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# Regulation of Adenosine Cyclic 3',5'-Monophosphate and Guanosine Cyclic 3',5'-Monophosphate Levels and Contractility in Bovine Tracheal Smooth Muscle

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SUMMARY

KATSUKI, SHOJI & MURAD, FERID (1977) Regulation of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol. Pharmacol.*, 13, 330-341.

The effects of various agents on cyclic 3',5'-AMP and cyclic 3',5'-GMP levels and mechanical activity of bovine tracheal smooth muscle were examined. Carbachol, acetylcholine, and histamine caused muscle contraction and increased cyclic GMP levels several fold in a dose-dependent manner; contraction preceded the increase in cyclic GMP. Serotonin and high $K^+$ concentrations contracted muscle to the same degree as carbachol, while cyclic GMP increases were smaller than that due to carbachol. The effects of carbachol and histamine were blocked by atropine and diphenhydramine, respectively. The calcium ionophore A-23187 also increased cyclic GMP levels and caused contraction. Guanylate cyclase activators—sodium azide, hydroxylamine, sodium nitrite, nitroglycerin, and sodium nitroprusside—increased cyclic GMP levels and relaxed tracheal smooth muscle. None of these agents had any effect on cyclic AMP levels. The presence of $Ca^{2+}$ in the incubation medium was not required for cyclic GMP increases with these latter agents. However, it was required in order to see increases in cyclic GMP with carbachol, histamine, and A-23187. Adrenergic agonists relaxed muscle preparations and increased cyclic AMP levels 2-3-fold. Both these effects were blocked by propranolol. Inhibitors of cyclic nucleotide phosphodiesterases relaxed muscle preparations and were associated with increases in both cyclic AMP and cyclic GMP. Prostaglandins $E_1$ and $F_{2\alpha}$ had little or no effect on mechanical activity or cyclic nucleotide levels. The addition of 1 mM 8-bromo-cyclic GMP or 8-bromo-AMP relaxed muscle preparations. A variety of other nucleotides had no effect. These results support the hypothesis that cyclic AMP plays a role in relaxation of tracheal muscle induced by adrenergic agents. However, with a variety of other agents, we found that contraction or relaxation occurred with increases either in cyclic GMP alone or in both cyclic AMP and cyclic GMP. Thus the roles of cyclic AMP and cyclic GMP in smooth muscle mechanical activity remain obscure.

## INTRODUCTION

The effects of many hormones and drugs are thought to be mediated through al-

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tered intracellular accumulation of cyclic nucleotides. It has been shown that *beta* adrenergic agonists and prostaglandins increase cyclic 3′,5′-AMP levels in several tissues (1–8), and many experiments support the view that cyclic AMP regulates relaxation of smooth muscle (5, 7–9).

On the other hand, cyclic 3′,5′-GMP has been found to be increased by choline esters, histamine, serotonin, bradykinin, $K^+$, and ionophores in many tissues (7, 8, 10–14). These agents cause smooth muscle contraction in many preparations. Since their effects are antagonistic to *beta* adrenergic agonists, it has been postulated that cyclic GMP plays a role in smooth muscle contraction (7, 8, 11, 14–16). Tracheal and bronchial smooth muscle were classified by Lands *et al.* (17) as a $beta_2$ adrenergic receptor system and have been used in many pharmacological experiments. However, there are few studies describing the regulation of cyclic AMP and cyclic GMP levels in this tissue, and even fewer studies correlating cyclic nucleotide levels and tracheal smooth muscle motility (6–8). In our previous experiments using guinea pig tracheal rings as a model of bronchial smooth muscle (6, 7), we examined the effects of various agents on the accumulation of cyclic nucleotides. Since this preparation is quite heterogeneous, it was difficult to correlate observed changes of cyclic nucleotides with the expected alterations in guinea pig tracheal smooth muscle motility.

The experiments reported here were performed with relatively homogeneous bovine tracheal smooth muscle preparations. The effects of catecholamines, prostaglandins, choline esters, histamine, and other agents on cyclic AMP and cyclic GMP accumulation and muscle motility were examined. Inhibitors of cyclic nucleotide phosphodiesterases and activators of guanylate cyclase (18) were also examined for their effects on cyclic nucleotide accumulation and muscle contraction and relaxation. Based on these experiments, the regulation of cyclic AMP and cyclic GMP accumulation and their possible role(s) in muscle contraction and relaxation are discussed. Some of these experiments were previously presented in abstract form (19, 20).

## MATERIALS AND METHODS

### *Preparations of Bovine Tracheal Smooth Muscle*

Bovine tracheas obtained from a local abattoir were placed in chilled Krebs-Ringer-bicarbonate solution containing glucose and pyruvate and brought to the laboratory. The solution contained NaCl, 118.5 mM; KCl, 4.74 mM; $MgSO_4$, 1.18 mM; $KH_2PO_4$, 1.18 mM; $CaCl_2$, 2.54 mM; $NaHCO_3$, 24.9 mM; glucose, 10 mM; and pyruvic acid, 1 mM. Smooth muscle was carefully separated from cartilage and connective tissue, cut into 1–2 × 5–10 mm strips (1–3 mg of protein), kept in chilled Krebs-Ringer-bicarbonate solution saturated with 95% $O_2$ and 5% $CO_2$, and used within 24 hr. The tissue segments were 90–95% smooth muscle (Fig. 1). Under these conditions basal levels and the responsiveness of tissues to changes in cyclic nucleotide levels and motility with various agents were quite constant.

