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Regulation of cAMP Metabolism in Mouse Parotid Gland by cGMP and Calcium

EILEEN L. WATSON, JEAN C. SINGH, COLIN MCPHEE, JOE BEAVO and KERRY L. JACOBSON

Departments of Oral Biology (E.L.W., J.C.S., K.L.J.) and Pharmacology (E.L.W., J.C.S., C.M., J.B., K.L.J.) and Research Center in Oral Biology (E.L.W.), University of Washington, Seattle, Washington 98195

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SUMMARY

The interaction of hormones acting via the mobilization of calcium and stimulation of cAMP levels in cells was examined by determining the effects of carbachol and forskolin on cAMP and cGMP accumulation in mouse parotid gland. Treatment of isolated acini with either carbachol (0.01 to 20 μM) or forskolin (1 μM) alone produced little or no increase in cAMP levels; carbachol, however, augmented the effect of forskolin on cAMP accumulation approximately 3- to 4-fold. The effects of carbachol on forskolin-stimulated cAMP levels were further augmented approximately 10-fold in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX) but not in the presence of "low K_m" cGMP-inhibited phosphodiesterase inhibitor milrinone. Augmentation of cAMP levels also occurred in the presence of carbachol plus the β-adrenergic agonist isoproterenol (0.01 μM). In either the presence or absence of forskolin, carbachol increased cGMP levels independently of the inclusion of MIX and in a fashion parallel to that observed for cAMP accumulation. In the presence of forskolin (1 μM), the concentration of carbachol that produced half-maximal effects on cAMP and cGMP levels was 0.62 and 0.72 μM, respectively. Similar values were obtained in the presence of MIX. Cyclic GMP levels were also enhanced by carbachol plus isoproterenol. Hydroxylamine, as well as dibutyryl-cGMP and 8-bromo-cGMP in combination with forskolin, mimicked the effects of carbachol plus forskolin on cAMP levels. LY83583 (6-anilino-5,8-quinolinedione), an agent that lowers cGMP by inhibiting guanylate cyclase, reduced basal levels of cGMP and also completely prevented the increase in cGMP caused by carbachol plus forskolin. In these experiments, however, the augmentation of forskolin-stimulated cAMP levels by carbachol was reduced by approximately 50%. Additional studies suggest that calcium is also required for carbachol augmentation of forskolin-stimulated cAMP accumulation by effects on the adenylate cyclase complex. Augmentation of cAMP levels by carbachol did not involve effects on cAMP degradation. The results suggest that, when cAMP synthesis is stimulated by forskolin or isoproterenol, the muscarinic agonist carbachol augments cAMP accumulation by mechanisms involving cGMP and calcium in mouse parotid gland.

cAMP and calcium play a major role in the regulation of exocrine secretion. In salivary tissue, the action of these intracellular messengers is known to be stimulatory. In parotid gland, muscarinic agonists act by mobilization of cellular calcium (1, 2), whereas β-adrenergic agonists, as well as the diterpine forskolin, cause activation of adenylate cyclase (3, 4). Recent evidence from mouse and rat parotid gland suggests that a synergistic stimulation of amylase release occurs by hormones acting via different pathways, i.e., the cAMP and calcium pathways (5, 6). In these tissues the synergistic effect on amylase release was found to be calcium dependent. In mouse parotid gland, increases in cytoplasmic calcium have been shown to be required for carbachol stimulation of cGMP levels (7). Recent data suggest that cGMP stimulates amylase release from mouse parotid gland (8) and, further, may be required for the muscarinic agonist augmentation of amylase release by forskolin (6). It was shown that augmentation of amylase release by carbachol in the presence of forskolin could be mimicked by agents that directly activate guanylate cyclase, i.e., hydroxylamine, and by dibutyryl- and 8-bromo-cGMP, each in combination with forskolin (6). In these same studies the cholinergic agonist carbachol was found to augment the cAMP response to forskolin. This was a surprising finding, because cholinergic agonists have only been reported to inhibit β-adrenergic-stimulated cAMP accumulation in parotid gland (1, 9, 10). In the present study, we examined several possible mechanism(s) underlying the augmentation of cAMP accumulation by carbachol in mouse parotid gland; data suggest that cGMP, protein kinase C, and calcium are involved.

Experimental Procedures

Materials. Trypsin inhibitor (type II-S), collagenase (type II), EGTA, carbachol, isoproterenol, MIX, hydroxylamine, dibutyryl cGMP, and 8-bromo-cGMP were purchased from Sigma Chemical Co.

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ABBREVIATIONS: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; KHB, Krebs-Henseleit bicarbonate; BSA, bovine serum albumin; MIX, 3-isobutyl-1-methylxanthine; G-kinase, cGMP-dependent protein kinase.

