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Relaxation of Pig Coronary Arteries by New and Potent cGMP Analogs that Selectively Activate Type Iα, Compared with Type Iβ, cGMP-Dependent Protein Kinase

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SUMMARY

Smooth muscle preparations of human aorta or pig coronary arteries contain nearly equal amounts of cGMP-dependent protein kinase isozymes (cGMP kinase Iα and Iβ). In order to understand the roles of these isozymes in relaxing vascular smooth muscle, several new cGMP analogs were synthesized and tested for potencies in activating each enzyme and in relaxing pig coronary arteries. Analogs modified with a derivatized phenylthio group at the 8-position were as much as 72-fold more potent in activating purified cGMP kinase Iα than cGMP kinase Iβ. Electron-donating substituents, such as hydroxy, amino, and methoxy, on the phenyl ring enhanced the potencies of these analogs in activating cGMP kinase Iα. The most potent of these cGMP analogs [8-(4-hydroxyphenylthio)-cGMP] was 17 times more potent (EC_{50} = 1.1 μM) as a muscle relaxant than the most efficacious analog tested previously. Among derivatives with an 8-halo group, 8-iodo-cGMP was the most potent compound (K_a = 9 nM for Iα and 122 nM for Iβ) for both Iα and Iβ. Analogs modified at the 1,N^2-position or at both the 1,N^2- and 8-positions of cGMP were highly potent for activating both isozymes. Within this group, 8-I-β-phenyl-1,N^2-etheno-cGMP had K_a values of 22 nM and 17 nM for cGMP kinase Iα and Iβ, respectively, whereas the K_a values of cGMP were 110 nM and 250 nM for the two isozymes. 8-I-β-phenyl-1,N^2-etheno-cGMP was the most potent muscle relaxant tested, with EC_{50} of 0.4 μM. For all cGMP analogs tested, there was a positive correlation between potency for activation of cGMP kinase Iα and that for relaxation of pig coronary arteries. Assuming that the kinase assay conditions yielded a cyclic nucleotide specificity similar to that which would exist in intact cells, it was concluded that the cGMP kinase Iα isozyme mediates the relaxation of pig coronary artery smooth muscle caused by cGMP elevation. However, an additional role for cGMP kinase Iβ in the relaxation process could not be ruled out.

Cyclic nucleotides have been implicated as mediators of vascular smooth muscle relaxation in response to β-adrenergic agents, nitrovasodilators, atrial natriuretic peptide, adenosine, acetylcholine, and specific phosphodiesterase inhibitors (1–4). These effects could be mediated by cAMP kinase, cGMP kinase, or both. The cAMP and cGMP kinases are homologous proteins, although there are several differences between them that might provide for specific functions (1). Our recent studies using cyclic nucleotide analogs to relax smooth muscle have indicated a definite role for cGMP kinase, but not for cAMP kinase (5). Further evidence for a cGMP kinase role is the observation by Cornwell and Lincoln (6) that restoration of this kinase to cGMP kinase-depleted rat aortic smooth muscle cells restores the calcium-lowering responsiveness to cGMP. Vascular smooth muscle contains two cGMP kinase isozymes, termed type Iα (cGMP kinase Iα) and type Iβ (cGMP kinase Iβ), which are present in approximately equal amounts in bovine aorta (7). Although type Iβ is similar to type Iα in several respects, including the presence of two different binding sites (site 1 and site 2) for cGMP, this form has several properties quite different from those of the well described type Iα of bovine lung and type II of intestinal brush border (8).

According to the criteria set forth by Sutherland and coworkers (9), intracellular events modulated by changes in cyclic nucleotide levels should be mimicked by the extracellular addition of that cyclic nucleotide and/or its analogs. The binding site requirements of the specific cyclic nucleotide receptor would dictate, to a great extent, the usefulness of various cyclic

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ABBREVIATIONS: cAMP kinase, cAMP-dependent protein kinase; cGMP kinase, cGMP-dependent protein kinase; 1,N^2-PET, β-phenyl-1,N^2-etheno; 1,N^2-β-NET, β-naphthyl-1,N^2-etheno; IBMX, 3-isobutyl-1-methylxanthine; PKI-5–24, synthetic cAMP-dependent protein kinase inhibitor.

nucleotide analogs as promoters of that physiological response. Because the cGMP analog specificities of the type Iα and Iβ cGMP kinases are known to be different, it is possible to test these analogs in intact tissues with some ability to predict their rank order of potencies if they act on either of the kinases. New analogs of cGMP have been synthesized, in the present study, to probe the roles of these isozymes in relaxing pig coronary arteries.