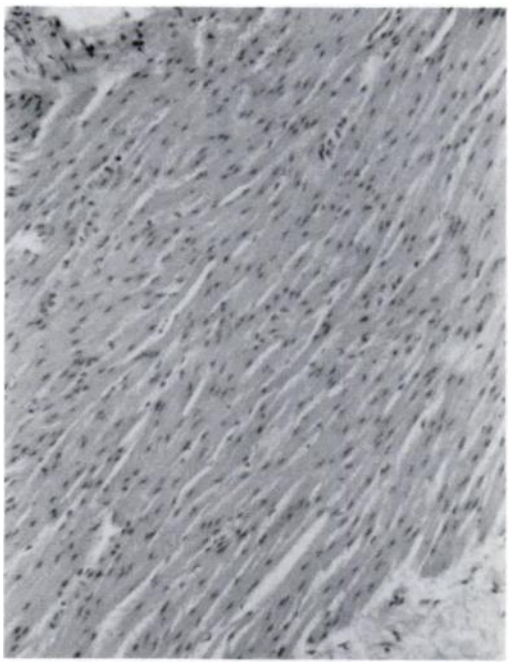

FIG. 1. *Microscopic section of representative bovine tracheal smooth muscle segment*

Sections were stained with hematoxylin and eosin. × 100.

*Incubation of Muscle Strips*

Incubation of strips was performed in two ways. When muscle movement and cyclic nucleotide levels were measured simultaneously, method A was used. Where only cyclic AMP and cyclic GMP levels were measured, method B was used.

*Method A*. Strips were hung in organ baths (20 ml) and equilibrated for 2 hr in Krebs-Ringer-bicarbonate at 37° with 95% $O_2$–5% $CO_2$. The medium was replaced several times during this incubation. Contraction or relaxation of muscle strips was measured isometrically, using a Grass force displacement transducer. During incubation muscles relaxed and had no inherent tone. Strips were loaded with less than 200 mg of tension before addition of various agents. At appropriate times after the addition of agents (about 20 $\mu$l), the bath fluid was rapidly drained and strips were frozen by clamping with a small metal clamp cooled in Dry Ice–acetone. Contraction is expressed as an increase in tension. In order to measure relaxation, contraction was first induced by 0.1 $\mu$M carbachol, since preparations did not have inherent tone. Relaxation is expressed as the percentage decrease in 0.1 $\mu$M carbachol-induced tension. Neither cyclic GMP nor cyclic AMP levels were changed by 0.1 $\mu$M carbachol (see RESULTS).

*Method B*. One end of the muscle strip was tied to wire for easy handling of the strip, hung in the bath, and incubated in the same way as in method A. However, tension was not applied to these muscle segments. The incubation was stopped by removing the wire and muscle from the bath and freezing between blocks of Dry Ice. Detailed experimental maneuvers are indicated in each figure or table. There were no differences in the cyclic nucleotide responses to agents under the two incubation conditions.

*Cyclic AMP and Cyclic GMP Measurements*

Frozen muscle strips were homogenized in 6% trichloracetic acid containing 0.2 pmole of cyclic [$^3$H]AMP. Supernatant fractions were extracted with ether, and a portion of the extract was acetylated before radioimmunoassay for cyclic nucleotides (21). Some of the sample was assayed for tritium, and it was assumed that the recovery of cyclic GMP was the same as that of cyclic AMP (about 95%).

Radioimmunoassay for cyclic AMP and cyclic GMP was performed according to the method of Steiner *et al.*, with some modification (22, 23). The sensitivity of the cyclic AMP radioimmunoassay was 0.05 pmole for cyclic AMP and 0.005 pmole for acetylated cyclic AMP. Cross-reactivity to cyclic GMP was less than 0.1%, and to ATP, 0.001%. The sensitivity and cross-reactivity of the cyclic GMP radioimmunoassay have been reported previously (21, 22). Muscle extracts were not purified prior to assay, since values were similar after purification on Dowex 1-chloride (AG1-X2, 50–100 mesh) columns as described previously (7).

Precipitable protein in trichloracetic acid was determined by the method of Lowry *et al.* (24), using bovine serum albumin as standard. Cyclic nucleotide values are presented as picomoles per milligram of protein. Statistical analyses were performed with Student's *t*-test.

*Materials*

Cyclic [$^3$H]AMP (specific activity, 27.5 Ci/mmole) was obtained from New England Nuclear. The $^{125}$I-labeled tyrosine methyl esters of succinyl cyclic AMP and succinyl cyclic GMP were prepared by the method of Hunter and Greenwood (25) and purified by column chromatography on Sephadex G-10 as described by Steiner *et al.* (23). Prostaglandins $E_1$ and $F_{2\alpha}$ were kindly supplied by the Upjohn Company. The ionophore A-23187 was supplied by Eli Lilly and Company and was solubilized according to Prince *et al.* (26). Nitroglycerin was extracted with ethanol from Nitrostat (Parke, Davis). The same concentration of ethanol was added to control experiments. 1-Methyl-3-isobutylxanthine was obtained from Searle and Company. 8-Bromo derivatives of various nucleotides, sodium nitroprusside, and sodium azide were obtained from Sigma. Other materials were obtained as described previously (6, 7, 18).