Forskolin was purchased from Calbiochem Co. Forskolin was dissolved in 95% ethanol as a stock solution. LY83583 (6-anilino-5,8-quinolinedione) was generously supplied by Eli Lilly and Co. and trypsin was obtained from Difco Co. cAMP and cGMP radioimmunoassay kits were purchased from Immunonuclear and New England Nuclear, respectively. Purified G-kinase was generously supplied by Dr. T. Lincoln, University of South Alabama.

Isolation of mouse parotid acini. Small groups of isolated mouse parotid cells (acini) were prepared by a modification of the method of Kanagasuntherum and Randle (11). Parotid tissue was obtained from seven mice/experiment in the manner described by Watson *et al.* (2). All glassware used in preparation of acini was siliconized with a 1% aqueous solution of Prosil-28 (PRC Research Chemicals, Inc.). Glands were trimmed and sliced in KHB solution, pH 7.4, containing 0.9 mM Mg^{2+} and 1.28 mM Ca^{2+} and continuously gassed with 95% O_2/5% CO_2. The slices were transferred to 20 ml of the above medium, containing 1 mg/ml trypsin (Difco), and incubated at 37° for 10 min with shaking at 90 times/min. Tissue was separated from the enzyme solution by centrifugation at 100 × *g* for 3 min (Sorvall RC2-B centrifuge). Tissue was resuspended in 20 ml of Ca^{2+}/Mg^{2+}-free KHB solution containing 2 mM EGTA and 1 mg/ml trypsin inhibitor (Sigma type II-S). Incubation was continued with shaking for 5 min. Following centrifugation, the cells were washed with complete KHB solution and further digested in complete KHB solution, containing 1 mg/ml collagenase (Sigma type II or type V), at 37° for 45 min. Mechanical shearing, consisting of pipetting up and down 11 times through polypropylene pipettes, determined the final degree of cell dispersion. The dispersed cell suspension was passed through two layers of nylon mesh and centrifuged at 120 × *g* for 3 min. Acini were washed twice more with complete KHB solution, containing 4% BSA, pH 7.4, and were suspended in complete KHB solution, containing 0.1% BSA, to the desired concentration.

Cyclic nucleotide determinations. For cAMP and cGMP determinations, isolated acini were suspended 1:200 (w/v) in KHB (pH 7.4) containing 0.1% BSA, with and without various phosphodiesterase inhibitors. Cell suspensions (1500 μl) were initially incubated with the phosphodiesterase inhibitors MIX and milrinone for 5 min at 37° and then further incubated with and without secretogogue for another 5 min. At the end of the 5 min, incubations were terminated by addition of an equal volume of ice-cold 10% trichloroacetic acid. [^{3}H]cAMP was added to monitor recoveries. The samples were centrifuged at 2500 × *g* for 30 min at 4°. The resulting pellets were dissolved in 1 ml of 2 N NaOH and assayed for protein according to the method of Lowry *et al.* (12). The supernatants were extracted 6 times with 5 ml of water-saturated ether to remove trichloroacetic acid. The samples were taken to dryness by lyophilization and cAMP was determined by the radioimmunoassay procedure of Steiner *et al.* (13). Samples were acetylated for cGMP determinations according to the method of Harper and Brooker (14). The cross-reactivity of the cGMP antiserum with cAMP at the highest levels of cAMP produced in the experiments was <0.01%; the cross-reactivity of the cAMP antiserum with cGMP was also <0.01%. Results were calculated as pmol of cAMP or fmol of cGMP per mg of protein.

Preparation of membranes. Parotid glands were removed from Swiss Webster mice in the manner previously described by Watson *et al.* (2). Membranes were prepared by homogenization of glands in ice-cold buffer containing 0.25 M sucrose, 10 mM Tris·HCl, and 10 mM $MgCl_2$ at pH 7.5. Homogenates were centrifuged at 20,000 × *g* at 4° for 20 min. Pellets were washed once with the above buffer, recentrifuged, and resuspended in 10 mM Tris·HCl, 6 mM $MgCl_2$, pH 7.5. Fresh membranes were utilized in all experiments.

Measurement of adenylate cyclase activity. Adenylate cyclase was assayed by the methods of Chiu *et al.* (15) and Krishna *et al.* (16). Membranes were incubated, in a final volume of 0.1 ml, in a reaction mixture containing 25 mM Tris·HCl buffer, pH 7.5, 6 mM $MgCl_2$, 3 mM mercaptoethanol, 0.0075% BSA, 1 mM cAMP, [α-^{32}P]ATP (1 mCi, 1 μM), 5.4 mM phosphoenolpyruvate, and 8 μg of pyruvate kinase. The concentration of free calcium corresponding to each addition of $CaCl_2$ to the cyclase assays was determined as previously described (17). Assays were initiated by addition of enzyme (50–100 μg), carried out at 37° for 10 min, and terminated by addition of 0.1 ml of a solution containing 10 mM ATP and 1 mM [^{3}H]cAMP (10,000 cpm) at pH 7.4. The adenylate cyclase assay was linear with respect to time and membrane protein concentration. Assays were performed in duplicate, and activity was expressed as pmol of cAMP/mg of protein/10 min. Protein was determined by the method of Lowry *et al.* (12).

