0026-895X/88/040506-12$02.00/0

MOLECULAR PHARMACOLOGY, 34:506-517

Relaxation of Vascular and Tracheal Smooth Muscle by Cyclic Nucleotide Analogs That Preferentially Activate Purified cGMP-Dependent Protein Kinase

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Received December 5, 1987; Accepted July 11, 1988

SUMMARY

Cyclic nucleotide analogs were used to study relaxation of pig coronary arteries and guinea pig tracheal smooth muscle in an attempt to determine the roles of cAMP- and cGMP-dependent protein kinases (cA-K and cG-K). In pig coronary artery strips, cGMP analogs were generally more effective than cAMP analogs in promoting relaxation of K^+-induced contractions. Significant relaxation of this tissue was caused primarily by those cyclic nucleotide analogs that had high affinities for purified cG-K but not for cA-K. The low potencies of cA-K-specific analogs, as compared with cG-K-specific analogs, could not be readily explained by either unusually high susceptibilities to phosphodiesterases or low partition coefficients. The most potent cGMP analog, 8-(4-chlorophenylthio)-cGMP, exhibited a very slow reversibility of its relaxant effects in the intact tissue, consistent with its strong resistance to hydrolysis by phosphodiesterases measured *in vitro*. Pig coronaries contained atypically high levels of cGMP and cG-K, implying a potentially important role of this enzyme in smooth muscle function.

Carbamylcholine-induced contractions of guinea pig tracheal segments were more sensitive than K^+-induced pig coronary artery contractions to relaxation by cyclic nucleotide analogs. Consequently, the number of analogs that could be studied was significantly expanded. The cGMP analogs were again generally more potent, and the effectiveness of both cGMP and cAMP analogs in relaxing this preparation correlated with the K_a of the analogs for *in vitro* activation of cG-K, but not cA-K. A particularly strong correlation was observed when the effects of analogs modified only at the C-8 position were examined. A known target enzyme of cA-K, phosphorylase, was not activated by cG-K-specific analogs but was activated by high concentrations of the cA-K-specific analogs. Studies using cyclic nucleotide analogs support a role for cG-K, but not for cA-K, in decreasing smooth muscle tone.

Several lines of evidence have implicated cyclic nucleotides as mediators of smooth muscle relaxation in response to stimuli as diverse as β-adrenergic agents, nitrovasodilators, atrial natriuretic peptide, adenosine, acetylcholine, and specific phosphodiesterase inhibitors (1–4). The mechanism whereby elevations in cAMP or cGMP levels could mediate these effects remains an enigma, although there is considerable evidence that each could play a role. With isoproterenol, epinephrine, and adenosine, there has been a strong temporal correlation as well as an agonist concentration dependence in the reduction of smooth muscle tone and elevation of cAMP levels (1, 5–7). The ability of dibutyryl-cAMP to relax a variety of smooth muscle preparations has provided additional evidence that cAMP could play a role, although a systematic study of the effects of other cAMP analogs in these tissues has not been done (8). A role for cGMP in smooth muscle relaxation is a more recent proposal (9, 10). Numerous investigators have reported relaxation associated with significant elevation of cGMP, but not of cAMP, in smooth muscle treated with nitrovasodilators (11–13). Subsequent studies with other vasodilators including atrial natriuretic peptide (14) and agents that cause release of endothelium-derived vasodilators (15–17) have demonstrated selective increases in cGMP levels and in cGMP-dependent protein kinase activation coincident with the mechanical effects of the vasodilators. Whether or not the cyclic nucleotide-dependent protein kinases function as the intracellular receptors for the cyclic nucleotides or whether another receptor exists for these compounds in smooth muscle has not been determined.

According to the criteria set forth by Sutherland and coworkers (18), cellular events modulated by changes in cyclic nucleotide levels should be mimicked by the extracellular addition of that cyclic nucleotide. The binding site requirements of the specific cyclic nucleotide receptor would dictate to a great extent the usefulness of various cyclic nucleotide analogs as promoters of that physiological response. If either of the cyclic

This work was supported in part by Research Grants HL-19325 and AM-15988 from the National Institutes of Health.

ABBREVIATION: IBMX, 3-isobutyl-1-methylxanthine.

nucleotide-dependent protein kinases is involved in smooth muscle relaxation, then the effectiveness of a given cyclic nucleotide analog could reflect the ability of that compound to activate the respective kinase. A consideration in analog studies involves the relative specificity of an analog for the two cyclic nucleotide-dependent protein kinases. Because both kinases can be activated by cAMP or cGMP analogs, it is possible that the physiological effects elicited by an analog may be attributable to either enzyme. When endogenously generated cAMP or cGMP is relatively high, the concentration of that nucleotide could be sufficient to activate either cyclic nucleotide kinase. The resistance of the analogs to cleavage by phosphodiesterases and their abilities to penetrate into the cell could also be important determinants in analog potency, but these factors have not been studied in this tissue previously.

Comprehensive studies of cyclic nucleotide specificities for kinase activation and for binding to the two different intrasubunit binding sites of both the purified cAMP- and cGMP-dependent protein kinases have been recently carried out (19, 20). The enzyme specificity studies have been expanded. It is now possible to test these analogs in intact tissues with some degree of predictability of their rank order of potencies if they act on either of the kinases. In order to achieve this, two types of smooth muscle have been used to perform a systematic analysis of the relaxation induced by 24 cyclic nucleotide analogs, some of which exhibit high specificity for one or the other of the two protein kinases.

Experimental Procedures

Materials

Carbamylcholine chloride was purchased from ICN (Irvine, CA). Heptapeptide substrates for cAMP-dependent protein kinase (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and for cGMP-dependent protein kinase (Arg-Lys-Arg-Ser-Arg-Ala-Glu) were obtained from Peninsula Laboratories (Belmont, CA). Dr. Tom Soderling (Howard Hughes Medical Institute, Vanderbilt University, Nashville, TN) provided a synthetic cAMP-dependent protein kinase inhibitor peptide (Ile-Ala-Ala-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile-His-Asp-Ile-Leu-Val-Ala-Ala), which was modelled after a protein sequence from the inhibitory region of the heat-stable protein kinase inhibitor of the cAMP-dependent protein kinase published by Cheng *et al.* (21). 8-Bromo-cAMP, 8-bromo-cGMP, N^6-benzoyl-cAMP, N^2-2′-*O*-dibutyryl-cGMP, N^2-monobutyryl-cGMP, 8-(4-chlorophenylthio)-cAMP, 8-(6-aminohexylamino)-cAMP, N^6-2′-*O*-dibutyryl-cAMP, N^6-monobutyryl cAMP, calf thymus type IIA histone, bovine serum albumin, *Crotalus atrox* 5′-nucleotidase, and IBMX were all purchased from Sigma Chemical Company (St. Louis, MO). Dr. Roland K. Robins of ICN Biomedicals kindly supplied the 8-methyl-cGMP, 8-butyryl-cGMP, 8-neopentyl-cGMP, 8-benzyl-cGMP, 8-carbamoyl-cGMP, and 8-(1-hydroxyethyl)-cGMP. [^{3}H]cAMP was from New England Nuclear (Boston, MA) and [^{3}H]cGMP was from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP was prepared according to the method of Walseth and Johnson (22).

Methods

Synthesis of cyclic nucleotide analogs. A number of cyclic nucleotide analogs were synthesized according to published procedures. These included 8-(4-chlorophenylthio)-cGMP (23), β-phenyl-1-N^2-etheno-cGMP, and 1-methyl-cGMP (24). After the syntheses the compounds were purified by Sephadex G-25 (superfine) chromatography in 50 mM ammonium bicarbonate at pH 7.8 (25). These cGMP analogs were retained on the Sephadex column and were well separated from cGMP as shown in Fig. 1. The analog 1-methyl-cGMP eluted at tube 13 just before cAMP (data not shown). Structures of the β-phenyl-1-N^2-etheno-cGMP and the 8-(4-chlorophenylthio)-cGMP were verified by mass spectral analysis, which indicated that the major peaks in the spectrum corresponded to the predicted molecular mass of the protonated and unprotonated forms of the analog. There was no evidence for any other cyclic nucleotide component in the preparation.

