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Carbachol mimics phorbol esters in its ability to enhance cyclic GMP production by STa, the heat-stable toxin of *Escherichia coli*

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STa, the heat-stable enterotoxin of *Escherichia coli*, stimulates membrane-bound guanylate cyclase in enterocytes, elevates cyclic GMP, and results in intestinal secretion of ions and fluid. Using the T84 colon carcinoma cell line as a model, Weikel et al. reported that phorbol esters enhance STa-stimulated cyclic GMP production by 60–140% [(1990) Infect. Immun. 58, 1402–1407]. In the present report we demonstrate that the acetylcholine analog carbachol enhanced toxin-stimulated cyclic GMP accumulation in intact T84 cells by 50–100% and that this effect was blocked by 10 μM atropine and 10 μM sphingosine. Pertussis toxin treatment of the T84 cells did not affect the subsequent response to carbachol. Carbachol, which elevates intracellular calcium in these cells, may act through protein kinase C to enhance cyclic GMP production.

Heat-stable enterotoxin; *Escherichia coli*; Guanosine 3′,5′-cyclic monophosphate; Phorbol ester; Carbachol; Protein kinase C; Muscarinic receptor

1. INTRODUCTION

Toxigenic *Escherichia coli* infections are the most common cause of traveller's diarrhea in adults as well as a leading cause of diarrhea in children in developing countries [1]. The small peptide toxin STa, the heat-stable toxin of *E. coli*, binds with high affinity to a receptor apparently distinct from particulate guanylate cyclase (GTP pyrophosphate lyase [cyclizing], EC 4.6.1.2) and stimulates the latter enzyme [2,3]. The T84 cell line is a human colon carcinoma cell line which responds to STa with a rise in cyclic GMP and to carbachol with a rise in intracellular calcium; both secretagogues result in chloride secretion [4–7]. Weikel et al. recently showed that phorbol esters, which alone have no effect on cyclic GMP levels, enhanced STa-stimulated cyclic GMP levels by 1.6–2.4-fold [8]. Since carbachol is likely to activate protein kinase C in T84 cells [7] we tested this cholinergic agonist for its ability to 'prime' T84 cells for STa-induced cyclic GMP accumulation in a way similar to phorbols.

2. MATERIALS AND METHODS

2.1. *Cell culture*

T84 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) at passage 50 and were grown for 25 additional passages as described previously [8,9].

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Abbreviations: STa, the heat-stable enterotoxin of *Escherichia coli* (this toxin has also been referred to as STp and ST-I by some authors); cyclic GMP, guanosine-3′,5′-cyclic monophosphate; G-protein, guanine nucleotide binding protein or guanine nucleotide regulatory protein

2.2. *Cyclic GMP accumulation in intact cells*

To cells grown in 24-well plates additions to various wells were made as follows: potential inhibitors (atropine, sphingosine), when present, were added first; next carbachol was added to a final concentration of 100 μM; within 2–5 min of the addition of carbachol the toxin STa was added to a final concentration of 0.5 μM (1 μg/ml). At time intervals of 1–60 min after the addition of STa, cyclic GMP accumulation was terminated by the addition of 0.5 ml of 0.2 M HCl. Cell protein was determined as described [9].

Sphingosine was prepared fresh for each use as an initial 2 mM stock in 55% ethanol, and the final concentration in the cell wells was 10–12.5 μM in 0.3% ethanol. Pertussis toxin [9,10] was the gift of Erik Hewlett, Univ. of Virginia, and was applied to the cells growing in standard medium at 1 μg/ml for 16 h before beginning an experiment. This dose and length of pertussis treatment was previously shown to result in 99% ADP-ribosylation of G-protein substrate in T84 cells [9] and also is sufficient to abolish somatostatin's inhibition of agonist-induced cyclic AMP generation in T84 cells (data not shown).

2.3. *Cyclic GMP radioimmunoassay*

Cell extracts in 0.1 M HCl were acetylated, then analyzed by automated radioimmunoassay as previously described [8,9,11]. Antibody to cyclic GMP was a gift of Joel Linden, as was ^{125}I-succinyl cyclic GMP tyrosine methyl ester prepared by the method of Patel and Linden [12].