Statistical analysis. The data are presented as the mean ± standard error. Statistical analysis was performed using a paired *t* test and a two-way analysis of variance. Differences with *p* values less than 0.05 were considered significant.

Results

To examine the mechanism of the synergistic interaction reported previously (6) between cholinergic agonists and agents that stimulate adenylate cyclase in mouse parotid gland, we first determined the effect of varying concentrations of carbachol on forskolin-stimulated cAMP accumulation. Data presented in Fig. 1A show that, in the absence of a phosphodiesterase inhibitor, carbachol alone had no detectable effect on cAMP accumulation. Carbachol, however, augmented forskolin-stimulated cAMP levels approximately 3-fold at 10 μM carbachol. Augmentation of the response to forskolin was maximal at 5 min (data not shown). The concentration of carbachol, determined in the presence of forskolin, that produced half-maximal activation was 0.62 μM.

In the presence of the phosphodiesterase inhibitor MIX (0.1 mM), carbachol further increased cAMP accumulation in the absence or presence of forskolin (1 μM) (Fig. 1B). Maximal cAMP levels were 60 pmol/mg of protein is the absence and 546 pmol/mg of protein in the presence of forskolin. As with the studies conducted in the absence of MIX, carbachol augmentation of forskolin-stimulated cAMP levels was maximal at 5 min, and the concentration of carbachol, determined in the presence of forskolin, that produced half-maximal potentiation was 0.83 μM. To show that the ability of carbachol to augment cAMP levels was not selective for forskolin, the effects of carbachol on isoproterenol-stimulated cAMP accumulation were also determined. Carbachol (10 μM) enhanced isoproterenol (0.01 μM)-stimulated cAMP levels, with a maximum increase of approximately 4-fold (Fig. 2).

In the next experiments, the effects of carbachol on cGMP accumulation in forskolin-stimulated acini were determined. Treatment of parotid acini with carbachol alone or with carbachol plus forskolin (1 μM) stimulated cGMP accumulation in the absence of MIX (Fig. 3A). In the presence of (0.1 mM) MIX, cGMP accumulation was further increased (Fig. 3B). The concentrations of carbachol, determined in the presence of forskolin, that produced half-maximal increases in cGMP levels in the absence or presence of MIX (i.e., 0.72 and 0.66 μM, respectively) were similar to the concentrations of carbachol that produced half-maximal augmentation of cAMP levels. Cyclic GMP levels were also noted to be significantly greater in acini exposed to carbachol (10 μM) plus forskolin than in acini exposed to carbachol (10 μM) alone. Similar results were obtained with carbachol (10 μM) plus isoproterenol (0.01 μM); however, the changes in cGMP levels were not as dramatic in the presence of isoproterenol. cGMP values were 687 ± 104 pmol/mg of protein for control acini, 936 ± 91 pmol/mg of protein for carbachol-treated acini, 836 ± 77 pmol/mg of pro-