Experimental Procedures

Materials. 8-Br-cGMP, cGMP, and IBMX were purchased from Sigma Chemical Company (St. Louis, MO). Heptapeptide substrate of cGMP kinase (RKRSRAE) and PKI-5–24 were obtained from Peninsula Laboratories (Belmont, CA). [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). 8-I-cGMP was initially supplied by Dr. B. Jastorff, University of Bremen (Germany), along with 8-Cl-cGMP and 8-benzylamino-cGMP. Later, 8-I-cGMP was prepared in the Vanderbilt laboratory. 2-Aminobenzenethiol, 4-aminobenzenethiol, 4-hydroxybenzenethiol, 3-chlorobenzenethiol, 2-naphthalenethiol, 3-bromobenzenethiol, 2,6-dichlorobenzenethiol, 4-methoxybenzenethiol, 3-methoxybenzenethiol, 2-methoxybenzenethiol, 2-bromobenzenethiol, 4-chlorobenzenethiol, 4-nitrobenzenethiol, 2-bromoacetophenone, 2-bromo-2′-acetonaphthone, 2-bromo-4′-methoxyacetophenone, 2-bromo-4′-chloroacetophenone, and 2-bromo-4′-nitroacetophenone were all purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

Synthesis of cGMP analogs. Synthesis of the cGMP analogs was achieved by using slight modifications of reported methods. 8-I-cGMP (compound **33**) was synthesized following the method described by Muneyama *et al.* (10) for the preparation of 8-I-cAMP. Beaman and Robins (11) have demonstrated the utility of introduction of halide into the 8-position of purine by treatment of the 8-thio or 8-alkylthio compound with molecular halogen in the presence of excess halide ion. 8-Thio-cGMP (compound **38**) was prepared by refluxing 8-Br-cGMP (compound **34**) and thiourea in methanol (12). When 8-thio-cGMP (compound **38**) was treated with an aqueous iodine-potassium iodide solution, 8-I-cGMP (compound **33**) was formed. The phenylthio derivatives were synthesized by reacting 8-Br-cGMP with various thiophenols, such as 2- and 4-amino-, 2- and 4-hydroxy-, 3- and 4-chloro-, 2- and 3-bromo-, 4-fluoro-, 4-nitro-, 2,4-dihydroxy-, and 4-, 3-, and 2-methoxybenzenethiols and β-naphthylenethiol, in the presence of either sodium acetate or sodium hydroxide (12). Reaction between cGMP or 8-Br-cGMP and α-bromoacetophenone, in the presence of 1,5-diazobicyclo[5.4.0]undec-5-ene, in dimethylsulfoxide, yielded 1,N^2-PET-cGMP (compound **29**) and 8-Br-1,N^2-PET-cGMP (compound **24**), respectively (13). Similar syntheses were performed using α-bromo-*p*-methoxyacetophenone and 2-bromo-2′-acetonaphthone, to furnish the respective 1,N^2-(4-CH_3O-PET)-cGMP (compound **23**) and 1,N^2-β-NET-cGMP (compound **25**). 8-I-cGMP was also reacted with α-bromoacetophenone, to yield 8-I-1,N^2-PET-cGMP (compound **28**). 8-Br-1,N^2-PET-cGMP was reacted with 4-hydroxybenzenethiol in the presence of sodium acetate to form 8-(4-hydroxyphenylthio)-β-phenyl-1,N^2-etheno-cGMP (compound **27**). All of these compounds were purified by Sephadex G-25 (superfine) chromatography in 50 mM ammonium bicarbonate (pH 7.8), at 4° (14). The structures of 12 cyclic nucleotide analogs (compounds **2, 4, 5, 14, 23, 24, 25, 26, 27, 28, 33,** and **37** of Table 1) were confirmed by mass spectra.