## RESULTS

### *Effects of Contractile Agents on Cyclic AMP and Cyclic GMP Levels*

Carbachol at 1 μM induced contraction of muscle preparations and elevated cyclic GMP several fold (Fig. 2). An increase in tension was apparent 10 sec after the addition of carbachol. However, a significant increase in cyclic GMP was not detected until 20 sec after addition of carbachol. Both contraction and the increase in cyclic GMP reached a plateau at 1-2 min. Increases in tension and cyclic GMP levels were maintained for at least 3 min; longer times were not examined. The effects of carbachol were dose-dependent (Fig. 3). However, 0.1 μM carbachol produced obvious contraction that was not accompanied by a significant increase in cyclic GMP. This concentration of carbachol did not increase cyclic GMP levels between 10 and 180 sec after addition (not shown). The dose response for cyclic GMP increases with carbachol was not altered with 5 mM theophylline included in incubations (not shown). High concentrations of carbachol (10 μM) produced maximal contraction and cyclic GMP increases, with a significant increase in cyclic AMP levels (Fig. 3). Decreases in cyclic AMP levels were not observed with a variety of carbachol concentrations between 10 and 180 sec of incubation.

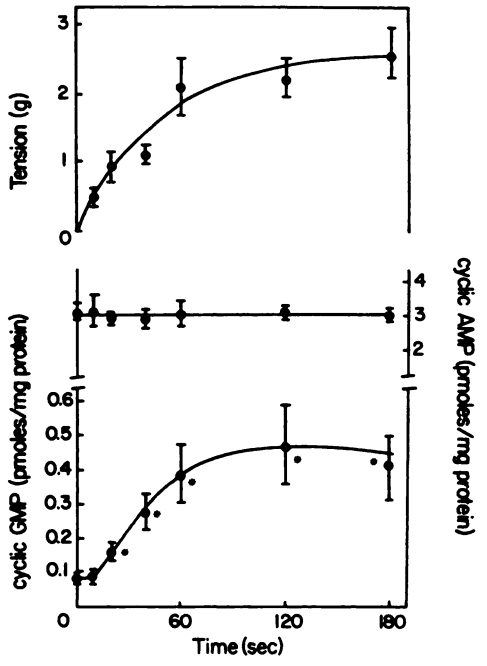


FIG. 2. *Time course of effect of carbachol on muscle tension and cyclic nucleotide levels*

The experiment was performed by method A, using 1 μM carbachol for the times indicated. Contraction and cyclic nucleotide levels were determined on the same muscle segments. Values are means ± standad errors of three to six segments.

* Significantly different ($p < 0.05$) from the zero-time values.

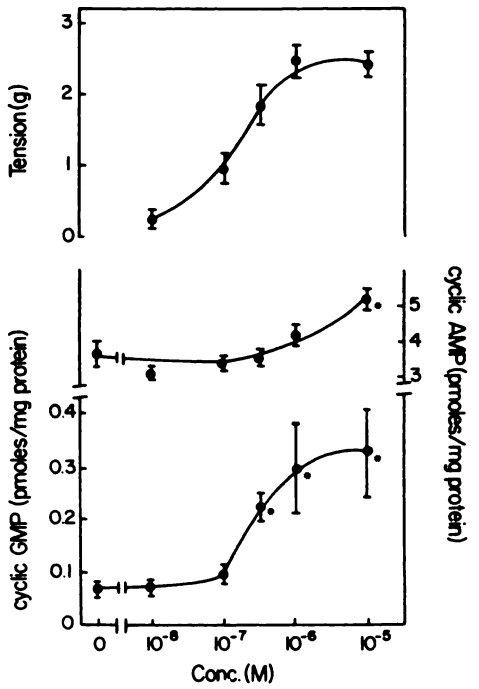


FIG. 3. *Dose-response curves for carbachol on muscle tension and cyclic nucleotide accumulation*

The experiment was performed by method A, using the concentrations of carbachol indicated with an exposure time of 2 min. Values are means ± standard errors of five or six segments.

* Significantly different ($p < 0.05$) from the controls without carbachol.

Histamine also increased contractility and cyclic GMP levels in a dose-dependent manner (not shown). In contrast to the effects of carbachol, there were no apparent differences in the concentrations required to produce these two effects. Significant increases in both contraction (about 30% of maximal contraction) and cyclic GMP levels (about 35% increases) occurred

with 0.1 μM histamine and 2 min of incubation. The concentrations of histamine that resulted in half-maximal increases in contraction and cyclic GMP levels were similar (1 μM). High concentrations of histamine (1 μM or greater) also increased cyclic AMP levels (from 3.5 to 5 pmoles/mg of protein). The contractile and cyclic GMP-increasing effects of carbachol were prevented by atropine, and those of histamine, by diphenhydramine, indicating that the effects are mediated by specific cholinergic and histaminergic receptors, respectively (not shown). The blockers themselves had no effect on contractility or cyclic nucleotide levels.

The effects of several agents that contract smooth muscle are summarized in Table 1. Serotonin (10 μM) and $K^+$ (80 mM) produced contraction to the same degree as 10 μM acetylcholine or 1 μM carbachol. The contractions produced were almost maximal. However, the increases in cyclic GMP levels with serotonin and high $K^+$ were much less than those with choline esters. A concentration of 80 mM $K^+$ also significantly increased cyclic AMP levels.