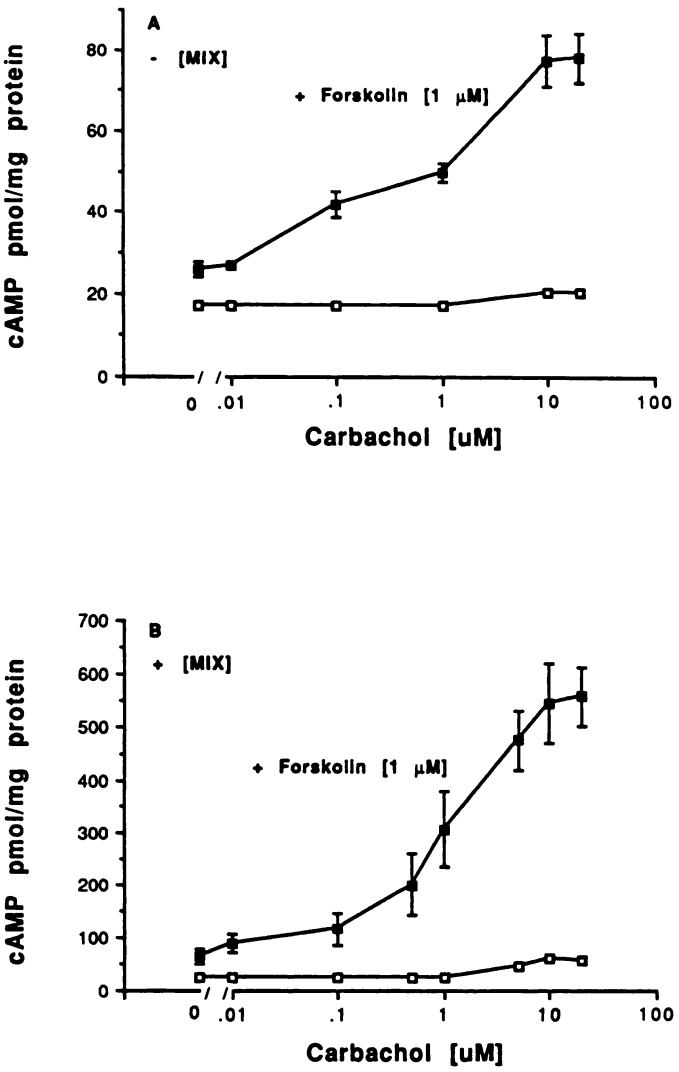


Fig. 1. Effect of increasing concentrations of carbachol, alone (□) and in combination with forskolin (1 μM) (■), on cAMP accumulation in isolated mouse parotid acini. A, In the absence of 0.1 mM MIX. B, In the presence of MIX. See Experimental Procedures for details. Control cAMP values were 17.0 ± 1.1 and 20.6 ± 3.1 pmol/mg of protein in the absence and presence of 0.1 mM MIX, respectively. cAMP accumulation stimulated by forskolin (1 μM) alone was 26.8 ± 1.9 and 64.7 ± 14.6 pmol/mg of protein, respectively, in the absence and presence of MIX. Each value represents the mean± standard error of six experiments determined in duplicate. For *symbols* without *error bars*, the standard error was smaller than the symbol.

tein for isoproterenol-treated acini, and 1420 ± 101 pmol/mg of protein for carbachol- plus isoproterenol-treated acini.

To determine whether other agents that increase cGMP levels could mimic the effects of carbachol in augmenting forskolin-stimulated cAMP accumulation, we examined the effects of hydroxylamine as well as dibutyryl- and 8-bromo-cGMP. MIX (0.1 mM) was included in the incubated media for these experiments. In the presence of hydroxylamine (25 μM to 1 mM), an agent that activates guanylate cyclase independently of calcium (18), cGMP levels increased in a dose-dependent manner (Fig. 4); forskolin potentiated the hydroxylamine response at 50 and 100 μM concentrations but did not significantly alter the maximal response. Although hydroxylamine alone did not have an appreciable effect on cAMP accumulation, it significantly augmented the stimulation by forskolin

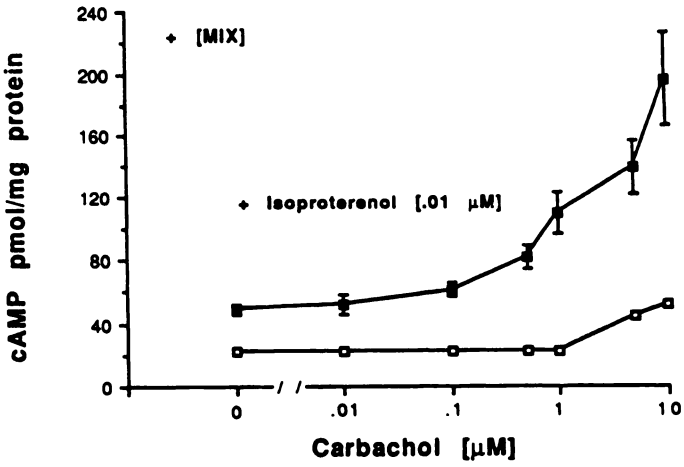


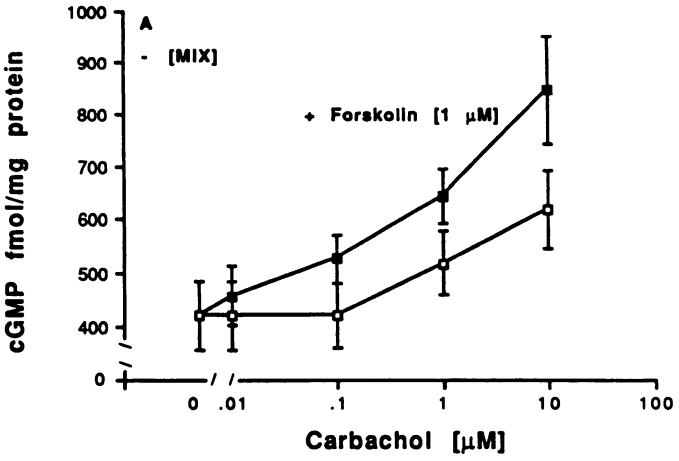
Fig. 2. Effects of increasing concentrations of carbachol, alone (□) and in combination with isoproterenol (0.01 μM) (■), on cAMP accumulation in isolated mouse parotid acini. MIX (0.1 mM) was present in the incubation media. See Experimental Procedures for details. Control cAMP values were 21.8 ± 1.6 pmol/mg of protein. Cyclic AMP accumulation stimulated by isoproterenol (0.01 μM) was 48.9 ± 3.1 pmol/mg of protein. Each value represents the mean ± standard error of five experiments determined in duplicate. For *symbols* without *error bars*, the standard error was smaller than the symbol.