Tissue studies. *Pig coronary arteries.* Pig coronary artery tissue was prepared essentially as described by Lorenz and Wells (26). Pig coronary arteries were obtained from a local slaughterhouse and packed in ice until dissection. A segment of the right coronary artery was dissected and placed in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 127 mM NaCl, 16 mM $NaHCO_3$, 4.74 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 2.54 mM $CaCl_2$, 10 mM glucose, and 1 mM pyruvate (buffer A). The buffer was bubbled continuously with 95% O_2/5% CO_2. Vessels were dissected in a way that disrupts functional endothelium (27). A small glass rod was inserted through each vessel and the vessel was carefully trimmed of all visible fat and fascia. Each artery was then cut into helical strips approximately 3 mm in width. The tissues were then stored at 0–4° overnight in the same buffer (with adjustment of the bicarbonate level to maintain pH 7.4 at this temperature) with aeration before use the following day. Small segments (1.5 cm in length, ~50 mg in weight) were cut from the spiral arterial strips and mounted by surgical threads in individual organ baths of 2.5 ml each at 37°. Two grams of tension were applied to the tissue for approximately 2 hr with periodic adjustments of tension until no further spontaneous relaxation was observed. Isometric tension was recorded by Statham UC2 isometric force transducers connected to Gould-Brush 2400 recorders. After the initial 2 hr of equilibration and several buffer changes, the tissue segments were sequentially contracted and relaxed by the addition and removal of Krebs-Ringer bicarbonate buffers containing 117 mM K^+ (buffer B), attained by substituting K^+ for Na^+ in buffer A. This treatment brought the tissues to a state of relatively constant responsiveness to the subsequent contractile stimulus. Contraction for the experimental studies was initiated by replacement of buffer A by the same Krebs-Ringer bicarbonate, in which the K^+ concentration had been increased to 20 mM K^+ (buffer C) and the Na^+ level reduced to 128 mM. After tension had stabilized (approximately 45 min later) at an apparent maximum for those conditions, analogs were added, unless otherwise specified in the text. The tension generated by these strips in response to the 20 mM K^+ challenge was typically 15–20% of the maximum tension generated by the 117 mM K^+-induced contraction (12–25 g). At the end of the analog treatment, 100 μM IBMX was added. The difference between the stable tension achieved before addition of any relaxants and the tension achieved after IBMX treatment was defined as 100% relaxation.

Guinea pig trachealis. Tracheal tissues were dissected from Hartley guinea pigs, either sex (400–450 g), that had been sacrificed by decapitation. Trachea were placed in buffer A and carefully trimmed of extraneous tissue before storage in Krebs-Ringer bicarbonate buffer at 0–4° with continuous aeration with 95% O_2/5% CO_2. Tracheal tissues were used within 1 day of sacrifice. The trachea were cut into rings ~1 mm in width, tied with surgical suture, and mounted in 2.5-ml tissue baths containing buffer A, with continuous aeration at 37°. The tracheal rings were then cut on the cartilage side and 2 g of tension was applied. Tension was measured as described above. Tissues were allowed to stabilize for 60–90 min without further tension adjustment. The trachealis muscle was then contracted with 0.1 μM carbamylcholine chloride. After achieving a stable contraction (after ~15 min) of ~40% of the maximum tension that could be generated by 10 μM carbamylcholine, the stimulus was removed by repeated exchanges with buffer A. After return to baseline tension, the muscle was contracted by a second addition of carbamylcholine and the cyclic nucleotide analogs were added after the tension had stabilized. IBMX (100 μM) was then added to achieve full relaxation of the tissue. The percentage of relaxation in the tissue was calculated as described for the pig coronary artery preparation. Tissues used for phosphorylase studies were allowed to contract for 40 min before addition of analogs.

Tissue extract preparations. Preparation of tissue extracts varied depending on the biochemical parameters to be measured. In order to

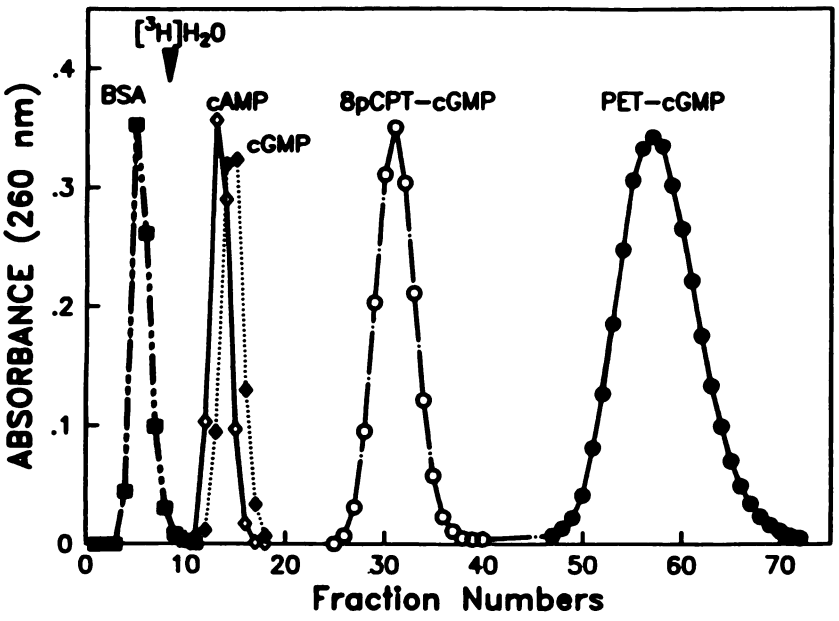

Fig. 1. Chromatography of nucleotides on Sephadex G-25. Standard solutions of cGMP (♦), cAMP (◇), β-phenyl-1-N^2-etheno-cGMP (●) (*PET-cGMP*), 8-(4-chlorophenylthio)-cGMP (○) (8pCPT-*cGMP*), [^{3}H]H_2O, and bovine serum albumin (□) were chromatographed on Sephadex G-25 (superfine) (0.9 × 13 cm) in 50 mM ammonium bicarbonate, pH 7.8, as described in Experimental Procedures.

study cyclic nucleotide concentrations and enzyme activities in either pig coronary arteries or guinea pig trachealis, the tissues were quickly rinsed in the appropriate buffer and frozen in liquid nitrogen immediately after the respective treatments. The tissues were stored at −70° until used for these analyses. The frozen tissue was powdered using a Wig-L-Bug dental amalgamator (Cresent Dental Manufacturing Co., Lyons, IL). Capsules and pestles used in the amalgamator had been precooled in liquid nitrogen. The capsule was then shaken in the Wig-L-Bug three times, for 20 sec each time, and the capsules were cooled in liquid nitrogen before and after each agitation. The powdered tissue was either used immediately or stored at −70°.

Purification of cGMP and cAMP in tissue extracts. To avoid interference by other metabolites in crude extracts, cGMP and cAMP were purified before assay. The powdered tissue (~200 mg) was homogenized in 700 μl of 10 mM KH_2PO_4 (pH 6.8), 20 mM EDTA, and 1 mM IBMX, using a motor-driven Dounce homogenizer with a Teflon pestle and a glass homogenizing tube (four up and down strokes). The resulting homogenate was heated for 5 min in a boiling H_2O bath, followed by centrifugation in a microfuge for 7 min. The supernatant fraction (0.5 ml) was combined with either 10 μl of 67 pM [^{3}H]cGMP or [^{3}H]cAMP and 3–4 mg of sucrose crystals. This mixture was chromatographed on a 0.9 × 13 cm Sephadex G-25 (superfine) column equilibrated in 10 mM KH_2PO_4 (pH 6.8) at 4°. As shown in Fig. 1, under these conditions cGMP was partially separated from cAMP but complete separation of the two nucleotides could be achieved by extending the column length. The elution position of ATP slightly preceded that of [^{3}H]H_2O. Fractions containing [^{3}H]cGMP or [^{3}H]cAMP tracer were analyzed for cyclic nucleotide content.

Cyclic nucleotide assays. *cGMP assay.* For cGMP determinations, a partially purified cGMP-dependent protein kinase, which was highly cGMP dependent, was prepared as described below. The high degree of cGMP dependence exhibited by this preparation was crucial for the sensitivity of the assay.

One pair of beef lungs (2.3 kg) was collected from a local slaughterhouse, and transported on ice to the laboratory and all subsequent procedures were performed at 0–4°. The lung was sectioned with a knife, passed through a meat grinder, and homogenized in a Waring blender in 4 volumes (ml/g) of 10 mM KH_2PO_4, 1 mM EDTA, 25 mM 2-mercaptoethanol at pH 6.8 (buffer D). The homogenate was centrifuged at 17,680 × *g* in a Beckman JA10 rotor for 30 min. The supernatant was filtered through glass wool and then mixed with DEAE-cellulose (DE-52) in a 5 to 1 volume ratio of supernatant to settled DEAE. This suspension was mixed vigorously and allowed to settle four times over a period of 1 hr and then allowed to sit overnight. The supernatant was decanted and the resin was washed batchwise three times with 3 liters of 50 mM NaCl in buffer D before being poured into a column (7.6 × 22 cm). The resin was washed further with 3 liters of the 50 mM NaCl buffer and the cGMP-dependent protein kinase was eluted with 4 liters of 200 mM NaCl in buffer D. Fractions of 500 ml each were collected and assayed for cGMP binding. The peak fractions (usually fractions 3 and 4) containing activity (235 pmol of cGMP bound per ml) were pooled, diluted 4-fold with H_2O, and applied onto a DEAE-cellulose column (100 ml) equilibrated with buffer D. The column was washed with 500 ml of buffer D containing 50 mM NaCl and then eluted with a 400-ml linear gradient (50–250 mM NaCl). One main peak of cGMP-dependent protein kinase activity, eluting at 150 mM NaCl, and a trailing shoulder were usually observed. Fractions of the leading edge of the main peak, which exhibited activity of highest cGMP dependence, were pooled (activity = 4 nmol of P_i/min/ml) and small aliquots were stored at −70°. The enzyme in the leading edge was recently shown (28) to contain mainly cGMP-free enzyme, whereas the trailing edge contains bound cGMP. The hand-thawed enzyme was used once for the cGMP assay.