Activation of cGMP kinase isozymes. The bovine lung cGMP kinase type Iα and the bovine aorta smooth muscle type Iβ were purified to homogeneity as previously described (15, 16). Neither isozyme was contaminated by the other in detectable amounts, by analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and neither contained detectable levels of phosphodiesterase activity. The molecular weights of the two isozymes are 76,331 for type Iα (17) and 77,803 for type Iβ (18). The phosphotransferase activities of the purified cGMP kinases were assayed for 15 min at 20° by the phosphocellulose paper method, as described by Roskoski (19). Ten microliters of 2–8 nM enzyme (final concentration of enzyme in the assay was 0.3–1.1 nM) were added to a 50-μl reaction mixture containing 20 mM Tris·HCl, pH 7.4, 20 mM magnesium acetate, 200 μM [γ-^{32}P]ATP, 100 μM IBMX, 130 μM heptapeptide substrate (RKRSRAE), 0.45 μM PKI-5–24, and 10 μl of analog. A 6–10-fold activation of the kinases by cGMP or its analogs was routinely obtained. The enzymes were essentially cGMP-free, as determined by cGMP assays (20). The concentration of cyclic nucleotide required for half-maximal activation was taken as the apparent activation constant (K_a).

Pig coronary arteries. Smooth muscle preparations from pig right coronary arteries were prepared as described by Lorenz and Wells (21) and were utilized for studies of relaxation essentially as described earlier (5). The ability of cyclic nucleotide analogs to relax K^+-induced

TABLE 1

Potencies of various cyclic nucleotide analogs as activators of cGMP-kinase Iα and Iβ and as relaxants of pig coronary arteries

The K_a and EC_{50} values are representative of results from two to four experiments.

Compounds[a]	K_a		EC_{50}
	Type Iα	Type Iβ	
	nM		μM
1. cGMP[b]	110	250	NA[c]
8-Phenylthio derivatives			
2. 8-(2,4-Di-OH-Ph-S)-cGMP	5	360	2.0
3. 8-(2-NH_2-Ph-S)-cGMP	7	1370	4.7
4. 8-(4-OH-Ph-S)-cGMP	14	250	1.1
5. 8-(4-NH_2-Ph-S)-cGMP	16	175	11.8
6. 8-(3-Cl-Ph-S)-cGMP	23	600	ND[d]
7. 8-β-Naphthyl-S-cGMP	27	360	11.5
8. 8-(3-Br-Ph-S)-cGMP	36	618	7.7
9. 8-(2-OH-Ph-S)-cGMP	40	565	ND
10. 8-(2,6-Di-Cl-Ph-S)-cGMP	44	650	21.6
11. 8-(4-CH_3O-Ph-S)-cGMP	44	660	14.0
12. 8-(2-Br-Ph-S)-cGMP	45	1800	26.0
13. 8-(4-Cl-Ph-S)-cGMP[b]	50	440	17.8
14. 8-(2,4,6-Tri-OH-Ph-S)-cGMP	51	ND	12.5
15. 8-(4-F-Ph-S)-cGMP	70	262	55.0
16. 8-(3-CH_3O-Ph-S)-cGMP	70	666	ND
17. 8-$C_6H_5CH_2$-S-cGMP[b]	85	ND	60.0
18. 8-Ph-S-cGMP	85	ND	ND
19. 8-(2-CH_3O-Ph-S)-cGMP	210	1500	ND
20. 8-(4-NH_2-Ph-S)-cAMP	1500	1008	ND
21. 8-(4-Cl-Ph-S)-cIMP[b]	1800	ND	ND
22. 8-(4-NO_2-Ph-S)-cGMP	2000	3530	ND
1,N^2-PET-cGMP derivatives			
23. 1,N^2-(4-CH_3O-PET)-cGMP[b]	12	12	11.4
24. 8-Br-1,N^2-PET-cGMP	13	9	0.7
25. 1,N^2-β-NET-cGMP	13	30	20.0
26. 8-Br-1,N^2-PMET-cGMP	15	ND	11.4
27. 8-(4-OH-Ph-S)-1,N^2-PET-cGMP	17	23	0.6
28. 8-I-1,N^2-PET-cGMP	22	17	0.4
29. 1,N^2-PET-cGMP[b]	26	20	11.0
30. 8-Br-1,N^2-β-NET-cGMP	54	3000	50.0
31. 8-Br-1,N^2-(4-NO_2-PET)-cGMP	185	219	ND
32. 1,N^2-(4-Cl-PET)-cGMP	480	3750	ND
8-Halo-cGMP derivatives			
33. 8-I-cGMP	9	122	25.0
34. 8-Br-cGMP[b]	26	210	20.0
35. 8-Cl-cGMP	31	460	ND
Miscellaneous			
36. 8-(6-Aminohexylthio)-cGMP	32	ND	ND
37. 8-(2-Hydroxyethylthio)-cGMP	76	ND	ND
38. 8-Thio-cGMP[b]	100	180	ND
39. 8-Amino-cGMP[b]	445	ND	ND
40. 8-Benzylamino-cGMP[b]	3400	>200 μM	ND