(Fig. 5). Both dibutyryl-cGMP (50 μM) and 8-bromo-cGMP (50 μM) also enhanced forskolin-stimulated cAMP accumulation approximately 3- to 4-fold (Table 1).

Previous studies in other tissues have shown that LY83583 can inhibit basal and hormone-stimulated cGMP levels, presumably by inhibiting guanylate cyclase (19, 20). In mouse parotid acini, LY83583 did not significantly reduce basal cGMP levels but completely prevented the increase in cGMP levels caused by carbachol plus forskolin (Table 2). In these experiments, the specificity of the action of LY83583 was demonstrated by its lack of effect on basal levels on cAMP, and forskolin-stimulated cAMP levels; however, LY83583 reduced carbachol plus forskolin-induced increases in cAMP accumulation by approximately 50%.

Because stimulation of muscarinic receptors leads to the mobilization of calcium (1, 2), experiments were conducted to determine whether the effects of carbachol on forskolin-stimulated cAMP accumulation were dependent on calcium in the extracellular media. Results presented in Table 3 show that enhancement of forskolin-stimulated cAMP accumulation by carbachol was almost completely inhibited in calcium-free media. On the other hand, augmentation of the isoproterenol response by forskolin was not affected by removal of extracellular calcium. To further determine whether the effects of calcium as well as cGMP are involved in the synthesis of cAMP, we examined the effects of these compounds on adenylate cyclase activity. Neither 8-bromo-cGMP (5 μM) nor purified G-kinase (up to 0.5 μM) altered basal or forskolin-stimulated enzyme activities. Adenylate cyclase activities were 402 ± 31, 455 ± 53, and 446 ± 34 pmol/mg of protein for basal, cGMP-treated, and G-kinase-treated membranes in the absence of forskolin and 1061 ± 41, 1125 ± 32, and 1180 ± 52 pmol/mg of protein in the presence of forskolin (1 μM), respectively.

Calcium at 0.01 to 1 μM concentrations, however, was found to enhance forskolin-stimulated cyclase activity (Fig. 6). Significant enhancement occurred with calcium concentrations up to 0.6 μM. To determine whether augmentation of cAMP levels



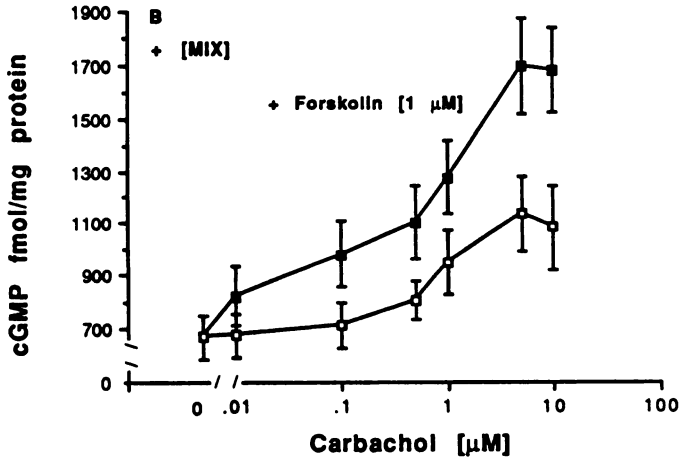


Fig. 3. Effect of increasing concentrations of carbachol, alone (□) and in combination with forskolin (1 μM) (■), on cGMP accumulation in mouse parotid acini. A, In the absence of 0.1 mM MIX. B, In the presence of 0.1 mM MIX. See Experimental Procedures for details. Control cGMP values were 422.8 ± 64 and 668.8 ± 80 fmol/mg of protein in the absence and presence of MIX, respectively. Forskolin (1 μM) alone had no detectable effect on cGMP levels. Each point represents the mean ± standard error of six experiments determined in duplicate.

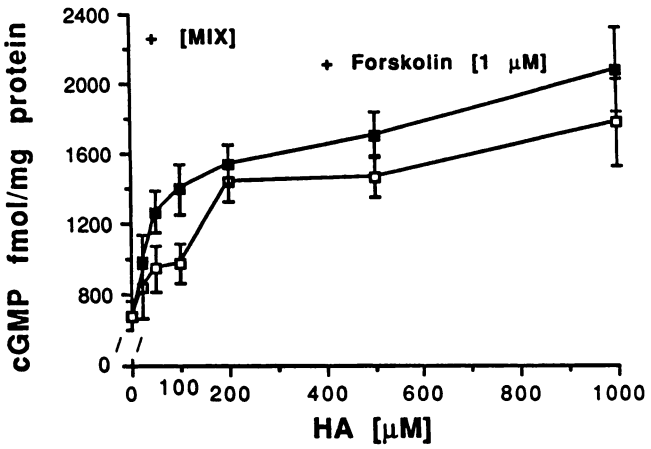


Fig. 4. Effect of increasing concentrations of hydroxylamine (*HA*), alone (□) and in combination with forskolin (1 μM) (■), on cGMP accumulation in mouse parotid acini. MIX (0.1 mM) was present in the incubation media. See Experimental Procedures for details. Control cGMP values were 670.0 ± 96 fmol/mg of protein. Forskolin (1 μM) had no detectable effect on cGMP accumulation. Each point represents the mean ± standard error of five experiments determined in duplicate.