A standard curve of known cGMP concentrations ranging from 0.156 to 10 nM cGMP was constructed, using the cGMP-dependent protein kinase described above. The fractions or standards (200-μl volumes) were added to 20 μl of cGMP-dependent protein kinase assay mixture as described below. The reaction was started by the addition of 20 μl of thawed kinase (diluted 2.5-fold in buffer D containing 1 mg/ml bovine serum albumin and 10 mM 2-mercaptoethanol) and the mixture was incubated overnight at 0°. Aliquots (50 μl) of the reaction mixture were then spotted on phosphocellulose papers that were washed and counted as described previously (29). The cGMP in the fraction was determined from the standard curve and corrected for the recovery (~50% in peak fraction) of [^{3}H]cGMP tracer in the fractions. The sensitivity of the assay was about 100-fold greater at 0° than at 30° as seen in Fig. 2, thereby allowing for assay of samples with very low concentrations of cGMP. The high specificity of the cGMP-dependent protein kinase assay under these conditions eliminated interference in the assay by any cAMP in the samples. The K_a for cAMP activation of cGMP-dependent protein kinase at 0° was approximately 0.1 μM (not shown).

cAMP assay. The cAMP content of samples was determined by a cAMP-dependent protein kinase activation assay as described by this laboratory (30). This assay was similar to that described above for cGMP. There was no interference by cGMP in the samples.

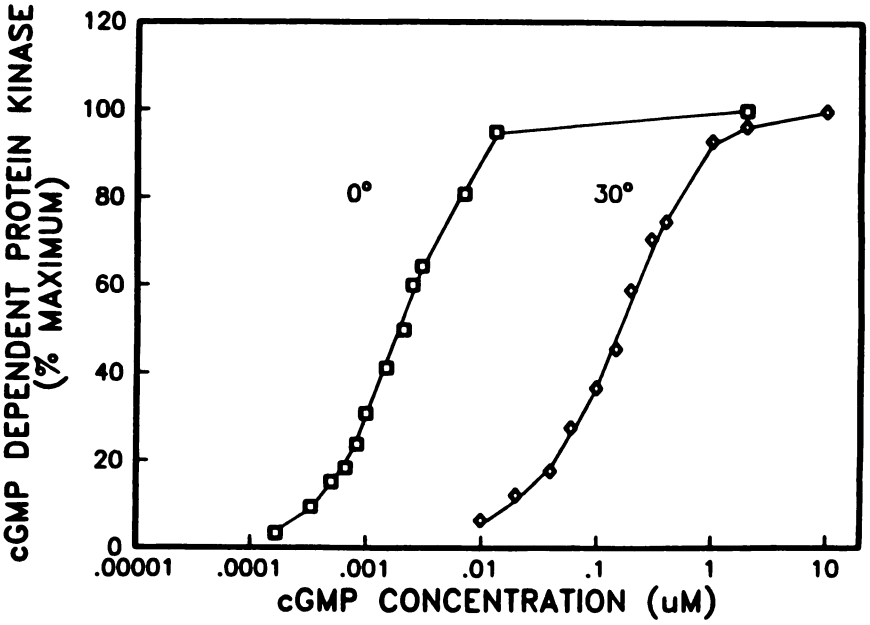

Fig. 2. Assay of cGMP by protein kinase activation at 0° and 30°. A frozen aliquot of partially purified bovine lung cGMP-dependent protein kinase prepared as in Methods was thawed by hand warming. The assay was done as described in Methods except that the incubation volume was 160 μl at 0° (□) and 70 μl at 30° (◇), and the final enzyme concentration was 0.42 nM at 0° and 1.2 nM at 30°. The enzyme concentrations were calculated on the basis of a specific activity of 5 μM P_i incorporated into heptapeptide per min per mg enzyme at 30°. The incubation time was 18 hr at 0° and 10 min at 30°. Total activity was 22.2 and 3452 pmol per min per ml of undiluted enzyme at 0° and 30°, respectively. The cGMP concentration indicated is the final concentration in the incubation mixture.

Protein kinase activities. For cAMP-dependent protein kinase and cGMP-dependent protein kinase measurements in extracts, the powdered pig coronary artery was suspended in 10 mM KH_2PO_4, 1.0 mM EDTA, 150 mM NaCl, pH 6.8, at 0–4°, and quickly homogenized in a Dounce homogenizer, using a motor-driven chilled Teflon pestle. For determination of the cAMP-dependent protein kinase activity ratio, the tissue was homogenized in 10 mM KH_2PO_4, 10 mM EDTA, 150 mM NaCl, and 0.1 mM IBMX. The homogenates were then centrifuged at 11,600 rpm for 5 min at 0–4° and the supernatant fractions were assayed. Cyclic AMP-dependent protein kinase was assayed as previously described, using a specific heptapeptide substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) (29) in the presence and absence of 2 μM cAMP. Cyclic GMP-dependent protein kinase was assayed in the presence and absence of 2 μM cGMP, using the same procedure except that this assay included 150 μM of a heptapeptide (Arg-Lys-Arg-Ser-Arg-Ala-Glu) that was shown by Glass and Krebs (31) to be a relatively specific substrate for cGMP-dependent protein kinase. Specific inhibition of cAMP-dependent protein kinase activity in the cGMP-dependent protein kinase assay was achieved by including 15 μM synthetic peptide inhibitor of the cAMP-dependent protein kinase (21). Kinase reactions were stopped by spotting aliquots of the reaction mixture onto Whatman P-81 phosphocellulose papers, which were washed in 75 mM phosphoric acid according to the method of Roskoski (29).

Separation of the isozymic forms of the cAMP-dependent protein kinase was done using the medial layer from 10 fresh pig coronary arteries. After dissection these layers were homogenized with an Ultraturex homogenizer in buffer D and centrifuged at 10,000 rpm for 30 min at 0–4°. The supernatant fraction was chromatographed on DEAE-cellulose using a linear gradient of 0–0.4 M NaCl to separate the types I and II cAMP-dependent protein kinase isozymes as previously described (32).

[^{3}H]cAMP binding assay. The cAMP binding assay (33) included 50 μl of homogenate, 50 μl of 50 mM KH_2PO_4, pH 6.8, containing 1 mM EDTA, 2 M NaCl, 0.5 mg/ml type IIA histone, 1 μM unlabeled cGMP, and 1 μM [^{3}H]cAMP. Samples were incubated at 30° for 45 min before the addition of 1 ml of cold 10 mM KH_2PO_4/1 mM EDTA. This mixture was immediately transferred to a premoistened 0.45-μm Millipore filter in a Millipore filtration device, the tube was rinsed with another 1-ml aliquot of cold KH_2PO_4/EDTA, and then the filter was rinsed with eight 1-ml aliquots of cold buffer. The filters were dried and counted in a nonaqueous scintillant.

[^{3}H]cGMP binding assay. The assay was done essentially as described by Corbin and Doskeland (34). The assay mixture was the same as that for the cAMP binding assay except that the NaCl was omitted and labeled 1 μM [^{3}H]cGMP and 1 μM unlabeled cAMP were included. Coronary artery extract (50 μl) was combined with assay mix (50 μl) and the mixture was incubated at 30° for 30 min. Two milliliters of cold saturated $(NH_4)_2SO_4$ was added to each tube and samples were kept at 0–4° until filtered by vacuum filtration to separate bound [^{3}H]cGMP from free [^{3}H]cGMP. The 0.45-μm Millipore filter paper was wet with 1 ml of cold water, then rinsed with 2 ml of the cold $(NH_4)_2SO_4$ solution. The sample was then applied to the filter and the sample tube was washed with 2 ml of additional $(NH_4)_2SO_4$ solution. The paper was washed with two more rinses of 2 ml each of the cold $(NH_4)_2SO_4$. The papers were dried, dissolved in 2 ml 2% sodium dodecyl sulfate, and counted in aqueous scintillant.

Analog activation of cyclic nucleotide-dependent protein kinases. Bovine lung cGMP-dependent protein kinase was purified to homogeneity using 8-(6-aminohexylamino)-cAMP-Sepharose according to a published procedure (34). This enzyme preparation was used to measure the K_a of analogs for activation (34). The partially purified isozymes of cAMP-dependent protein kinase were prepared from rat and rabbit skeletal muscle and bovine heart; the K_a for analog activation was determined as previously described (19).