[a] Ph, phenyl; S, thio; 1,N^2-PMET, β-phenyl-α-methyl-1,N^2-etheno.
[b] Syntheses of these compounds were reported earlier (12, 13, 27).
[c] NA, not active; cGMP did not show any activity.
[d] ND, not determined.

tension in pig coronary arteries was examined. All tissues were adjusted to 2 g of tension before experiments were begun. Isometric tension was measured by a Narco myograph tension adjuster connected to Gould RS3400 recorders. Contractions were initiated by replacement of Krebs-Ringer buffer containing 5.9 mM K^+ with the same buffer containing 20 mM K^+ and proportionately reduced Na^+. The tension achieved with 20 mM K^+ was only 13–20% of the maximum tension generated by 117 mM K^+. Tension generated in the tissue was allowed to stabilize before addition of the analog. Various concentrations of analogs were added to the muscle preparations, in order to achieve different degrees of relaxation. For instance, four different concentrations, which produced 15%, 30%, 50%, and 65% relaxation, would be used to ensure a linear response range. After the tissue relaxed in response to each concentration of analog and the force stabilized, 100 μM IBMX was added. Relaxation in response to 100 μM IBMX was taken as 100% relaxation. EC_{50} values were the concentrations of analogs that reduced the force by 50% of this value, as described earlier (5, 21). The difference between the force at the time of addition of the analog and the force after relaxation was used to calculate percentage of relaxation. The maximum effects of potent analogs were equal to that of IBMX. However, maximum relaxation could not be achieved with a few of the less potent analogs, due to the large amounts of analog that would have been required.

DEAE-Sephacel chromatography of crude extracts of human aorta and pig coronary arteries. Frozen human aortic tissue was obtained from the Vanderbilt Autopsy Laboratory and stored at −70°. The tissue was partially thawed in the cold room, and 3.5 g of tissue were minced and combined with 50 ml of buffer containing 10 mM KH_2PO_4, 1 mM EDTA, and 25 mM 2-mercaptoethanol (KPEM). The tissue was then homogenized in a Cuisinart Mini-mate Plus chopper/grinder (5 × 10 sec), and the homogenate was centrifuged at 10,000 × *g* for 30 min at 4°. The supernatant fraction was filtered through glass wool and then applied to a DEAE-Sephacel column (0.9 × 7 cm) that had been pre-equilibrated in KPEM buffer containing 0.05 M NaCl. The column was then washed with 10 column volumes of the same buffer before elution of the enzyme with a linear 50-ml NaCl gradient (50–300 mM), and 0.8-ml fractions were collected.

Fresh pig hearts were obtained from a local slaughterhouse, and the right coronary arteries were prepared essentially as previously described (5, 21). DEAE-Sephacel chromatography of extracts of these tissues was conducted using a protocol similar to that for human aorta.

cGMP kinase assay using crude extracts. The assay for cGMP kinase activity in the fractions of crude extracts was similar to that for the purified enzymes described above, except for specific modifications in the reaction mixture. Ten microliters of enzyme fraction were added to 40 μl of the same reaction mixture described above, except that 0.9 μM PKI-5-24 was included, and the assay was performed in the presence and absence of 10 μM cGMP. The assay proceeded at 20° for 30 min and was terminated by spotting of an aliquot of the reaction mixture onto phosphocellulose paper, as described above.