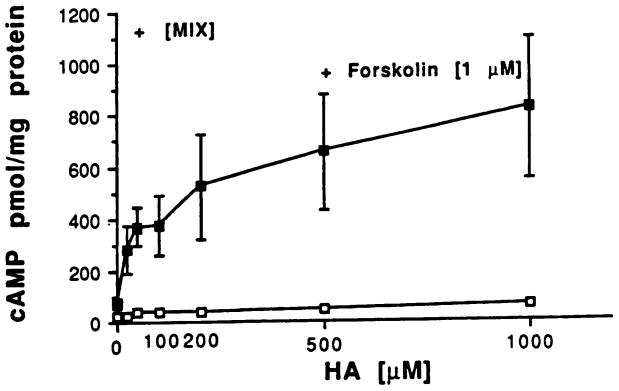


Fig. 5. Effect of increasing concentrations of hydroxylamine (*HA*), alone (□) and in combination with forskolin (1 μM) (■), on cAMP accumulation in mouse parotid acini. MIX (0.1 mM) was present in the incubation media. See Experimental Procedures for details. Control cAMP values were 26.2 ± 3.4 pmol/mg of protein. Cyclic AMP accumulation stimulated by forskolin (1 μM) was 65.1 ± 29.9 pmol/mg of protein. Each point represents the mean ± standard error of five experiments determined in duplicate.

also might involve inhibition of cAMP degradation, we first examined the effects of milrinone, a selective inhibitor of "low K_m" cGMP-inhibited phosphodiesterase (21), on cAMP levels induced by forskolin (1 μM) and carbachol (1 μM) plus forskolin (1 μM). Milrinone (0.01 to 100 μM) had no significant effect on cAMP accumulation induced by these agents. Cyclic AMP degradation was also assessed directly by the method described by Meeker and Harden (22). Adenylate cyclase was activated with isoproterenol (1 μM) in the presence of forskolin (1 μM), with subsequent inhibition of isoproterenol-induced synthesis by propranolol (10 μM) in the absence or presence of carbachol (10 μM). The rate constants for cAMP degradation were 0.44 and 0.47 min^{-1} in the absence and presence of carbachol, respectively (Fig. 7).

Discussion

β-Adrenergic agonists as well as forskolin have been shown to potentiate amylase release in response to calcium-dependent

TABLE 1

Effect of cGMP derivatives on cAMP accumulation in mouse parotid acini

Values represent the mean ± standard error of four experiments determined in duplicate. Acini were incubated at 37° for 5 min with forskolin or with forskolin plus a derivative of cGMP. MIX (0.1 mM) was present in the incubation media. See Experimental Procedures for details.

Drug	cAMP
	pmol/mg of protein
None	14.1 ± 0.8
Forskolin (1 μM)	27.5 ± 1.7
Dibutyryl-cGMP (50 μM)	13.8 ± 0.7
8-Bromo-cGMP (50 μM)	14.4 ± 0.6
Dibutyryl-cGMP (50 μM) + forskolin (1 μM)	87.2 ± 7.4
8-Bromo-cGMP (50 μM) + forskolin (1 μM)	99.6 ± 15.5

TABLE 2

Effect of LY83583 on augmentation of carbachol-stimulated cAMP and cGMP accumulation by forskolin

Values represent the mean ± standard error of seven experiments determined in duplicate. Acini were preincubated at 37° for 5 min with LY83583 before addition of carbachol and forskolin. MIX (0.1 mM) was present in the incubation media.

Drug	cAMP	cGMP
	pmol/mg of protein	*fmol/mg of protein*
None	17.2 ± 1.9	503.0 ± 49.8
LY83583 (10 μM)	14.5 ± 2.6	435.1 ± 41.6
Forskolin (1 μM)	54.1 ± 6.1	542.0 ± 45.0
Forskolin (1 μM) + LY83583 (10 μM)	49.1 ± 3.2	490.7 ± 23.3
Carbachol (10 μM)	38.7 ± 0.6	820.7 ± 54.1
Carbachol (10 μM) + LY83583 (10 μM)	36.0 ± 1.4	375.9 ± 43.3
Carbachol (10 μM) + forskolin (1 μM)	378.2 ± 81.4	1124.3 ± 81.6
Carbachol (10 μM) + forskolin (1 μM) + LY83583 (10 μM)	183.8 ± 24.4	395.7 ± 40.6

TABLE 3

Carbachol enhancement of forskolin-stimulated cyclic AMP accumulation in the absence and presence of Ca^{2+}

Mouse parotid acini were incubated at 37° for 30 min, in a calcium-containing (1.28 mM) buffer or a calcium-free buffer containing 0.5 mM EGTA, before exposure to drugs. MIX (0.1 mM) was present in the incubation media. Cyclic AMP accumulation in the presence and absence of forskolin (1 μM) was 53.6 and 56.8 pmol/mg of protein, respectively. The results are expressed as the mean ± standard error of three experiments.