The ionophore A-23187 facilitates transport of divalent cations and causes contraction of smooth muscle (26–29). A-23187 contracted tracheal smooth muscle preparations and increased cyclic GMP levels. This agent required about 1 min before the onset of contraction, and at this time increases in cyclic GMP were observed (Table 1).

### *Effects of Adrenergic Agents*

Since bovine tracheal smooth muscle did not have inherent tone in these experiments, it was necessary to contract preparations with 0.1 μM carbachol in order to examine the relaxing effects of adrenergic agents. The concentrations required for 50% relaxation were 0.01 μM for *l*-isoproterenol, 0.6 μM for *l*-epinephrine, 1 μM for *l*-norepinephrine, and 10 μM for *l*-phenylephrine (not shown). Relaxation and the cyclic AMP increase induced by *l*-isoproterenol were dependent upon both time and concentration. *l*-Isoproterenol (1 μM) resulted in 50% relaxation and a 100% increase in cyclic AMP levels within 20 sec. Plateaux for both effects occurred within 1–2 min (not shown). Theophylline at 5 mM increased basal cyclic AMP and cyclic GMP levels 2–3-fold in 5 min. It also markedly increased the effect of 1 μM *l*-isoproterenol on cyclic AMP accumulation, from 2.5-fold to 5-fold, and decreased the concentration of *l*-isoproterenol required for half-maximal accumulation of cyclic AMP, from 0.03 μM to 0.01 μM (not shown).

Other adrenergic agonists at concentrations that were maximally effective in relaxation increased cyclic AMP levels to the

TABLE 1

*Effects of several contractile agents on cyclic nucleotide levels*

Incubations were performed by method A. Tracheal smooth muscle segments were incubated with agents for the times indicated. *N* indicates the number of tissue segments examined.

| Addition | Incubation time | *N* | Tension | Cyclic GMP | Cyclic AMP |
|---|---|---|---|---|---|
| | *min* | | *g* | *pmoles/mg protein* | |
| Experiment 1 | | | | | |
| None | 2 | 11 | 0 | 0.095 ± 0.008 | 5.78 ± 0.33 |
| Serotonin (10 μM) | 2 | 11 | 1.99 ± 0.23 | 0.128 ± 0.011[a] | 6.74 ± 0.34 |
| $K^+$ (80 mM) | 2 | 11 | 1.91 ± 0.14 | 0.149 ± 0.016[a] | 7.96 ± 0.56[a] |
| Acetylcholine (10 μM) | 2 | 11 | 2.07 ± 0.23 | 0.433 ± 0.045[a] | 6.68 ± 0.39 |
| Carbachol (1 μM) | 2 | 11 | 2.10 ± 0.21 | 0.297 ± 0.045[a] | 6.31 ± 0.47 |
| Experiment 2 | | | | | |
| Control solvent | 1 | 3 | 0 | 0.097 ± 0.019 | 3.36 ± 0.39 |
| A-23187 (10 μM) | 1 | 5 | 0.1 ± 0.05 | 0.196 ± 0.025[a] | 3.76 ± 0.48 |
| Control solvent | 3 | 3 | 0 | 0.088 ± 0.013 | 3.37 ± 0.70 |
| A-23187 (10 μM) | 3 | 5 | 1.04 ± 0.38 | 0.296 ± 0.077[a] | 4.20 ± 0.45 |

[a] $p < 0.05$ compared with control.

TABLE 2

*Effects of adrenergic agonists on carbachol-induced contraction and cyclic nucleotide levels*

Incubations were performed by method A. Contraction was induced with 0.1 μM carbachol for 2 min, and adrenergic agents were added at the concentrations indicated for an additional 1 min. Values are means ± standard errors of five observations.

| Addition | Relaxation | Cyclic GMP | Cyclic AMP |
|---|---|---|---|
| | % | *pmoles/mg protein* | |
| None | | 0.105 ± 0.014 | 3.21 ± 0.14 |
| Carbachol | | 0.094 ± 0.014 | 2.87 ± 0.12 |
| Carbachol + *l*-isoproterenol (0.1 μM) | 95 ± 3 | 0.102 ± 0.011 | 7.28 ± 0.33[a] |
| Carbachol + *l*-epinephrine (1 μM) | 98 ± 2.5 | 0.099 ± 0.007 | 6.70 ± 0.46[a] |
| Carbachol + *l*-norepinephrine (10 μM) | 95 ± 2 | 0.087 ± 0.015 | 7.80 ± 0.49[a] |
| Carbachol + *l*-phenylephrine (0.1 mM) | 100 | 0.083 ± 0.014 | 6.25 ± 0.29[a] |

[a] $p < 0.001$ compared with control.

