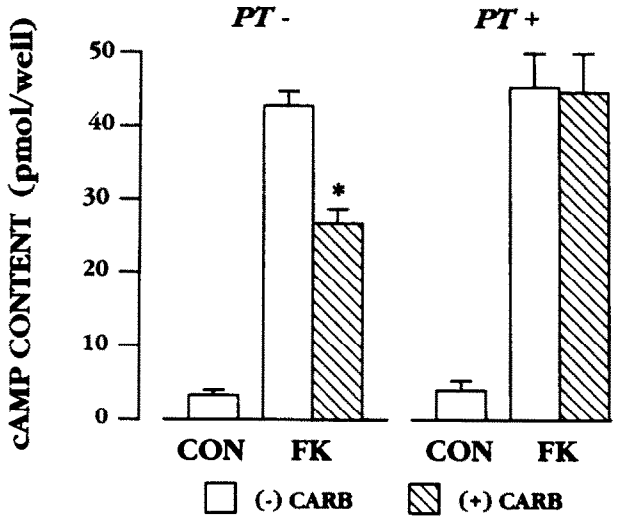

Fig 2. Effect of PT pretreatment on carbachol (CARB, 10^{-4} mol/L) inhibition of forskolin (FK, 10^{-4} mol/L)-induced cAMP production in D cells. Means ± SE from four separate experiments are shown. CON, Control. * $P < .01$ versus FK alone.

Table 1. Effects of Carbachol on PG- or TPA-Induced Increases in Membrane-Associated Protein Kinase C Activity

Agents	Carbachol (10^{-4} mol/L)	Membrane-Associated Protein Kinase C Activity (pmol/min · mg Protein)
Control	–	30.6 ± 3.5
	+	45.5 ± 3.6*
PG (10^{-7} mol/L)	–	54.5 ± 4.3*
	+	56.2 ± 4.0*
TPA (10^{-7} mol/L)	–	68.5 ± 6.8*
	+	65.6 ± 7.0*

NOTE. Values are means ± SE from four separate dog preparations.
* Significantly different from control ($P < .01$). No data obtained with carbachol were significantly different from their respective values with PG or TPA alone ($P > .05$).

RESULTS AND DISCUSSION

We observed that carbachol inhibited SLI release induced by forskolin but following PT pretreatment, carbachol potentiated the stimulatory effect of forskolin on SLI secretion (Fig 1). This unmasked stimulatory action of carbachol is of great interest but will not be discussed in this manuscript as it was the subject of a previous publication.[13] Since the ability of carbachol to inhibit forskolin-induced increases in cAMP production was blocked by PT pretreatment (Fig 2), muscarinic inhibition of SLI release appears to involve Gi-mediated inhibition of adenylate cyclase. However, our data indicate that the inhibitory action of muscarinic agonists on SLI secretion may also occur at a point distal to the production of cAMP since dibutyryl cAMP-induced SLI release itself could be inhibited by carbachol in a fashion preventable with PT (Fig 1). Moreover, we observed that stimulation of SLI release by pentagastrin (PG) and 12-0-tetradecanoyl-phorbol-13-acetate (TPA), processes that we have previously demonstrated to be mediated via membrane inositol phospholipid turnover and/or protein kinase C activation,[5] could also be inhibited by carbachol via a PT-sensitive pathway (Fig 1). Because stimulation of inositol phospholipid turnover is followed by the activation of protein kinase C and increases in $[Ca^{2+}]i$,[14,15] we measured the effect of carbachol on stimulant-induced protein kinase C activity and $[Ca^{2+}]i$ in D cells. We observed that although carbachol had no effect on PG- or TPA-induced increases in protein kinase C activity in the membrane (Table 1), it attenuated the increase in D cell $[Ca^{2+}]i$ induced by PG (Fig 3). Furthermore, after PT pretreatment, the inhibitory action of carbachol on the increase in $[Ca^{2+}]i$ by PG was abolished (Fig 3). Together with our previous data that PG-induced enhancement of inositol phospholipid turnover is not influenced by carbachol,[13] our study indicates that carbachol activates an inhibitory mechanism that predominates over the stimulatory action of protein kinase C on SLI secretion via mechanisms that have yet to be defined but presumably are distal to the phosphorylation of cellular proteins.

The potential sites for the inhibitory action of muscarinic agonists on D cells are numerous. Some inhibitory actions

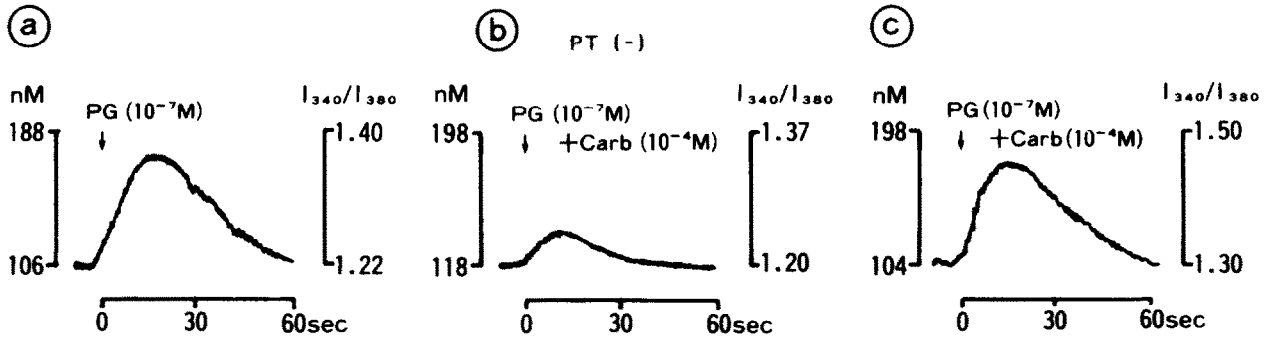

Fig 3. Effects of carbachol (CARB, 10^{-4} mol/L) on PG (10^{-7} mol/L)-induced increase in D cell $[Ca^{2+}]i$ in the presence of 1.5 mmol/L extracellular Ca^{2+} with or without PT (200 ng/mL) pretreatment for 4 hours. (a) PG alone without PT pretreatment; (b) PG plus carbachol without PT pretreatment; (c) PG plus carbachol with PT pretreatment. (Reprinted with permission.[18])

are mediated by Gi. These include inhibition of adenylate cyclase activity, inhibition of cAMP action, inhibition of effects resulting from protein kinase C activation, and inhibition of increases in $[Ca^{2+}]i$. Since carbachol's inhibitory action on TPA-stimulated SLI secretion cannot be ascribed to inhibition of $[Ca^{2+}]i$ mobilization or membrane-associated protein kinase C activity, it may result from action at some distal site at which various signal transduction pathways converge to stimulate D cells. The mechanisms for such an activity is unknown but may involve such basic processes as phosphoprotein dephosphorylation or ion channel gating, both of which are known to be G-protein–dependent actions of muscarinic agonists in other systems.[16,17]

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