Calculation of the intracellular molar concentrations of cyclic nucleotide-dependent protein kinases. The intracellular molar concentrations of the cyclic nucleotide-dependent protein kinases were determined in crude extracts in two ways. First, the measured protein kinase activities were compared with the known specific catalytic activities of the respective purified enzymes. The specific activity of pure cGMP-dependent protein kinase under these conditions was determined in each assay of crude extracts. The specific activity for the catalytic subunit of cAMP-dependent protein kinase was 15 μmol of P_i/min/mg. The concentrations of these two enzymes were also assayed by measuring the specific binding of [^{3}H]cAMP or [^{3}H]cGMP to the respective proteins in the extracts as described previously in this section. Tissue weight was assumed to be 50% intracellular water. Four cyclic nucleotide binding sites were assumed per holoenzyme molecule for both kinases (34, 35).

Phosphodiesterase activity. Frozen powdered tissue (500 mg) from pig coronary arteries was homogenized in 2 ml of 20 mM NaH_2PO_4, pH 6.8, 10 mM 2-mercaptoethanol, pH 6.8. After centrifugation at 12,000 × *g* for 20 min at 0–4°, the supernatant fraction was assayed for cyclic nucleotide phosphodiesterase activity. The assay contained 50 mM Tris, pH 7.5, 0.33 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, and various concentrations of cyclic nucleotides at 30°. Two

types of phosphodiesterase experiments were conducted. In one set of experiments, the ability of a variety of cyclic nucleotide analogs to inhibit the hydrolysis of 0.1 μM [^{3}H]cAMP was studied. These experiments were conducted at 30° for 20 min and the reactions were terminated by the addition of 20 μl of a stop mixture containing 10 mM cGMP, 10 mM cAMP, 50 mM EDTA, 30 mM theophylline, and 100 mM Tris, pH 7.5. *Crotalus atrox* snake venom (200 μg) was added for another 10 min at 30° and this reaction was stopped by the addition of 1 ml of 15 mM EDTA, 0.1 mM adenosine, and 0.1 mM guanosine. The mixture was chromatographed over (QAE)-Sephadex as previously described (36) and the effluent was counted in a Triton-based scintillant. In the second set of phosphodiesterase experiments, the hydrolysis of selected analogs by the coronary extracts was examined directly using three concentrations of the respective analogs (0.1, 1, and 10 μM). The reaction mixtures were incubated at 30° for 30 min and 2.5 hr. The reaction was terminated by the addition of IBMX and EDTA to a final concentration of 0.15 mM and 2 mM, respectively. The samples were then placed in a boiling water bath for 7 min, followed by centrifugation in a Beckman TJ-6 centrifuge at 2800 rpm for 10 min. The supernatants were stored at −20° before being assayed using the kinase assays described previously. The extent of cGMP analog hydrolysis was assayed using cGMP-dependent protein kinase activation and a standard curve generated by various concentrations of that particular analog. The hydrolysis of cAMP analogs was assessed similarly, using the cAMP-dependent protein kinase.

Phosphorylase assay. Phosphorylase activity was determined by measuring the incorporation of [^{14}C]glucose-1-phosphate into glycogen in the presence of 100 μM 5′-AMP or 80 μM caffeine as previously described (37). Individual frozen guinea pig trachealis muscle strips were suspended in 0.6 ml of buffer containing 40 mM β-glycerophosphate, 10 mM EDTA, 20 mM sodium fluoride, and 10 mM 2-mercaptoethanol at pH 6.8. Tissues were then pulverized by two × 10-sec Ultraturex treatments at 0–4°. The samples were centrifuged in a refrigerated Beckman desktop centrifuge for 10 min and the supernatant fractions were assayed for phosphorylase activity.

Protein assay. Protein was measured by the method of Bradford (38).

Determination of partition coefficients. Partition coefficients of the cyclic nucleotides were determined using 1-butanol or 1-pentanol as the organic phase and 0.05 M KH_2PO_4, pH 7.4, as the aqueous phase. The organic and aqueous phases were thoroughly mixed in a separatory funnel and allowed to separate into the respective phases by standing at room temperature overnight. The cyclic nucleotide analogs were diluted to a final concentration of 0.05 M, using the aqueous phase prepared as described above. The nucleotide mixture (1 ml) was then combined with the appropriate organic phase (3 ml) and vigorously agitated on a Vortex mixer for 30 sec. The samples were then centrifuged and the aqueous phase was used to determine the absorbance at 260 nm for each nucleotide before and after the organic extraction. The partition coefficient was determined by the ratio of the quantity of analog removed from the aqueous phase divided by the amount of analog remaining in the aqueous phase after extraction.

Results

Studies of Pig Coronary Arteries

Comparison of potency of cyclic nucleotide analogs to relax the tissues and to activate cyclic nucleotide-dependent protein kinases. The ability of cyclic nucleotide analogs to relax pig coronary artery segments was examined. Contractions were initiated by replacement of Krebs-Ringer buffer containing 5.9 mM K^+ with the same buffer containing 20 mM K^+. The tension generated under these conditions represented 13–20% of the maximum tension generated by 117 mM K^+. The tension was allowed to stabilize before the addition of analogs. After the tissue had partially relaxed in response to each analog and the tension had stabilized, 100 μM IBMX was added to achieve full relaxation. A typical tracing of these events is shown in the upper panel of Fig. 3.

The relative potencies of a number of cyclic nucleotide analogs in promoting relaxation and in activating cGMP-dependent protein kinase are listed in Table 1. Preincubation of the tissue with several of these analogs resulted in a decrease in baseline tension and either diminished or blocked contractile responses induced by 20 mM K^+ (not shown). Many of the analogs that were most effective in activating the cGMP-dependent protein kinase were also some of the most potent compounds in relaxing the vascular smooth muscle. With one exception [8-(4-chlorophenylthio)-cAMP], cAMP analogs were much less effective than cGMP analogs in inducing relaxation. Because 8-(4-chlorophenylthio)-cAMP activated the cGMP-dependent protein kinase as well as the cAMP-dependent protein kinase at low concentrations, its effects on relaxation could be explained by its ability to activate the former. The decreases in tension induced by the analogs were relatively slow, concentration-dependent, and reversible. With increasing concentrations of analogs, the relaxation occurred more rapidly. For instance, the time required for complete relaxation induced by 8-(4-chlorophenylthio)-cGMP varied markedly with the concentrations of analog used (33 μM required 65 min, 65 μM required 40 min, and 130 μM required 33 min). In order to demonstrate reversal after analog treatment, the buffer in the baths was exchanged at least six times (buffer A) over a 1-hr period before recontracting the strips with buffer C. In response to this challenge, the coronary strips, which had been relaxed by the addition of cyclic nucleotide analogs, contracted with the same force as they did before analog exposure. In the tissues treated with 8-(4-chlorophenylthio)-cGMP, the effect persisted for a much longer period of time, despite repeated exchanges of buffer A. After relaxation with this compound (30 μM), 3 hr with 15 buffer changes was required to reverse the effect. The differences in refractoriness might be explained by different relative susceptibilities of these analogs to hydrolytic cleavage by endogenous phosphodiesterases or to the abilities of the analogs to diffuse out of the cell.

Several of the more potent analogs induced a partial relaxation of a contraction produced by 117 mM K^+. Muscle contraction by 117 mM K^+ represented the maximum tension that could be generated in the tissue. The ability of the analogs to effect only a partial relaxation under these conditions was not unusual. Miller and Wells (39) found that, using maximally contracted tissues, even high levels of isoproterenol (10 μM), a potent physiological vasodilator, induced only a partial relaxation.

Partition coefficients of analogs. The effectiveness of an analog in inducing a cellular response could be related to a number of factors, including its ability to penetrate the cell membrane to obtain access to the relevant enzymes. Although the properties necessary for a compound to traverse the plasma membrane are unclear, the ability to partition into lipids may be important, expecially if the agent partitions nearly completely into either aqueous or lipid phases. Therefore, the partition coefficients of the cyclic nucleotide analogs were determined using either *n*-butanol or *n*-pentanol as the extracting solvent. The analogs are listed in Table 2 in order of decreasing ability to activate the cGMP-dependent protein kinase. Although a number of the most potent analogs effi-