Results and Discussion

8-Substituted phenylthio-cGMP analogs. Previous work (5) demonstrated that 8-(4-chlorophenylthio)-cGMP (compound **13**) was a potent activator of the cGMP kinase type Iα. This was further investigated by synthesis of several analogs with substituents on the phenyl ring of 8-phenylthio-cGMP. The most potent compound in this series for activating cGMP kinase Iα was 8-(2,4-dihydroxyphenylthio)cGMP (compound **2**) (K_a = 5 nM), which was 22-fold more active than cGMP. In contrast, the monohydroxylated derivative, 8-(4-hydroxyphenylthio)-cGMP (compound **4**), was only 8-fold more potent than cGMP as an activator of this isozyme, and the amino derivative had about the same potency as the monohydroxy derivative. Based on these results, we examined whether there was a positive correlation between the electron-donating nature of the substituent and kinase activation constants for cGMP kinase Iα. Based on measurements of acidity of 4-substituted benzoic acids, the electron-donating nature of various substituents, in decreasing order, is as follows: amino > hydroxy > methoxy > bromo > chloro > nitro > fluoro (22). It can be seen in Table 1 that the rank order of potencies of analogs containing these substituents was in partial agreement with that which would be predicted by a favorable effect of electron donation. The introduction of an electron-withdrawing substituent (nitro group) on the phenyl ring (compound **22**) yielded a very poor activator of cGMP kinase (K_a = 525 nM for Iα and 3530 nM for Iβ).

The 2-amino group of cGMP is important for high affinity binding to cGMP kinase, as suggested from the results of earlier cyclic nucleotide analog studies (23), by molecular modeling of cGMP binding sites (24), and from results of site-directed mutagenesis (25). When the 2-amino group in 8-(4-chlorophenylthio)-cGMP was removed, to yield 8-(4-chlorophenylthio)-cIMP (compound **21**), the resulting compound was a poor activator of the kinase (K_a = 1.8 μM for Iα); however, it was 5-fold more active than cIMP (K_a = 10 μM for Iα) itself in activating cGMP kinase. This suggested that the appended substituent enhanced analog binding. Most of the compounds in the group of 8-phenylthio analogs were poor activators of cGMP kinase Iβ. This allowed for high type Iα/type Iβ specificity in some cases. For example, 8-(2,4-dihydroxyphenylthio)-cGMP (compound **2**) was 72-fold more specific for type Iα than for type Iβ (Fig. 1).

The role of the phenyl ring in the aforementioned compounds was studied by synthesizing several nonaromatic sulfides and testing them on type Iα (Table 1, Miscellaneous). These compounds included 8-(6-aminohexylthio)-cGMP (compound **36**) (K_a = 32 nM), 8-(2-hydroxyethylthio)-cGMP (compound **37**) (K_a = 76 nM), and 8-thio-cGMP (compound **38**) (K_a = 100

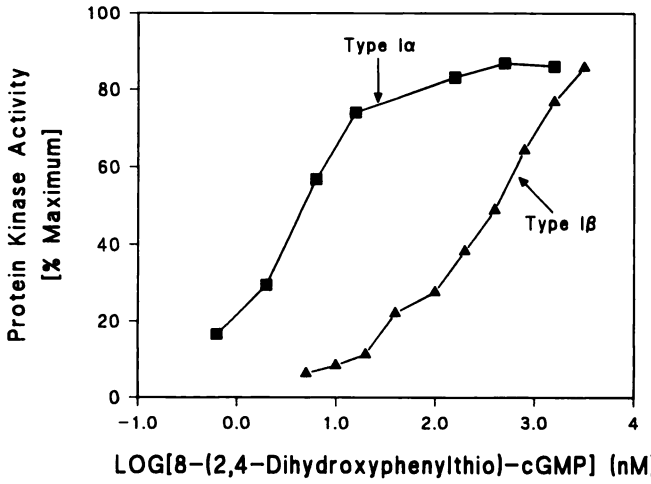

Fig. 1. Effect of 8-(2,4-dihydroxyphenylthio)-cGMP concentration on activation of cGMP kinases Iα and Iβ. Incubations (70 μl) were carried out for 15 min at 20° in the presence of 20 mM Tris·HCl, pH 7.4, 20 mM magnesium acetate, 0.2 mM [^{32}P]ATP, and 2 nM cGMP kinase Iα (or Iβ). Fifty microliters of reaction mixture were spotted on phosphocellulose paper and washed with dilute phosphoric acid before counting. The activation constants were determined as described in Experimental Procedures. The data were plotted as log [8-(2,4-dihydroxyphenylthio)-cGMP] (nM) versus the kinase activity (percentage of maximum). Maximum kinase activity was that obtained in the presence of 14 μM cGMP. The sources of the enzymes are described in Experimental Procedures.

nM). 8-Phenylthio-cGMP (compound **18**) activated type Iα with approximately the same potency (K_a = 85 nM) as did 8-thio-cGMP or 8-(2-hydroxyethylthio)-cGMP. These results suggested that an aromatic ring on sulfur might not be necessary for favorable interaction with the enzyme.