2.4. *Materials*

Purified STa prepared by the method of Dreyfus et al. [13] was a gift of Donald C. Robertson, Univ. of Kansas. Carbachol, atropine, sphingosine, 3-isobutyl-1-methylxanthine, and other reagents were obtained form Sigma Chemical Co. (St. Louis, MO), and cell culture media and supplies were from Gibco (Grand Island, NY).

2.5. *Data analysis*

The data shown are expressed as means ± SD, and the Student's *t*-test was used to test statistical significance.

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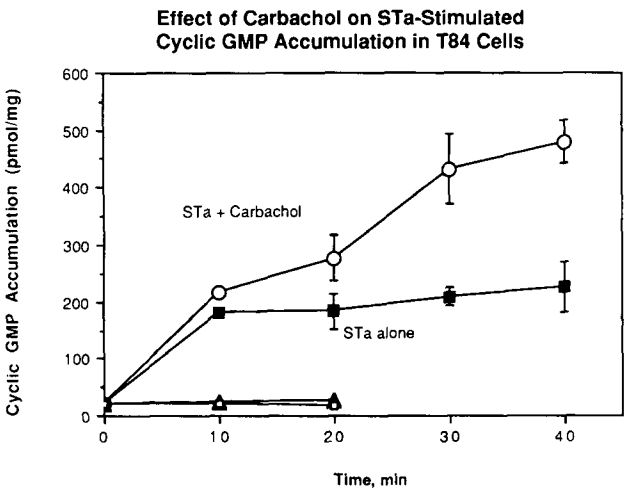

Fig. 1. Effect of carbachol on STa-stimulated cyclic GMP accumulation in intact T84 cells. Cells were incubated in medium containing 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine as described in section 2. STa alone (final concentration 0.5 μM, a maximal dose, ■), carbachol alone (final concentration 100 μM, ▲), normal saline alone (□) or carbachol plus STa (○) were added as indicated. When carbachol and STa were both present the carbachol was added 3 min before STa. This experiment is typical of 8 such experiments. In 2 other experiments cyclic GMP levels at 3–15 min did not differ in cells treated with STa alone vs STa plus carbachol.

3. RESULTS

The time course of cyclic GMP accumulation in intact T84 cells in response to toxin and carbachol is shown in Fig. 1. Carbachol alone (100 μM) did not alter cyclic GMP levels compared to control cells throughout a 45 min incubation. STa (0.5 μM) added alone produced its expected increase in cyclic GMP, but the combination of STa plus carbachol caused an even further increase in cyclic GMP levels which was statistically significant by 20 min. In 11 separate experiments, the addition of carbachol plus STa produced a mean increase in cyclic GMP of 78% over that seen with STa alone at the 45 min time point (range, 30–100% increase). Despite this, carbachol does not stimulate guanylate cyclase activity in T84 cell homogenates or membranes (data not shown).

T84 cells were next treated with atropine to test whether carbachol, a nonselective cholinergic agonist, was acting via muscarinic receptors in the T84 cell line. In 3 experiments 10 μM of the muscarinic antagonist atropine completely blocked the effect of 100 μM carbachol (Table I, upper portion).

Muscarinic effects on intracellular calcium concentrations are believed to result from an interaction of the receptor via a guanine nucleotide regulatory protein (G-protein) with a phosphatidylinositol-specific phospholipase C [14–16]. The G-protein coupled to muscarinic receptors has been reported to be susceptible to pertussis toxin in some systems but not others [16]. T84 cells fully intoxicated with pertussis toxin (see section 2) were tested for their ability to respond to carbachol plus STa with an increase in cyclic GMP (Table I, lower portion). These experiments showed that carbachol's ability to enhance STa-stimulated cyclic GMP accumulation was not diminished in pertussis-treated T84 cells.