TABLE 3

*Effects of l-isoproterenol and carbachol on muscle contraction and cyclic nucleotide levels*

Experiments were performed by method A. *l*-Isoproterenol (1 μM) was added 30 sec prior to the addition of carbachol (1 μM). Incubation was performed for 2 min with carbachol and 2.5 min with *l*-isoproterenol. Values are means ± standard errors of six observations.

| Addition | Tension | Cyclic GMP | Cyclic AMP |
|---|---|---|---|
| | *g* | *pmoles/mg protein* | |
| None | 0 | 0.096 ± 0.012 | 3.57 ± 0.15 |
| Carbachol | 2.71 ± 0.28 | 0.275 ± 0.038[a] | 3.75 ± 0.17 |
| *l*-Isoproterenol | 0 | 0.087 ± 0.019 | 8.55 ± 0.40[a] |
| *l*-Isoproterenol + carbachol | 0.95 ± 0.28 | 0.402 ± 0.091[a] | 9.24 ± 0.40[a] |

[a] $p < 0.05$ compared with control.

same degree as *l*-isoproterenol without altering cyclic GMP levels (Table 2). The effects of the *alpha* adrenergic agonist *l*-phenylephrine can be attributed to its weak *beta* adrenergic properties. This agent did not contract but relaxed the preparation, and was associated with increases in cyclic AMP and no change in cyclic GMP. The effects of 1 μM *l*-isoproterenol on cyclic AMP accumulation and relaxation were prevented with a 10 μM concentration of the *beta* adrenergic blocker propranolol, but not the *alpha* adrenergic blocker phenoxybenzamine (not shown).

*l*-Isoproterenol (1 μM) decreased the contractile response produced by 1 μM carbachol without diminishing the accumulation of cyclic GMP induced by carbachol (Table 3). Similarly, 1 μM carbachol did not decrease the accumulation of cyclic AMP without or with 1 μM *l*-isoproterenol. Thus the accumulations of cyclic AMP and cyclic GMP appear to be regulated independently of one another.

### *Effects of Phosphodiesterase Inhibitors and Prostaglandins*

Inhibitors of cyclic nucleotide phosphodiesterase increased both cyclic AMP and cyclic GMP (Table 4). At the concentrations and time tested, these compounds relaxed carbachol-induced contraction more than 60%. $PGE_1$[2] increased cyclic AMP slightly. Cyclic GMP was not changed by either $PGE_1$ or $PGF_{2\alpha}$. Neither $PGE_1$ nor $PGF_{2\alpha}$ at 5 μg/ml contracted or relaxed muscle preparations (not shown).

### *Effects of Guanylate Cyclase Activators*

We have observed that sodium azide and hydroxylamine are potent activators of guanylate cyclase (18) and produce cyclic GMP accumulation in slices of rat liver, cerebral cortex, and cerebellum, (30).

[2] The abbreviations used are: $PGE_1$, prostaglandin $E_1$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

TABLE 4

*Effects of phosphodiesterase inhibitors and prostaglandins on cyclic nucleotide levels*

Incubations were performed by method B. Agents were present for 5 min in experiment 1 and 2 min in experiment 2. *N* indicates the number of observations. Values are means ± standard errors.

| Addition | *N* | Cyclic GMP | Cyclic AMP |
|---|---|---|---|
| | | *pmoles/mg protein* | |
| Experiment 1 | | | |
| None | 3 | 0.047 ± 0.007 | 3.29 ± 0.39 |
| Papaverine (0.5 mM) | 3 | 0.120 ± 0.012[a] | 6.26 ± 0.57[a] |
| 1-Methyl-3-isobutylxanthine (0.1 mM) | 3 | 0.177 ± 0.012[a] | 10.5 ± 0.83[a] |
| Theophylline (5 mM) | 3 | 0.130 ± 0.012[a] | 7.68 ± 0.39[a] |
| Experiment 2 | | | |
| None | 6 | 0.098 ± 0.008 | 4.03 ± 0.21 |
| $PGE_1$ (5 μg/ml) | 6 | 0.107 ± 0.009 | 5.19 ± 0.33[a] |
| $PGF_{2\alpha}$ (5 μg/ml) | 6 | 0.098 ± 0.010 | 4.21 ± 0.33 |

[a] $p < 0.05$ compared with control.

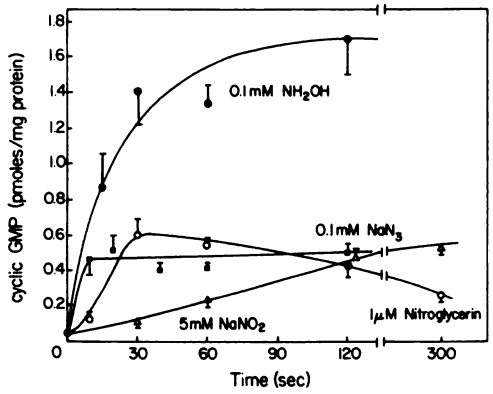


FIG. 4. *Time course of effects of $NaN_3$, nitroglycerin, hydroxylamine, and $NaNO_2$ on cyclic GMP accumulation*

Incubations were performed by method B. $NaN_3$ (0.1 mM), nitroglycerin (1 μM), hydroxylamine (0.1 mM), or $NaNO_2$ (5 mM) was present for the times indicated. Values are means ± standard errors of triplicate incubations. All the points are significantly different ($p < 0.05$) from the zero-time values.