Medium	Drug	cAMP
		pmol/mg of protein
Ca^{2+}-containing buffer	None	18.4 ± 2.1
	Carbachol (10 μM) + forskolin (1 μM)	325.5 ± 60.4
	Isoproterenol (1 μM) + forskolin (1 μM)	2168.0 ± 304.6
Ca^{2+}-free buffer	None	22.6 ± 2.6
	Carbachol (10 μM) + forskolin (1 μM)	52.1 ± 5.1
	Isoproterenol (1 μM) + forskolin (1 μM)	2116.0 ± 386.2

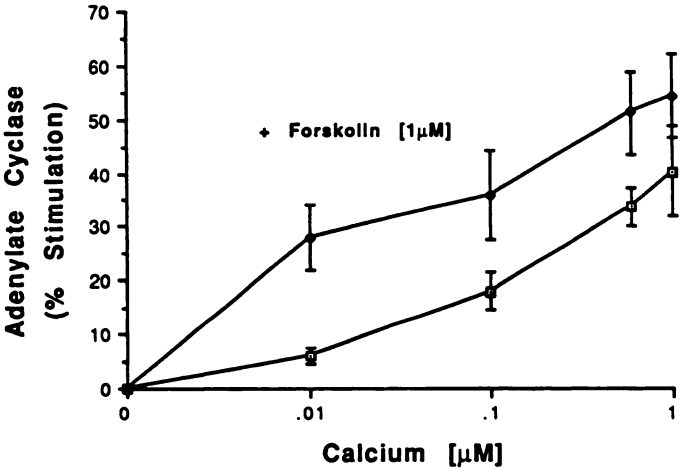


Fig. 6. Effect of calcium ions (0.01 to 1 μM) on basal (□) and forskolin (1 μM)-stimulated (♦) adenylate cyclase activity. Activity is presented as a percentage of stimulation over basal or forskolin-stimulated values of 390.7 ± 32.1 and 796.1 pmol/mg of protein, respectively. See Experimental Procedures for details. Values represent the mean ± standard error of six experiments determined in duplicate.

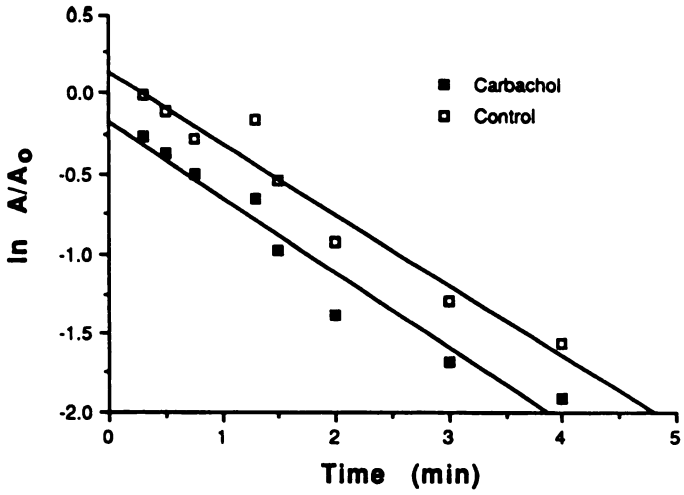


Fig. 7. Effect of carbachol on cAMP degradation. Acini were incubated in the presence of isoproterenol (1 μM) plus forskolin (1 μM) for 5 min. Synthesis was then stopped by the addition of propranolol (10 μM), in the absence (□) or presence (■) of carbachol (10 μM). Data are plotted as the natural logarithm of cyclic AMP levels (A) at the indicated times divided by the initial level (A_0) of cAMP at the time of propranolol, with or without carbachol, addition. Rate constants for cAMP degradation were 0.44 and 0.47 min^{-1} in the absence and presence of carbachol, respectively. Values represent the mean of five experiments.

agonists in several tissues, including pancreas and salivary gland, by a mechanism involving calcium (6, 23–25). However, in mouse parotid gland it was shown that augmentation of forskolin-stimulated amylase release by the calcium-dependent agonist carbachol may also involve cAMP and cGMP (6). In the present study we examined the mechanism(s) by which carbachol acts to augment forskolin to increase cAMP levels in mouse parotid acini. It is clear from our data, as well as those of others, that the effects of cholinergic agonists on cAMP levels in cells may vary in both direction and mechanism, depending on tissue and species. In most tissues, muscarinic agonists such as carbachol have been shown to inhibit cAMP accumulation (22, 26, 27). In rat parotid gland, muscarinic agonists have also been shown to inhibit β-adrenergic isoproterenol-stimulated cAMP accumulation (1, 9, 10). Inhibition of cAMP accumulation was not noted in mouse parotid gland at the concentrations of agonists utilized. Although the potentiating effects of carbachol on cAMP accumulation in mouse parotid gland appear to be different from data previously reported for other tissues, including rat parotid gland, similar effects have been noted in neuroblastoma cells (28) and in cat submandibular gland (29).