One of the best studied potential inhibitors of protein kinase C is sphingosine [17,18]. Since carbachol was hypothesized to be acting via protein kinase C the effect of this lipid on STa- and carbachol-stimulated cyclic GMP accumulation was tested. Figure 2 demonstrates that while 10 μM sphingosine had no effect on cyclic GMP accumulation induced by STa alone (columns 3 and 7), this concentration of sphingosine blocked the

Table I

Effect of atropine and pertussis toxin on carbachol-enhanced, STa-stimulated cyclic GMP accumulation in intact T84 cells

Test agent	Experiment no	Ratio of cyclic GMP accumulation observed with carbachol + STa compared to STa alone	Ratio of cyclic GMP accumulation observed with test agent + carbachol + + STa compared to STa alone	P value
Atropine (10 μM)	1	1.40	0.96	
	2	1.30	0.94	
	3	1.54	0.90	
	Mean	1.41 ± 0.1	0.93 ± 0.03	0.03[a]
Pertussis toxin (1 μg/ml × 16 h)	1	1.40	1.25	
	2	1.30	1.25	
	3	1.65	1.65	
	4	1.30	1.42	
	5	1.64	1.33	
	Mean	1.46 ± 0.17	1.38 ± 0.17	0.34[a]

Experiments measuring cyclic GMP in intact T84 cells in response to STa, STa plus carbachol, and either atropine or pertussis toxin are combined in this table. Data from the 45 min time point only are shown. To allow comparisons of different experiments, cyclic GMP levels observed under various experimental conditions are expressed as a ratio to that observed with STa alone.

[a]*P* value by paired, one-tailed *t*-test

enhancement in cyclic GMP by carbachol (columns 4 and 5).

4. DISCUSSION

Following the observation of Weikel et al. that phorbol esters 'prime' T84 cells for STa-stimulated cyclic GMP accumulation [8], we investigated whether a more physiologic stimulus would have a similar effect. We chose to test the acetylcholine analog carbachol since it was known that T84 cells respond to carbachol with a rise in intracellular calcium, an intracellular messenger which can potentiate the activation of protein kinase C. Huott et al. had previously examined the cyclic GMP response of T84 cells to STa vs STa plus carbachol for 15 min and did not note an increase in cyclic GMP with the addition of carbachol [5]. Based on our data, however, 15 min is too early to see an effect of carbachol (Fig. 1 and other experiments not shown). The reason for the slow onset of the carbachol effect is not completely clear since the calcium rise induced by carbachol is rapid (within 1 min) and transient (offset within 5 min [7]). Phorbol ester treatment of T84 cells causes a rapid (within 10 min) increase in guanylate cyclase as assayed in broken cell preparations* while peak enhancement in cyclic GMP levels requires 45–60 min in intact cells [8]. Thus there is a lag in the appearance of cyclic GMP changes in these whole-cell experiments done in the presence of phosphodiesterase inhibitor. Another hypothesis is that carbachol treatment stabilizes toxin-stimulated guanylate cyclase in an active state, a phenomenon which has been demonstrated in the case of protein kinase C activation of adenylate cyclase from GH_4Cl pituitary cells [19].

The action of carbachol is mediated via muscarinic receptors as shown by the atropine experiments (Table I). We did not choose to further define the muscarinic receptor subtype in the T84 cell, but preliminary data in the T84 cell** suggest that the intracellular calcium rise is mediated by a receptor which would be designated M_3 in recent classifications [15,16]. In other tissues other muscarinic subtypes have been linked to phosphoinositide hydrolysis [20,21]. Muscarinic receptors coupled to inhibition of adenylate cyclase operate via the pertussis-sensitive G-proteins, but muscarinic receptors coupled to phosphoinositide hydrolysis and elevation of intracellular calcium in other animal species and tissues have more often been pertussis-insensitive [15]. Thus the lack of effect of pertussis toxin in these human intestinal cells is consistent with previous reports.