These agents increased cyclic GMP levels and relaxed carbachol-induced contraction. Relaxation occurred immediately after addition of these agents, and cyclic GMP accumulation was also quite rapid (Fig. 4 and Table 5). Ten seconds after addition cyclic GMP was increased maximally by 0.1 mM sodium azide. Nitroglycerin, sodium nitrite, and sodium nitroprusside also increased cyclic GMP levels and relaxed muscle preparations (Fig. 4 and Table 5). The time course of cyclic GMP accumulation at maximally effective concentrations was somewhat different with each of these agents (Fig. 4). However, in all instances cyclic AMP levels were not altered (Table 5) and increases in cyclic GMP were associated with relaxation. Of these agents nitroglycerin was the most potent in increasing cyclic GMP levels, with effects at concentrations as low as 0.1 μM (Table 5). Theophylline increased the accumulation of cyclic GMP induced with sodium azide or nitroglycerin (Table 5). Effects of nitroglycerin on cyclic GMP accumulation in uterine strips and arterial segments have been reported previously (31, 32). Nitroglycerin and sodium nitroprusside also increased guanylate cyclase activity in cell-free preparations from several tissues (33).

### *Effects of Calcium Ion in Incubation Medium*

The omission of calcium ion from the incubation medium had little or no effect on cyclic GMP levels. However, the effects of carbachol, histamine, and A-23187 on cyclic GMP accumulation were markedly diminished without $Ca^{2+}$ (Fig. 5). The effects of sodium azide, hydroxylamine, and nitroglycerin on cyclic GMP accumulation were similar with and without $Ca^{2+}$ included in the incubation medium (Fig. 6). These observations suggest that there are at least two mechanisms by which cyclic GMP accumulation can occur. One is dependent upon external calcium ion, and

TABLE 5

*Effects of $NaN_3$, nitroglycerin, nitroprusside, and theophylline on carbachol-induced contraction and cyclic nucleotide levels*

Methods were similar to those of Table 3 in experiments 1-3. Carbachol (0.1 μM) was added for 2 min to induce contraction. $NaN_3$, nitroglycerin, and sodium nitroprusside were added at the concentrations indicated. $NaN_3$ and nitroglycerin were present for 30 sec, and sodium nitroprusside, for 60 sec. Values are means ± standard errors of three to five observations. Experiment 4 was performed by method B, and theophylline (5 mM), when present, was added 5 min before $NaN_3$ or nitroglycerin.

| Addition | Relaxation | Cyclic GMP | Cyclic AMP |
|---|---|---|---|
| | % | *pmoles/mg protein* | |
| **Experiment 1** | | | |
| None | | 0.097 ± 0.012 | 5.02 ± 0.67 |
| Carbachol | | 0.124 ± 0.008 | 5.05 ± 0.90 |
| Carbachol + $NaN_3$ (1 μM) | 33 ± 7 | 0.173 ± 0.018[a] | 4.65 ± 0.47 |
| Carbachol + $NaN_3$ (10 μM) | 73 ± 2 | 0.418 ± 0.028[a] | 5.07 ± 0.76 |
| Carbachol + $NaN_3$ (0.1 mM) | 88 ± 2 | 0.653 ± 0.075[a] | 4.96 ± 0.45 |
| **Experiment 2** | | | |
| None | | 0.143 ± 0.010 | 3.46 ± 0.29 |
| Carbachol | | 0.164 ± 0.002 | 3.19 ± 0.11 |
| Carbachol + nitroglycerin (0.1 μM) | 3 ± 3 | 0.199 ± 0.021[a] | 2.80 ± 0.10 |
| Carbachol + nitroglycerin (0.3 μM) | 28 ± 5 | 0.306 ± 0.027[a] | 2.75 ± 0.20 |
| Carbachol + nitroglycerin (1 μM) | 70 ± 5 | 0.741 ± 0.075[a] | 3.21 ± 0.18 |
| **Experiment 3** | | | |
| None | | 0.092 ± 0.022 | 2.76 ± 0.13 |
| Carbachol | | 0.120 ± 0.007 | 2.82 ± 0.15 |
| Carbachol + nitroprusside (10 μM) | 77 ± 13 | 0.987 ± 0.183[a] | 2.72 ± 0.29 |
| **Experiment 4** | | | |
| None | ND[b] | 0.068 ± 0.007 | 2.94 ± 0.10 |
| $NaN_3$ (10 μM) | ND | 0.533 ± 0.053[a] | 3.66 ± 0.28 |
| Nitroglycerin (1 μM) | ND | 0.305 ± 0.044[a] | 3.12 ± 0.09 |
| Theophylline | ND | 0.167 ± 0.019[a] | 6.09 ± 0.58[a] |
| Theophylline + $NaN_3$ (10 μM) | ND | 1.725 ± 0.064[a,c] | 5.31 ± 0.09[a] |
| Theophylline + Nitroglycerin (1 μM) | ND | 1.034 ± 0.098[a,c] | 5.02 ± 0.27[a] |

[a] $p < 0.05$ compared with controls with no additions.

[b] Not determined.

[c] $p < 0.01$ compared with $NaN_3$ or nitroglycerin alone.