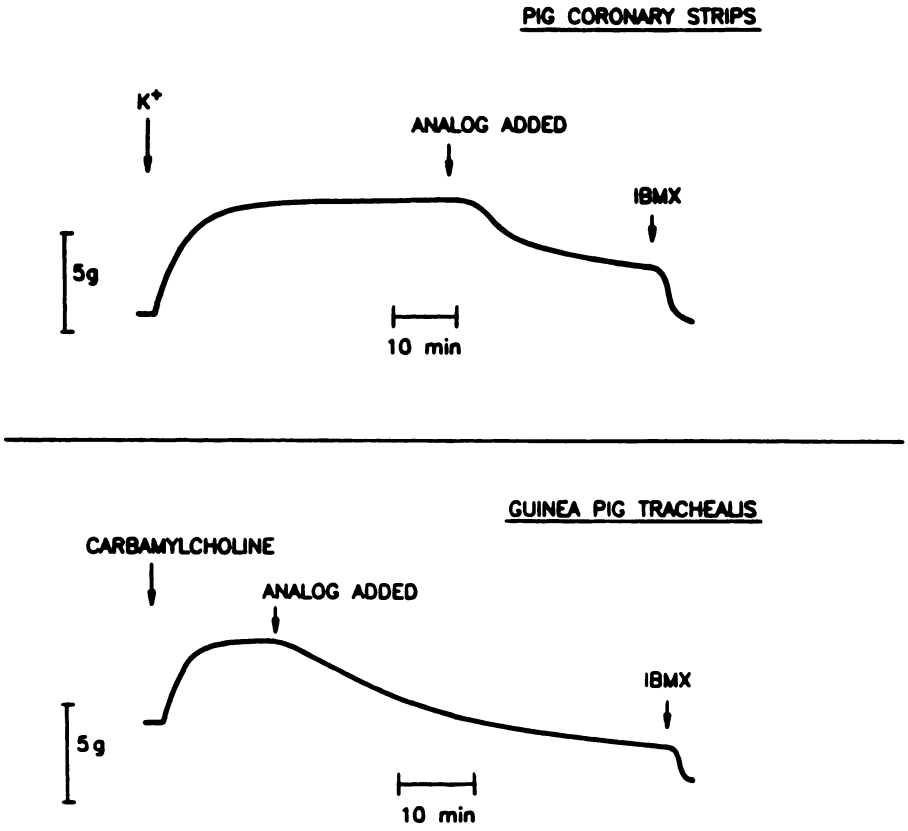

Fig. 3. Tension recordings of contraction and relaxation of a pig coronary artery strip and a guinea pig tracheal ring. *Upper*, pig coronary artery strip contracted with 20 mM K^+ as described in Experimental Procedures. *Lower*, guinea pig trachael ring contracted with 0.1 μM carbamylcholine. Relaxation profile upon analog addition was typical for a number of analogs.

TABLE 1

Comparison of cyclic nucleotide analog potencies in relaxing pig coronary artery and K_a of the analogs for cGMP-dependent protein kinase

The K_a for analog activation of purified bovine lung cGMP-dependent protein kinase was determined as described in Experimental Procedures. The EC_{50} is the concentration of analog producing a 50% decrease in 20 mM K^+-induced tension in the coronary strip. Complete relaxation of the tissue after analog treatment was achieved by the addition of 100 μM IBMX. These measurements were used in calculating tissue tension changes as described in Experimental Procedures. The cAMP-dependent protein kinase isozymes, types I and II, were from rabbit muscle and bovine heart, respectively. cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase.

Analog	Potency in activating cGK, K_a	Potency in relaxing muscle, EC_{50}	Potency in activating cAK, K_a	
			I	II
	μM	μM	μM	
8-Bromo-cGMP	0.025	47	2.8	12.5
β-Phenyl-1,N^2-etheno-cGMP	0.027	22	16.5	8.7
8-(4-Chlorophenylthio)-cGMP	0.050	18	0.74	1.3
8-Benzylthio-cGMP	0.085	60	3.0	3.0
6-Thio-cGMP	0.085	80	ND[a]	ND
8-(4-Chlorophenylthio)-cAMP	0.200	30	0.007	0.009
1-Methyl-cGMP	0.297	3700	49	40.2
N^6-Benzoyl-cAMP	1.0	>600	0.098	0.200
N^2-Monobutyryl-cGMP	2.1	2630	50.0	25.0
8-Thiomethyl-cAMP	3.4	1200	0.130	0.090
8-Thioethyl-cAMP	3.4	500	0.03	ND
8-(6-Aminohexylamino)-cAMP	4.0	>600	0.083	0.750
8-Bromo-cAMP	5.8	1670	0.065	0.053
cIMP	10.0	>>1000	0.64	0.92
N^2-2′-O-dibutyryl-cGMP	19.0	>>1000	2	1

[a] ND, not determined.

ciently partitioned into the organic phase, this factor was not correlated to the effectiveness of the analogs in promoting relaxation of the muscle strip. Both N^2-monobutyryl-cGMP and N^2,2′-O-dibutyryl-cGMP had high partition coefficients in *n*-butanol (29.3 and 3.2, respectively) but were weak in promoting relaxation. In contrast, 8-bromo-cGMP and 1-methyl-cGMP had much lower partition coefficients (1.2 and 0.4) and yet these compounds were much more potent agents in decreasing tension. Comparison of the partition coefficients of cAMP analogs with those of cGMP analogs did not provide an explanation for the impotence of the cAMP compounds. Although 8-bromo-cAMP partitioned into the *n*-butanol more efficiently than did 8-bromo-cGMP (1.6 versus 1.2), the 8-bromo-cGMP was 34 times more potent as a relaxing agent. In a study by Beebe *et al.* (40), consideration of the partition coefficients alongside other parameters such as K_a for analog activation of cAMP-dependent protein kinase and analog resistance to hydrolysis by phosphodiesterase proved very useful in explaining the pattern of analog effectiveness. However, in the present study there appeared to be no obvious relationship between abilities of the analogs to partition into organic solvents and their abilities to initiate the relaxation response.

Interaction of analogs with cellular phosphodiesterases. The ability of cyclic nucleotide analogs to promote a cellular response is likely to be related in part to the interaction of analogs with cellular phosphodiesterases. Analogs that are hydrolyzed efficiently may not achieve concentrations sufficient to activate the cyclic nucleotide-dependent protein kinases and those that are very resistant to hydrolysis could be more potent activators of the system. Furthermore, analogs that compete well for the catalytic sites of the phosphodiesterases could increase endogenous cyclic nucleotides by protecting

TABLE 2

Cyclic nucleotide analog partition coefficients and inhibition of cAMP phosphodiesterase activity

I_{50} for crude cAMP phosphodiesterase activity from pig coronary arteries was determined as described in Experimental Procedures in the presence of 0.1 μM [^{3}H]cAMP, 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.33 mg/ml bovine serum albumin, and 0.1 mM $CaCl_2$. An average of eight concentrations of each analog was used to determine the concentration of analog required to produce 50% inhibition of cAMP hydrolysis.

Analog	Partition Coefficient		cAMP PDE, I_{50}
	n-Pentanol/Aqueous	*n*-Butanol/Aqueous	
			μM
8-Bromo-cGMP	0.11	1.15	24
β-Phenyl-1-N^2-etheno-cGMP	8.27	ND[a]	24
8-(4-Chlorophenylthio)-cGMP	4.06	ND	100
8-Benzyl-thio-cGMP	3.57	8.44	152
cGMP	ND	0.27	1.1
8-(4-Chlorophenylthio)-cAMP	14.97	ND	32
8-Carbamoyl-cGMP	ND	0.30	20.5
1-Methyl-cGMP	0.07	0.42	2.2
8-Thio-cGMP	ND	0.32	7.2
8-Methyl-cGMP	ND	0.47	60.8
8-(6-Aminohexylamino)-cAMP	ND	3.69	>200
1-Amino-cGMP	ND	0.21	15.3
8-Butyryl-cGMP	ND	3.40	6.4
8-Hydroxy-cGMP	ND	0.02	ND
8-Benzyl-cGMP	ND	4.74	43.2
N^2-Monobutyryl-cGMP	ND	3.19	24
8-Thioethyl-cAMP	ND	3.05	14
8-Thiomethyl-cAMP	ND	1.78	20
8-Bromo-cAMP	0.3	1.6	5.0
8-(1-Hydroxylethyl)-cGMP	ND	0.30	ND
2-Fluoro-cIMP	ND	0.02	ND
cIMP	0.02	0.11	1.1
8-Neopentyl-cGMP	ND	6.46	43
N^2-2-O'-dibutyryl-cGMP	7.63	29.31	250
N^6-Benzoyl-cAMP	ND	2.98	ND

[a] Not determined.

them against hydrolysis. Two approaches were used to assess the contribution of phosphodiesterase activity in the pattern of analog effectiveness in intact tissue. In the first study, the abilities of analogs to inhibit the hydrolysis of a low concentration (0.1 μM) of [^{3}H]cAMP by phosphodiesterases in crude extracts from pig coronary arteries were used as an estimation of their interaction with cAMP phosphodiesterase catalytic sites. The I_{50} for an analog was calculated from a curve based on ~8 different concentrations of the analog. As shown in Table 2, 8-bromo-cGMP, β-phenyl-1-N^2-etheno-cGMP, N^2-monobutyryl-cGMP, 8-thioethyl-cAMP, and 8-thiomethyl-cAMP inhibited endogenous cAMP phosphodiesterases at similar concentrations. However, 8-bromo-cGMP and β-phenyl-1-N^2-etheno-cGMP were significantly more potent than N^2-monobutyryl-cGMP and 8-thioethyl-cAMP as promoters of muscle relaxation (Table 1). Likewise, the same two analogs were much more potent as activators of the purified cGMP-dependent protein kinase than were N^2-monobutyryl-cGMP, and 8-thiomethyl-cAMP (Table 1).