The present studies indicate that at least part of the synergistic effects of carbachol on forskolin-stimulated cAMP accumulation are caused by carbachol-stimulated increases in cGMP levels. An agent such as hydroxylamine that directly activates guanlyate cyclase independently of calcium (18) was found to mimic the effects of carbachol. It is recognized that this drug may have other actions in the cell in addition to activating guanylate cyclase. However, the ability of derivatives of cGMP, i.e., 8-bromo-cGMP and dibutyryl-cGMP, to mimic the effects of hydroxylamine and carbachol on forskolin-stimulated cAMP accumulation suggests that the increases in cAMP accumulation are due to specific alterations in cGMP and are consistent with the possibility that augmentation of cAMP levels may be mediated through a cGMP-dependent

kinase. The effects of the compound LY83583 provide additional evidence for a causal association between changes in cGMP levels and changes in cAMP levels.

From the data presented in Table 2, cGMP appears to be only partially responsible for the observed enhancement of cAMP levels by carbachol and forskolin. Data are presented in Table 3 and Fig. 6 suggesting that calcium ions are also required for carbachol enhancement of forskolin-stimulated cAMP and mediate the increase in cAMP levels by affecting cAMP synthesis.

The mechanism(s) by which cGMP affects cAMP accumulation is not clear. Results with hydroxylamine suggest that elevation of cGMP levels is insufficient to elevate cAMP levels in mouse parotid gland unless forskolin is present. This suggests that an additional intracellular regulatory mechanism may be required for the observed augmentation. The results are consistent with our findings that cGMP and G-kinase alone did not affect basal adenylate cyclase activity. Moreover, cGMP and G-kinase also failed to directly augment forskolin-stimulated adenylate cyclase activity. These findings suggest that cGMP acting via G-kinase may be acting indirectly to enhance adenylate cyclase activity. One possibility is that cGMP may be acting to increase calcium mobilization and, thus, increase $[Ca]_i$. cGMP-dependent kinase has been reported to enhance calcium current in snail neurons (30) and cGMP has been shown to increase calcium uptake into mouse parotid acini.[1] Increases in calcium are known to activate adenylate cyclase via calmodulin in mouse parotid gland (17) and in the present study calcium, at concentrations that stimulate adenylate cyclase, enhanced forskolin-stimulated cAMP accumulation. If cGMP affects cAMP accumulation via calcium, then an additional mechanism(s), perhaps related to protein kinase, would be required to explain the sensitization induced by carbachol. It is not likely that cGMP directly inhibits a phosphodiesterase, inasmuch as we were unable to demonstrate an effect of milrinone, a selective inhibitor of cGMP-inhibited phosphodiesterase (21), on cAMP accumulation. Further, more direct studies of rates of cAMP degradation in intact parotid cells failed to show that carbachol enhances cAMP degradation (Fig. 7).

An interesting finding from these studies was that carbachol plus either forskolin or isoproterenol enhanced both cAMP and cGMP levels. We can only speculate about the mechanism involved in the enhancement of cGMP accumulation. The data would suggest that enhancement may involve an interaction of one of the products of muscarinic receptor stimulation, i.e., protein kinase C or calcium, with guanylate cyclase. Protein kinase C has been shown to directly stimulate guanylate cyclase activity (31) and to be involved in α_1-adrenergic amplification of β-adrenergic-stimulated cGMP response (32). Further studies will be required to address this issue.

In conclusion, the results show that agents such as carbachol augment forskolin-stimulated cAMP accumulation in mouse parotid acini. Further, the effects on cAMP accumulation appear to be related to combined effects on cGMP and calcium. The results suggest that one possible role for cGMP in the mouse parotid gland may be in cAMP metabolism. This may be physiologically important, because in mouse parotid gland secretion is regulated by agonists that stimulate cAMP and cGMP synthesis. It remains unclear, however, whether the effects of cGMP on cAMP synthesis are due to indirect effects via calcium ions on adenylate cyclase activity or via other regulatory mechanisms in the cell.

[1] Watson, E. L., unpublished observations.

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Send reprint requests to: Eileen L. Watson, Ph.D., Department of Oral Biology, SB-22, University of Washington, Seattle, WA 98195.