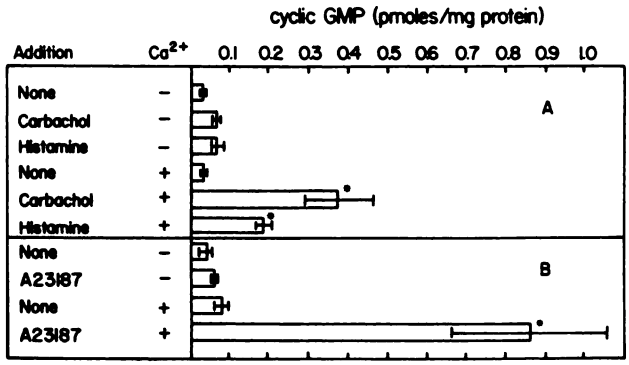


FIG. 5. *Effects of carbachol, histamine, A-23187, and $Ca^{2+}$ on cyclic GMP accumulation*

Tissues were incubated in Krebs-Ringer-bicarbonate medium for 90 min. The medium was then changed to one without or with 3.17 mM $CaCl_2$ and 1 mM EGTA, as indicated. Tissues were incubated for an additional 30 min before the addition of 1 μM carbachol or histamine for 2 min or 10 μM A-23187 for 3 min. Values are means ± standard errors of triplicate incubations.

* Significantly different ($p < 0.05$) from controls.

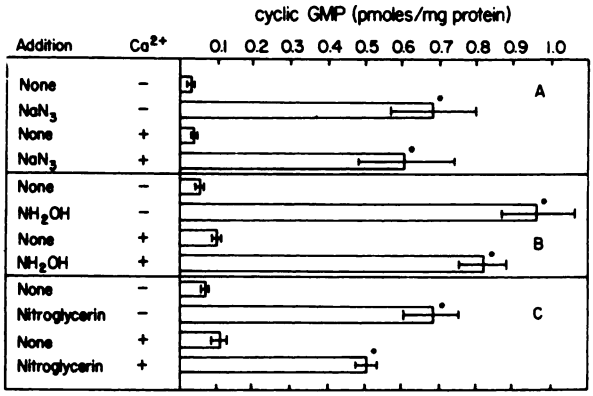


FIG. 6. *Effects of $NaN_3$, $NH_2OH$, nitroglycerin, and $Ca^{2+}$ on cyclic GMP accumulation*

Experiments were similar to those of Fig. 5, without and with $Ca^{2+}$ in the second incubation. As indicated, 0.1 mM $NaN_3$ or $NH_2OH$ was present for 1 min, and 1 μM nitroglycerin, for 30 sec. Values are means ± standard errors of triplicate incubations.

* Significantly different ($p < 0.05$) from controls.

the other is apparently independent of calcium in the medium. We have reported similar observations with incubations of rat cerebral cortex slices (30). The increase in cyclic GMP levels in rat renal cortex incubations with carbachol also requires calcium ion, while the effect of sodium azide does not (34).

### *Effects of Cyclic Nucleotides and Their Derivatives on Muscle Contractility*

Cyclic AMP, cyclic GMP, $N^6$,$O^{2'}$-dibutyryl cyclic AMP, 8-bromo-cyclic GMP, 8-bromo-cyclic AMP, 8-bromo-GMP, and 8-bromo-AMP were tested. When added alone, none of the compounds contracted muscle at concentrations ranging from 10 μM to 1 mM. With carbachol-induced contraction only 8-bromo-cyclic GMP and 8-bromo-AMP at 1 mM caused relaxation. The onset of relaxation by 8-bromo-cyclic GMP occurred within 1–2 min of addition and required several minutes to reach completion. Relaxation by 8-bromo-AMP was weaker (20–40% of maximal relaxation).

## DISCUSSION

From previous studies with various smooth muscle systems hypotheses have been developed suggesting that cyclic AMP accumulation is associated with relaxation while cyclic GMP accumulation is related to contraction. The experiments reported here were conducted in order to examine the effects of various agents on cyclic AMP and cyclic GMP accumulation in tracheal smooth muscle and correlate their changes with altered mechanical activity. In our previous experiments with cyclic nucleotide accumulation in incubations of guinea pig tracheal rings, contractility was not examined (6, 7). Our previous results and others are also difficult to correlate with expected mechanical activity, in view of the cellular heterogeneity of tracheal ring and other preparations. In the present studies cyclic nucleotide accumulation and mechanical activity of relatively homogeneous preparations of bovine tracheal smooth muscle (Fig. 1) were examined simultaneously. The use of the acetylated radioimmunoassay modification of Harper and Brooker (21) permitted such studies with as little as 10–20 mg of tissue.

Levels of cyclic AMP were increased with adrenergic agonists, cyclic nucleotide phosphodiesterase inhibitors, and high $K^+$ concentrations. These observations are similar to earlier reports with other smooth muscle preparations (1–8, 31, 35). Effects of *l*-isoproterenol on cyclic AMP accumulation and muscle relaxation were blocked by propranolol but not phenoxybenzamine, and were augmented by theophylline. Phenylephrine also increased cyclic AMP levels and produced relaxation

of carbachol-contracted muscle. These effects are probably attributable to some weak *beta* adrenergic activity of phenylephrine. Prostaglandins had little or no effect on mechanical activity or cyclic nucleotide levels. Thus receptors for *alpha* adrenergic agonists and prostaglandins in our bovine tracheal smooth muscle preparations either are absent or are present in small amounts.