In a second series of experiments, the hydrolysis of several analogs by the phosphodiesterases in pig coronary artery extracts was studied directly, using the respective cyclic nucleotide-dependent protein kinase assays as described in Experimental Procedures. Three concentrations of analogs (0.1, 1, 10 μM) were used in these studies. Cyclic GMP analogs (Table 3) appeared to be somewhat more resistant to hydrolysis by phosphodiesterases and 8-(4-chlorophenylthio)-cGMP, in particular, was very resistant to hydrolysis, which may contribute to

TABLE 3

Hydrolysis of cyclic nucleotide analogs by a crude supernatant fraction of pig coronary artery

The rate of cAMP and cGMP hydrolysis was measured at earlier time points and mathematically corrected to a 30-min rate to provide comparable numbers. For the studies with cAMP, 30-min incubation at 10 μM cAMP caused a 47% loss in cAMP; at 1 μM a 15-min incubation at 10 μM cAMP caused a 47% loss in cAMP; at 1 μM a 15-min incubation caused 37% hydrolysis. For studies with cGMP, 10 min of incubation reduced 10 μM cGMP by 45% and a 3 min incubation with 1 μM cGMP resulted in a hydrolysis of 40% of the substrate. The experimental methods used in determining the extent of hydrolysis are described in Experimental Procedures.

Analog	EC_{50}	30 min			2.5 hr		
		10 μM	1 μM	0.1 μM	10 μM	1 μM	0.1 μM
	μM	% hydrolyzed					
cAMP		47	74	100			
cGMP		100	100	100			
8-(4-Chlorophenylthio)-cAMP	30	23	26	13	42	77	41
8-(4-Chlorophenylthio)-cGMP	18	0	0	0	0	0	0
8-Bromo-cAMP	1670	6	22	45	46	76	90
8-Bromo-cGMP	47	12	8	4	10	4	9
8-Thioethyl-cAMP	500	9	8	45	24	59	100
8-Thiomethyl-cAMP	ND[a]	0	20	6	15	39	53

[a] No detectable relaxation at 500 μM.

the very slow reversal of the relaxation induced by 8-(4-chlorophenylthio)-cGMP noted above. However, the ineffectiveness of cAMP analogs as compared with cGMP analogs in relaxing the intact muscle apparently could not be adequately explained by unusual affinities of the cAMP analogs for endogenous phosphodiesterases. The most important factor in the pattern of analog-induced relaxation appeared to be the potencies with which the analogs activated the cGMP-dependent protein kinase.

Tissue levels of cyclic nucleotide-dependent kinases and cyclic nucleotides. Because no correlation was observed between the ability of an analog to induce relaxation of the smooth muscle and the K_a of that analog for the purified cAMP-dependent protein kinase, it was important (a) to determine that the cAMP-dependent protein kinase was present in these pig coronary arteries and, if so, (b) to show that the smooth muscle kinase demonstrated the pattern of analog specificity and sensitivity observed with the purified enzyme from other tissues.

The estimates of the concentrations of the cyclic nucleotide-dependent protein kinases are shown in Table 4. According to these data, the molar intracellular concentration of cAMP-dependent protein kinase was 0.13 or 0.21 μM, depending on whether the estimate was based on kinase activity or [^{3}H]cAMP binding. The estimate of the concentration of the cGMP-dependent protein kinase in this tissue was 0.10 or 0.15 μM, respectively. However, by both estimates the molar ratio of cGMP-dependent protein kinase to cAMP-dependent protein kinase (~0.74) was considerably higher than the molar ratio of these enzymes in most mammalian tissues (41).

The concentrations of cAMP and cGMP in boiled extracts from control arterial segments were determined as described in Experimental Procedures. The intracellular cAMP concentration was estimated to be 0.42 μM as compared with 0.09 μM cGMP. These concentrations were very similar to the concentrations of the respective kinases determined by independent procedures. Because the holoenzyme forms of the cyclic nucleotide-dependent protein kinases contain four binding sites each for cyclic nucleotides, the molar concentrations of cAMP and cGMP binding sites in the cell are 4 times greater than those

TABLE 4

Intracellular concentrations of cyclic nucleotide-dependent kinases and cyclic nucleotides in pig coronary arteries

Pig coronary artery extracts were prepared and assayed as described in Experimental Procedures and the respective enzymes were assayed using the specific cGMP- or cAMP-dependent protein kinase assays and the specific [^{3}H]cAMP or [^{3}H]cGMP binding assays. Calculations of intracellular concentrations of the enzymes were based on these assays in conjunction with the known presence of four cyclic nucleotide binding sites per holoenzyme molecule or from the specific activity of the purified kinases assayed in parallel. Nucleotide concentrations were assayed as described in Experimental Procedures. The tissue weight was assumed to be 50% intracellular water.

	Intracellular concentration
	μM
cAMP-dependent protein kinase holoenzyme	
Assayed by cAMP binding activity	0.21
Assayed by cAMP kinase activity	0.13
cGMP-dependent protein kinase holoenzyme	
Assayed by cGMP binding activity	0.15
Assayed by cGMP kinase activity	0.10
cAMP	0.42
cGMP	0.09

listed in Table 4. The relatively high ratio of cAMP to cAMP-dependent protein kinase in this tissue despite a low activity ratio (r = 0.21 in control tissues) could be explained by the observation of Cobb *et al.* (42); these workers found that in bovine heart a significant portion of the cAMP-dependent protein kinase holoenzyme contains bound cAMP. Because binding of two cAMP molecules to the holoenzyme can occur without activation of the catalytic subunit, it is possible that in the smooth muscle cells, as well as in other cell types, much of the enzyme may contain bound cAMP in the basal state. The cellular concentration of cAMP was not elevated by treating the tissues with β-phenyl-1-N^2-etheno-cGMP or 8-(4-chlorophenylthio)-cGMP (not shown).

On DEAE-cellulose chromatography, the type I form of cAMP-dependent protein kinase eluted at 0.08 M NaCl and the type II isozyme eluted at 0.20 M NaCl. Type I and type II peaks contained 33% and 67% of the total cAMP-dependent protein kinase, respectively, in this tissue as determined by the average of both kinase (30% versus 70%) and cAMP binding (36% versus 64%) activities. The K_a values for activation of type I and type II by 8-(6-aminohexylamino)-cAMP, N^6-benzoyl-cAMP, 8-thiomethyl-cAMP, and 8-bromo-cAMP were very similar to that for cAMP-dependent protein kinases from other tissues (Table 5). Therefore, the weak effects of cAMP analogs in initiating relaxation in tissues could not apparently be attributed to unusual analog specificities of the cAMP-dependent protein kinases in these tissues as compared with enzymes from other tissue sources.

Studies of Guinea Pig Trachea

Comparison of potency of cyclic nucleotide analogs to relax the tissues and to activate cyclic nucleotide-dependent protein kinases. The studies with analogs were extended to trachaelis smooth muscle in order to determine whether cGMP analogs caused relaxation of nonvascular as well as vascular smooth muscle and to determine whether the pattern of analog effects were the same.

A typical pattern for contraction of trachealis muscle is shown in Fig. 3, lower panel. The overall rank order of analog potency in trachealis muscle was the same as that found in the coronary arteries (Table 6). However, the actual concentration of analogs required to effect 50% relaxation of the trachealis muscle was significantly lower than required to relax the arterial muscle, despite the fact that the contraction in the tracheal tissues averaged a higher percentage (30–40%) of the maximum tension that could be achieved by higher concentrations of carbamylcholine. The contraction elicited in the coronary arteries by 20 mM K^+ was ~15–20% of the maximum possible tension in that tissue, so that according to these criteria the coronary arteries should have been more sensitive to analog effects. The greater potency of the analogs in this tissue could be due to differences in tissue thicknesses, levels of endogenous phosphodiesterases, or a variety of other factors. The increased sensitivity of the trachealis preparation allowed the use of a greater number of analogs because previous experiments with the coronary arteries were limited by the availability of adequate quantities and concentrations of the analogs.

The relationship between the K_a of 10 C-8 analogs in activating cGMP-dependent protein kinase *in vitro* and the analog concentrations required to cause 50% relaxation is illustrated in Fig. 4. The data were analyzed by linear regression analysis. There was clearly a very close correlation between the potencies of the analogs in causing relaxation of the contracted muscle and the potencies of the analogs in activating cGMP-dependent protein kinase *in vitro*. In this instance, the correlation coefficient was 0.95. The effects of three less potent analogs [8-benzyl-cGMP, 8-thiomethyl-cAMP, and 8-(1-hydroxyethyl)-cGMP], which are not represented in this plot, were also correlated with their abilities to activate cGMP-dependent protein kinase. When these three analogs were included with the 10 shown in Fig. 4, the correlation coefficient was 0.94. Although the ability of a cyclic nucleotide analog to activate cGMP-dependent protein kinase seemed to be a major deter-

TABLE 5

Analog activation of pig coronary artery type I and type II cAMP-dependent protein kinase isozymes

The type I and type II isozymes of cAMP-dependent protein kinase from pig coronary arteries were separated by DEAE-cellulose chromatography as described in Experimental Procedures. The isozymes from rat and rabbit skeletal muscle and bovine heart were prepared as previously described (19). The K_a values for each analog in activating the respective isozymes were determined as described in Experimental Procedures.