8-Halo-cGMP analogs (Table 1). 8-Br-cGMP (compound **34**) is 4.3-fold more potent than cGMP in activating purified cGMP kinase Iα and is 7.9 times more selective for interacting with site 1 of type Iα than with site 2 (23). This compound is also one of the most potent analogs in promoting relaxation of tracheal and vascular smooth muscle tissue (5). From an analysis of all C-8-modified analogs and from molecular modeling, it was hypothesized that the potency of 8-Br-cGMP, especially for site 1 binding, may be determined by several factors. These include the spatial tolerance of large substituents at C-8, preference of this binding site for the *syn* conformation of cyclic nucleotides, and the electron-withdrawing/donating properties of the substituent. In order to address these possibilities systematically, two additional 8-halo-cGMP derivatives (compounds **33** and **35**) were synthesized and tested for their potencies in activating both cGMP kinases Iα and Iβ. The potencies of all of these halo compounds in activating both isozymes were in the following order: 8-I-cGMP > 8-Br-cGMP > 8-Cl-cGMP. The chloro group is less bulky than the bromo group but has greater electron-withdrawing power, whereas the iodo group is bulkier than chloro or bromo but has less electron-withdrawing power. It was, thus, concluded that a bulky halogen substituent with low electron-withdrawing power favors the activation of the cGMP kinases.

Modification of 1,N^2-PET-cGMP (Table 1, 1,N^2-PET-cGMP derivatives). The parent compound in this series of analogs was 1,N^2-PET-cGMP (23). 1,N^2-PET-cGMP (compound **29**) was 4 times more potent in activating cGMP kinase Iα (K_a = 26 nM) and 13 times more potent for Iβ (K_a = 20 nM) than was cGMP. This compound also potently promoted relaxation of smooth muscle.

Introduction of a methoxy group on the phenyl ring of 1,N^2-PET-cGMP yielded 1,N^2-(4-CH_3O-PET)-cGMP (compound **23**), which was 2-fold more potent than the parent compound for both cGMP kinases Iα and Iβ. However, 1,N^2-(4-Cl-PET)-cGMP (compound **32**) was a poor activator for the Iα isozyme (K_a = 480 nM) and a very weak activator of type Iβ (K_a = 3750 nM). Substituting the phenyl ring with a naphthalene group furnished a more potent analog (compound **25**) (K_a = 13 nM) than 1,N^2-PET-cGMP for type Iα.

Modification of both the 1,N^2- and 8-positions of cGMP (Table 1, 1,N^2-PET-cGMP derivatives). Because both cGMP binding sites of cGMP kinase Iα or Iβ are involved in activating each enzyme (23) and because of the positive cooperativity of the two cGMP binding sites, it was postulated that the combination of two positive features, one for each binding site, might yield more potent compounds. The initial compound in this category was 8-Br-1,N^2-PET-cGMP (compound **24**), and it proved to be a very potent activator for both cGMP kinase isozymes (K_a = 13 nM for Iα and 9 nM for Iβ). It was hypothesized that the 8-substituent of this compound might increase the affinity of the compound for site 1 (23) and be tolerated by site 2, whereas the 1,N^2-substituent might favor the binding to site 2 (23) and be tolerated by site 1. The proposed interaction between the 2-amino group of cGMP and the hydroxyl group of threonine in each binding site (24) was apparently not affected by substitution of one proton on the 2-amino group of cGMP with a large substituent. In fact, the presence of the PET moiety could have stabilized this hydrogen bond. A hypothetical model illustrating the binding of a potent disubstituted cGMP analog is shown in Fig. 2, emphasizing that ample space is available to accommodate large appended groups at the 1,N^2-position and 8-position.

It might be expected from the K_a values of 8-I-cGMP and 8-Br-cGMP that 8-I-1,N^2-PET-cGMP (compound **28**) would be more potent than 8-Br-1,N^2-PET-cGMP, but this analog, with a K_a of 22 nM for type Iα and 17 nM for type Iβ, was less potent than 8-Br-1,N^2-PET-cGMP in activating both cGMP kinases. A further structural modification was made to form 8-(4-hydroxyphenylthio)-1,N^2-PET-cGMP (compound **27**). This analog retained its potency in activating type Iα (K_a = 17 nM), whereas its potency in stimulating type Iβ (K_a = 23 nM) was less, compared with that of 8-Br-1,N^2-PET-cGMP. However, comparison of 8-(4-hydroxyphenylthio)-cGMP (compound **4**) (K_a = 250 nM) and 8-(4-hydroxyphenylthio)-1,N^2-PET-cGMP revealed that the introduction of the PET group into 8-(4-hydroxyphenylthio)-cGMP (compound **4**) increased the potency of activation of type Iβ.