Cyclic GMP levels in tracheal smooth muscle were increased by three groups of agents. One group of well-known smooth muscle contractile agents included choline esters, histamine, serotonin, the ionophore A-23187, and potassium ion. A second group included reported activators of guanylate cyclase sodium azide, hydroxylamine, sodium nitrite, sodium nitroprusside, and nitroglycerin (18, 33, 34). The third group comprised cyclic nucleotide phosphodiesterase inhibitors. The effects of agents in the first and second groups on cyclic GMP accumulation were clearly different with regard to their requirements for $Ca^{2+}$ in the medium. Increases in cyclic GMP levels with carbachol, histamine, and A-23187 required $Ca^{2+}$ in the incubation medium, while increases in cyclic GMP with sodium azide, hydroxylamine, and nitroglycerin did not. Increases in cyclic GMP levels in incubations of umbilical artery segments with choline esters, histamine, $K^+$, ionophore, and bradykinin also required $Ca^{2+}$ in the medium, while increases in cyclic GMP with serotonin did not (36). Increases in cyclic GMP levels in other tissues with carbachol, histamine, $K^+$, and norepinephrine also required $Ca^{2+}$ in the medium (13, 30, 34–36). These studies suggest that there are at least two mechanisms for cyclic GMP accumulation in these tissues. One is dependent upon extracellular $Ca^{2+}$ and the other is not. Several forms of guanylate cyclase with different kinetic properties and sizes are found in most tissues (37–39). The effects of $Ca^{2+}$ may be related to its stimulatory or inhibitory effects on the different forms of guanylate cyclase (37–39).

Although reciprocal alterations in cyclic AMP and cyclic GMP levels have been reported with some tissues (11, 16), clearly the accumulation of cyclic AMP and cyclic GMP in tracheal smooth muscle may occur quite independently of one another. Neither consistent parallel nor consistent reciprocal changes in cyclic AMP and cyclic GMP levels were observed with a variety of different agents.

A great deal of evidence has suggested that increases in cyclic AMP are responsible for relaxation of smooth muscle caused by a variety of agents. However, only our observations with *beta* adrenergic agonists are consistent with this hypothesis. The effects of these agents on cyclic AMP accumulation and relaxation of carbachol-induced contraction could not be dissociated from either time or dose. The increases in cyclic AMP that we observed under other conditions (high concentrations of carbachol, histamine, $K^+$, and $PGE_1$) were not associated with relaxation. Therefore increases in cyclic AMP may not be associated with relaxation, and relaxation is not always associated with increases in cyclic AMP. Examples of the latter were the effects of sodium azide, hydroxylamine, etc. on relaxation without alterations in cyclic AMP levels. Others have also observed dissociations of cyclic AMP accumulation and smooth muscle relaxation (4, 31, 32, 35). The presence of different functional pools of cyclic AMP in smooth muscle may be offered as an explanation for such observations (4, 31). However, additional studies are needed.

Clearly the studies reported here have pointed out several discrepancies with respect to the hypothesis that cyclic GMP accumulation is associated with smooth muscle contraction (16). Increases in contractility with carbachol preceded changes in cyclic GMP levels (10 vs. 20 sec). Also, increases in contractility occurred at lower concentrations (0.1 $\mu$M) of carbachol than those (0.3 $\mu$M) required to increase cyclic GMP levels. It has recently been reported that contraction of uterine segments with carbachol and of ductus deferens with norepinephrine also preceded changes in cyclic GMP levels (35, 40). Serotonin and high $K^+$ caused tracheal smooth muscle contraction to the same degree as carbachol. However, increases in cyclic GMP

levels with serotonin and depolarizing concentrations of $K^+$ were far less than those induced by choline esters.

Sodium azide, hydroxylamine, nitroglycerin, and sodium nitroprusside, agents that can increase guanylate cyclase activity in cell-free systems from several tissues (18, 30, 33, 34), increased cyclic GMP levels without altering cyclic AMP. All these agents were associated with tracheal smooth muscle relaxation. Diamond and co-workers (31, 32) have also reported that nitroglycerin increases cyclic GMP levels in rat myometrial and canine femoral artery segments and produces relaxation of these preparations. With the reservations of interpreting effects of exogenous cyclic nucleotides and their derivatives, 8-bromo-cyclic GMP also produced relaxation of tracheal smooth muscle.

Several cyclic nucleotide phosphodiesterase inhibitors increased both cyclic AMP and cyclic GMP accumulation 2–4-fold. However, these agents alone decreased carbachol-induced contraction. Phopshodiesterase inhibitors also increase both cyclic AMP and cyclic GMP in ductus deferens and relax this smooth muscle preparation (12, 13, 35). While theophylline increased the effects of *l*-isoproterenol on cyclic AMP accumulation, it also increased cyclic GMP accumulation due to sodium azide and nitroglycerin. High concentrations of $K^+$, carbachol, and histamine increased both cyclic AMP and cyclic GMP and induced contraction of tracheal smooth muscle. Thus examples exist in which both cyclic nucleotides or only cyclic GMP are increased and either relaxation or contraction is observed. These and other studies do not permit us to conclude that there is a simple association between cyclic GMP accumulation and mechanical activity of smooth muscle. It is noteworthy that those agents that required $Ca^{2+}$ for increases in cyclic GMP produced contraction of tracheal smooth muscle while those that did not need external $Ca^{2+}$ produced relaxation. These observations support the view that the two mechanisms which increase cyclic GMP levels could result in separate pools of cyclic GMP that are functionally different. This remains to be established, and additional studies are required to relate or dissociate cyclic GMP and mechanical activity of smooth muscle.

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