Analog	K_a for type I			K_a for type II		
	Pig coronary arteries	Rat skeletal muscle	Rabbit skeletal muscle	Pig coronary arteries	Rat skeletal muscle	Bovine heart
	nM					
8-(6-Aminohexylamino)-cAMP	67	125	83	395	1393	750
8-Bromo-cAMP	82	31	65	45	65	53
8-Thiomethyl-cAMP	100	130	32	35	100	ND[a]
cAMP	110	65	91	65	78	90
N^6-Benzoyl-cAMP	149	59	98	95	181	200

[a] ND, not determined.

TABLE 6

Analog effectiveness in relaxing guinea pig trachealis and in activating purified cGMP-dependent protein kinase

The K_a for analog activation of purified bovine lung cGMP-dependent protein kinase *in vitro* was determined as described in Experimental Procedures. The EC_{50} is the concentration of analog producing a 50% decrease in a 0.1 μM carbamylcholine-induced tension. Analog treatment was followed by the addition of 100 μM IBMX to determine total tension present in the individual tissues.

Analog	K_a	EC_{50}
	μM	μM
8-Bromo-cGMP	0.025	10
β-Phenyl-1,N^2-etheno-cGMP	0.027	11
8-(4-Chlorophenylthio)-cGMP	0.050	9
8-Benzylthio-cGMP	0.085	28
6-Thio-cGMP	0.085	350
8-(4-Chlorophenylthio)-cAMP	0.203	34
8-Carbamoyl-cGMP	0.220	32
1-Methyl-cGMP	0.297	250
8-Thio-cGMP	0.323	25
8-Methyl-cGMP	0.338	49
8-(6-Aminohexylamino)-cAMP	0.627	83
1-Amino-cGMP	0.688	960
8-Butyryl-cGMP	0.780	113
8-Hydroxy-cGMP	0.917	120
8-Benzyl-cGMP	1.080	103
N^2-Butyryl-cGMP	2.00	950
8-Thioethyl-cAMP	3.4	425
8-Thiomethyl-cAMP	3.5	169
8-Bromo-cAMP	3.5	482
8-(1-Hydroxyethyl)-cGMP	5.90	198
2-Fluoro-cIMP	6.90	780
N^6-Benzoyl-cAMP	10.00	100
cIMP	10.00	7500
8-Neopentyl-cGMP	13.00	524
N^2-2′-O-dibutyryl-cGMP	18.6	6200
N^6-Butyryl-cAMP	34.4	186
N^6-2′-O-Dibutyryl-cAMP	1200	313

minant in the potency of that compound to induce smooth muscle relaxation, the correlation was not perfect. For instance, although comparison of all modifications introduced in the pyrimidine portion of the nucleotide also revealed a positive relationship between the K_a for cGMP-dependent protein kinase and the potency in relaxation, the correlation was not so marked (correlation coefficient = 0.54). The particular positions modified in these nine analogs varied and the chemical characteristics of the substitutions also varied (*e.g.*, 6-thio-cGMP, 2-fluoro-cIMP, 1-methyl-cGMP, β-phenyl-1-N^2-etheno-cGMP). The correlation was weaker when these analogs were analyzed as a group, but the variation in the position and chemical character of the substitutions may amplify other contributing factors (such as lipophilicity, side group modifications once inside the cell, and compartmentalization) to analog effectiveness discussed previously. The correlation between the potencies of the analogs to cause relaxation and cGMP-dependent protein kinase activation was in contrast to the relationship observed when the same comparison was made with cAMP-dependent protein kinase (not shown). When all analogs were used in this analysis, the correlation coefficient was 0.022, and when only analogs derivatized at the 8-position were used, the correlation coefficient was still very low (0.21). Several cAMP analogs [8-thiomethyl-cAMP, 8-(4-chlorophenylthio)-cAMP, and 8-(6-aminohexyl)amino-cAMP] that induced relaxation of the trachealis did so at concentrations related to their affinities for the cGMP-dependent protein kinase rather than for the cAMP-dependent kinase (Fig. 4; Table 6). However, the potencies of other analogs (dibutyryl-cAMP and N^6-benzoyl-cAMP) were not correlated with their affinities for the cGMP-dependent enzyme.

Phosphorylase activation. If cAMP-dependent protein kinase of guinea pig trachealis muscle were being activated by addition of cyclic nucleotide analogs, it would be expected that the tissue phosphorylase would also be activated. The abilities of the analogs to induce relaxation in this tissue were therefore correlated with their effects on phosphorylase. After 45 min of contraction with 0.1 μM carbamylcholine, tissues were treated with two analogs, 8-bromo-cAMP and 8-bromo-cGMP, each at two different concentrations, in an effort to determine whether or not the cAMP-dependent protein kinase was activated under these conditions. To assure that the cAMP-dependent protein kinase was not already in an active state, the activity ratio of kinase was determined as described in Experimental Procedures in the presence and absence of cAMP. In control tissues, the activity ratio was 0.34 whereas this ratio was 0.77 in tissues

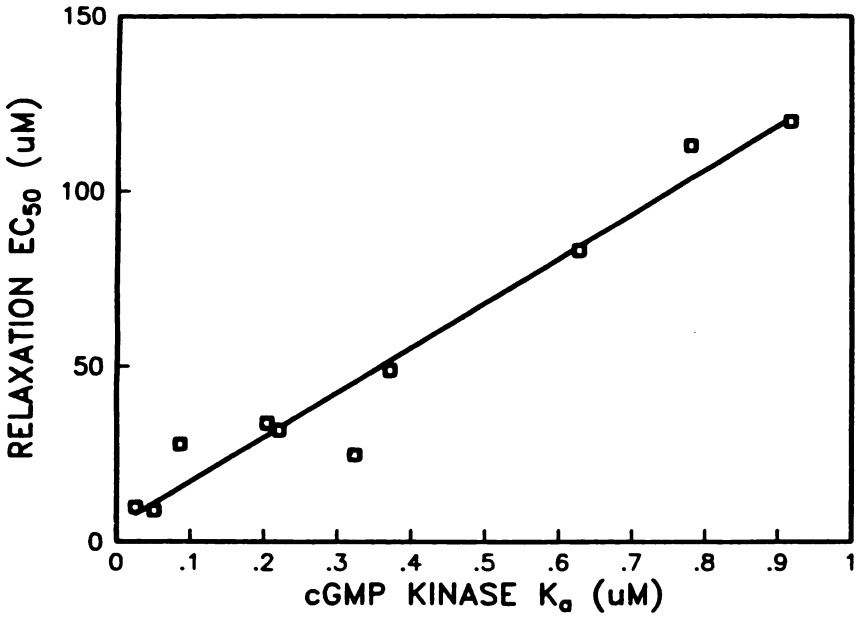

Fig. 4. Correlation of cyclic nucleotide analog potency in relaxation of guinea pig trachael strips with cGMP-dependent protein kinase K_a. Analog activation of cGMP-dependent protein kinase and tissue treatment was as described in Experimental Procedures. The curve was generated by a linear regression analysis program. Specific values for each analog are listed after the analog (K_a, EC_{50}). The analogs used were 8-bromo-cGMP (0.025 μM, 10 μM), 8-(4-chlorophenylthio)-cGMP (0.05 μM, 9 μM), 8-benzylthio-cGMP (0.085 μM, 28 μM), 8-(4-chlorophenylthio)-cAMP (0.20 μM, 34 μM), 8-carbamoyl-cGMP (0.22 μM, 32 μM), 8-thio-cGMP (0.32 μM, 25 μM), 8-methyl-cGMP (0.34 μM, 49 μM), 8-(6-aminohexylamino)-cAMP (0.63 μM, 83 μM), 8-butyryl-cGMP (0.78 μM, 113 μM), and 8-hydroxyl-cGMP (0.92 μM, 120 μM).

treated with 10 μM isoproterenol and 100 μM IBMX. At an analog concentration of 50 μM, 8-bromo-cGMP produced complete relaxation of the carbamylcholine-contracted trachealis muscle but phosphorylase was not activated (Table 7). 8-Bromo-cAMP at the same concentration caused little change in tissue tension and also failed to increase phosphorylase activity. That activation of cAMP-dependent protein kinase could cause phosphorylase activation was suggested by the finding that isoproterenol (10 μM) and IBMX (100 μM) caused a 42% increase in phosphorylase activity. It was therefore necessary to conduct the same experiments described above at a higher analog concentration (500 μM). In this instance, 8-bromo-cAMP produced approximately 50% relaxation of the muscle. Even at such excessively high levels, 8-bromo-cGMP did not increase phosphorylase activity, whereas 8-bromo-cAMP did so. These data implied that 8-bromo-cGMP did not cause relaxation by activating cAMP-dependent protein kinase, although 8-bromo-cAMP could have acted by this mechanism. Another analog, 8-(4-chlorophenylthio)-cAMP, at a concentration (35 μM) that caused ~60% decrease in tension in the tissue, also caused a 37% increase in phosphorylase activity, suggesting that it also activated cAMP-dependent protein kinase (not shown). However, because low concentrations of this analog activated both cyclic nucleotide-dependent kinases *in vitro* (Table 1), the muscle relaxation induced by 8-(4-chlorophenylthio)-cAMP could be due to activation of either of the cyclic nucleotide-dependent protein kinases.