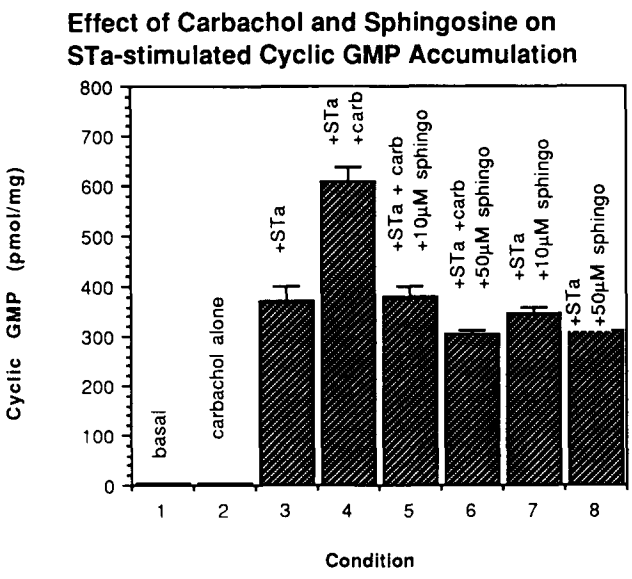

Fig. 2. Effect of carbachol and sphingosine on STa-stimulated cyclic GMP accumulation. Cyclic GMP levels were measured as described in section 2, 45 min after addition of 0.5 μM STa with other additions as indicated in the figure. Sphingosine was added approximately 20 min before carbachol and STa. Abbreviations: sphingo, sphingosine; carb, 100 μM carbachol. Wells receiving 10 μM sphingosine had a final concentration of 0.3% ethanol and those receiving 50 μM sphingosine had 1.5% ethanol. The experiment shown is representative of 4 such experiments.

Sphingosine has been reported to be a potent inhibitor of protein kinase C [17], but it is not specific because it also can inhibit several other protein kinases, including Ca^{2+}/calmodulin-dependent kinases, myosin light chain kinase, and cyclic nucleotide dependent protein kinases [18]. Thus the effect of sphingosine shown in Fig. 2 suggests but does not prove that the carbachol effect is mediated via protein kinase C.

Elevation of cyclic GMP levels in tissues in response to cholinergic agonists has been known for over 20 years [22,23], but the mechanism of signal transduction from receptor to cyclic nucleotide remains undefined. In many cell types the isoenzyme of guanylate cyclase responsible for cyclic GMP production in response to cholinergic agonists has been either undefined or soluble (cytosolic) guanylate cyclase based on its stimulation by calcium and its abundance in the tissue under study [24,25]. In the T84 cell and other intestinal tissues particulate or membrane-bound guanylate cyclase heavily predominates ([25,26] and J. Crane, unpublished data) and is the form stimulated by STa. The present report is, to our knowledge, the first to show that carbachol can enhance agonist-stimulated cyclic GMP production via the membrane-bound form of guanylate cyclase. In addition, the similarity of the effect of carbachol and the effect of phorbol esters in these cells suggests a common mechanism, possibly a phosphorylation of guanylate cyclase by protein kinase C.

*Crane, J.K., Guerrant, R.L., Linden, J. and Weikel, C.S. Phorbol esters potentiate guanylate cyclase stimulation by the heat-stable toxin of *Escherichia coli* (STa) and increase toxin binding sites, Mol. Pharmacol. (submitted for publication)

**Dharmsathaphorn, K., Beuerlein, G., Reinlib, L., Pandol, S., Donowitz, M. and Cohen, J. (1988) Gastroenterology 94, A98 (Abstr.)

Based on the present results showing an interaction between carbachol and STa on cyclic GMP levels, Levine et al. have investigated whether these two secretagogues also interact at the level of chloride ion secretion, measured as short-circuit current (I_{sc}) across T84 cell monolayers. The results confirm that carbachol and STa, when added simultaneously, induce a supra-additive or synergistic increase in I_{sc} compared to each agonist added alone***. These results may also provide an explanation for previous, poorly understood in vivo observations showing inhibition of STa-induced secretion in intestinal segments by cholinergic blockers in rats and cats [27]. Thus the interaction of carbachol and STa may have important physiologic implications, since the enteric nervous system and gastrointestinal hormones which raise intracellular calcium could affect one's susceptibility to diarrhea due to toxigenic *Escherichia coli*.

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