Discussion

The use of effector analogs to elucidate specific intracellular mechanisms is a well established approach to understanding metabolic processes. It has been used to discriminate between α- and β-adrenergic receptors and has proved important in better defining the physiological effects of these receptors (43). Phosphodiesterase inhibitors, many of which are substrate analogs of known specificities, have been used in intact tissues to develop a better understanding of the physiological function of a particular phosphodiesterase (44). In addition, by using a spectrum of cyclic nucleotide analogs, investigators in this laboratory have more clearly defined the role of cAMP-dependent protein kinase, and its isozymic forms, in a number of physiological processes including H4 hepatoma gene transcription, hepatocyte glycogenolysis, adipocyte lipolysis, and progesterone synthesis and LH receptor induction in human granulosa cells (45). However, to our knowledge, the study described in this paper is the first to use a diverse group of cyclic nucleotide analogs to describe a role for the cGMP-dependent protein kinase in a physiological process, i.e., smooth muscle relaxation. Perhaps equally important is that use of this same approach provides only limited support for a role of the cAMP-dependent protein kinase as mediator of the cyclic nucleotide analog effects in these tissues.

TABLE 7

Comparison of analog effects on phosphorylase activation and relaxation in guinea pig trachealis

Tissue strips from guinea pig trachea were contracted with 0.1 μM carbamylcholine before analog addition as described in Experimental Procedures. The extent of relaxation achieved by a given concentration of an analog was based on 10 separate determinations in tissues that were subsequently treated with 100 μM IBMX to determine the maximum extent of relaxation. Separate strips of trachea were used for preparation of the homogenates used for the determination of phosphorylase activation in the presence of 80 μM caffeine as described in Experimental Procedures. Isoproterenol and IBMX were used at final concentrations of 10 μM and 100 μM, respectively.

Analog	Concentration	Relaxation	Phosphorylase
	μM	%	% of control
Control			100
8-Bromo-cGMP	50	100	106
	500	100	90
8-Bromo-cAMP	50	0	113
	500	50	144
Isoproterenol + IBMX		100	142

Cyclic nucleotide analogs may prove particularly important in defining the participation of a particular protein in cGMP-mediated processes. There are at least three widely distributed intracellular proteins known to bind cGMP, namely, the cGMP-dependent protein kinase, the cGMP-stimulated cAMP phosphodiesterase, and the cGMP-binding protein-phosphodiesterase. Furthermore, specific tissues are known to contain novel cGMP binding proteins, such as the cGMP-dependent cation channel protein (46) and the cGMP-binding phosphodiesterase (47), which have been identified in photoreceptors. Because elevations in intracellular cGMP could evoke changes in the activity of any of these proteins, effects produced by either cGMP elevation or an exogenous cGMP analog should not be ascribed to the cGMP-dependent protein kinase without more thorough evidence. In this study, the order of potencies of the analogs for relaxation have no apparent relationship to the specificity of the purified cGMP-binding protein-phosphodiesterase[1] or the cGMP-stimulated cAMP phosphodiesterase (48). In the future, use of a variety of cyclic nucleotide analogs should be a valuable tool in evaluating the role of each of these proteins in intracellular events.

Much evidence has accumulated to support the interpretation that agents such as nitroglycerin that induce intracellular cGMP accumulation could cause smooth muscle relaxation by lowering cytoplasmic calcium. Such studies have included direct fluorimetric measurements of intracellular levels of calcium (49), determination of ^{45}Ca efflux and influx (50–52), changes in phosphorylase activity (53, 54), and varying sensitivities to agents such as nitroglycerin or 8-bromo-cGMP in the presence of varying extracellular calcium levels (8, 55). Possible mechanisms for the calcium lowering have included acceleration of calcium extrusion from the cell, reduction of calcium influx into the cell, increased intracellular sequestration of calcium in specific cellular substructures, or enhanced binding of calcium to proteins serving as calcium "sinks." Kobayashi and coworkers (49) have demonstrated increased calcium extrusion from cultured vascular smooth muscle cells treated with nitroglycerin. Using rabbit aorta, Collins *et al.* (56) have reported decreases in both calcium influx and the release of intracellular calcium in the presence of 8-bromo-cGMP and nitroglycerin and Lincoln and co-workers (57) have shown that the activity of the Ca^{2+}-activated ATPase from the rat aorta microsomal fraction is increased in the presence of the cGMP-dependent protein kinase. Therefore, the mechanism whereby increased levels of cGMP relate to smooth muscle relaxation and changes in calcium levels is unclear.

The data presented in this study clearly implicate the cGMP-dependent protein kinase as a major cGMP receptor in smooth muscle and the pattern of analog potencies implicates this protein as a specific mediator of the cGMP-modulated effects

[1] S. H. Francis and J. D. Corbin, unpublished results.

on smooth muscle tone. Although these results support a role for the cGMP-dependent protein kinase in this physiological response, the steps in smooth muscle relaxation that are activated subsequent to the putative cGMP-dependent protein kinase activation are not known. Greengard and co-workers (58) have demonstrated specific cGMP-stimulated phosphorylation of proteins in smooth muscle and Parks *et al.* (59) have presented data that indicate a single membrane form of kinase could be responsible for both cAMP- and cGMP-stimulated phosphorylation of specific membrane proteins in smooth muscle. Lincoln and Johnson (60) have reported a cGMP-specific phosphorylation of a 150,000-kDa protein in rabbit aortic microsomal fractions (60). The identities of these proteins have not been determined. The present study suggested that substrates that are specifically phosphorylated by cGMP-dependent protein kinase, and perhaps not by cAMP-dependent protein kinase, would be the best candidates for elucidating the mechanism whereby cyclic nucleotides induce relaxation in smooth muscle cells.

Our observations concerning the greater potency of a large number of cGMP analogs as compared with cAMP analogs in relaxing smooth muscle agrees well with previous reports primarily using 8-bromo-cyclic nucleotides. In rat ductus deferens, Schultz and co-workers (61) have reported a marked effect of 8-bromo-cGMP in reducing the response of that tissue to norepinephrine, whereas 8-bromo-cAMP is ineffective. Likewise, Napoli *et al.* (62) have shown that 8-bromo-cGMP induces relaxation in bovine coronary arterial strips whereas 8-bromo-cAMP is inactive. Using rat aortic strips contracted with norepinephrine, Lincoln (8) has demonstrated 8-bromo-cGMP to be an effective relaxant, whereas 8-bromo-cAMP had no effect. Furthermore, Vegesna and Diamond (63) have reported a dissociation between the ability of agents to elevate cAMP and the effects they produce on the contractile state of rabbit aortic rings. However, the role of cAMP as a second messenger in the mechanism by which vasodilation is induced by various agents has been supported by many investigations (1, 2, 6, 7, 44). These studies have shown a good correlation between activation of adenylate cyclase or increases in cAMP and cAMP-dependent protein kinase activity and vaso-relaxation by isoproterenol, phosphodiesterase "inhibitors," adenosine, forskolin, and prostaglandin E_2. Our results using cyclic nucleotide analogs provide little evidence for the participation of cAMP-dependent protein kinase in smooth muscle relaxation, but these results certainly do not eliminate the possibility that this enzyme may still play a pivotal role in this process. One should be cautious in drawing definitive conclusions because these results could be explained by a number of factors such as selective compartmentalization, varying rates of hydrolysis, or permeability barriers for the analogs.

By using a larger number of analogs, one may be able to predict the involvement of either the cGMP-dependent protein kinase and/or the cAMP-dependent protein kinase, as well as other cyclic nucleotide-binding proteins, in processes in which the role of the respective enzyme(s) is unclear. The use of analogs may be useful in studying adrenal steroidogenesis, renal sodium transport, intestinal mucosal secretion, cerebellar function, and choroid plexus secretory epithelium, where specific roles for cGMP have been suggested. The ability of a number of potent cGMP analogs, such as 8-bromo-cGMP, β-phenyl-1-N^2-etheno-cGMP, 8-(4-chlorophenylthio)-cGMP, and 1-methyl-cGMP, and less potent analogs, including cAMP analogs, to elicit the predicted physiological responses would strengthen the evidence for a role of the cGMP-dependent protein kinase in these tissues.

Acknowledgments

We thank Dr. Ian Blair for performing the mass spectral analysis on 8-(4-chlorophenylthio)-cGMP and β-phenyl-1-N^2-etheno-cGMP, Dr. Roland K. Robins for kindly supplying several of the cGMP analogs, Dr. Tom Soderling for supplying the protein kinase inhibitor, and Leslie Landiss for her skillful technical assistance. We also thank Odom's Sausage Co. for donation of pig hearts for this study and Mrs. Penny Stelling and Mrs. Cindy Reeder for assistance in the preparation of this manuscript.

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