Studies of pig coronary artery relaxation. The relative potencies of a number of cyclic nucleotide analogs in promoting relaxation and in activating cGMP kinases are listed in Table 1. Many of the analogs that were most effective in activating the cGMP kinases were also some of the most potent compounds in relaxing the vascular smooth muscle. The most potent compounds for relaxation among the cGMP analogs tested were 8-I-1,N^2-PET-cGMP (compound **28**), 8-Br-1,N^2-PET-cGMP (compound **24**), and 8-(4-hydroxyphenylthio)-cGMP (compound **4**) (EC_{50} = 0.4, 0.7, and 1.1 μM, respectively). Most of the other 8-phenylthio derivatives were also potent relaxants. A difference between the activity of 8-phenylthio derivatives and compounds substituted at the 1,N^2-position, such as 1,N^2-PET-cGMP and 8-Br-1,N^2-PET-cGMP, was that the effect of 8-phenylthio derivatives on relaxation persisted

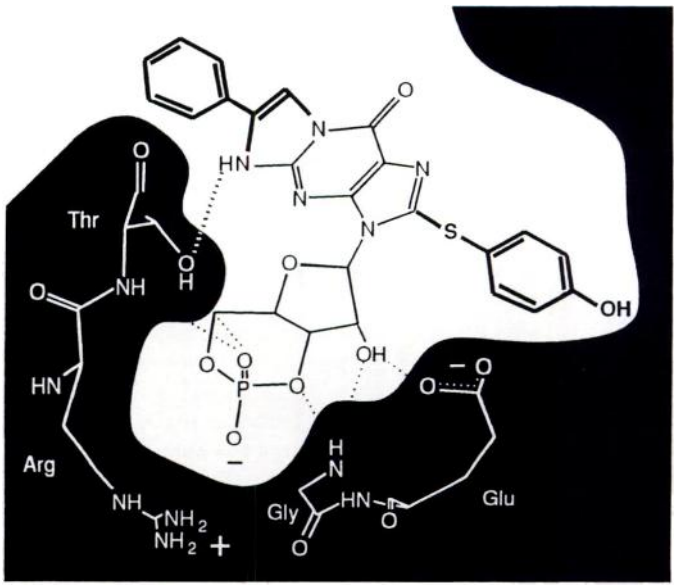

Fig. 2. Hypothetical model for potent disubstituted cGMP analog binding to cGMP kinase. The model is based on that predicted for the cGMP kinase binding site (25). *Dotted lines*, proposed hydrogen bonds. *Bold lines*, modifications done on cGMP.

for a very long time (3 hr), with more than 15 buffer changes being required to reverse the effect of the phenylthio derivatives that were tested. In contrast, the time required to reverse the effects of the PET derivatives that were tested was relatively short (<1 hr). This might be explained by different rates of analog degradation by intracellular phosphodiesterases. Analogs with bulky substituents in the 8-position are known to be resistent to breakdown by most phosphodiesterases (5).

It was observed that there was a strong positive correlation ($r = 0.9$) between the potencies of 8-phenylthio-cGMP analogs for activation of cGMP kinase Iα and for relaxing pig coronary arteries (Fig. 3). Even when all of the tested analogs were included in the analysis, the correlation was quite good ($r = 0.8$; data not shown). The correlation for cGMP kinase Iβ was poor either when the 8-phenylthio-cGMP analogs alone were included ($r = 0.13$) or when all of the analogs were included ($r = 0.22$). It was concluded from these results that cGMP kinase Iα mediates the relaxation of pig coronary artery smooth muscle in response to these analogs. An additional role for cGMP kinase Iβ could not be excluded, because analogs with relatively high specificity for this isozyme were not found. The data presented in this study implicated the cGMP kinase(s) as the major cGMP receptor in smooth muscle for mediating cGMP effects on muscle tone.

The excellent correlation between potencies of cGMP analogs in activating cGMP kinases and in relaxing smooth muscle suggested that the K_a value for cGMP kinase was by far the most important property of analogs to consider for stimulation of relaxation. However, cell-penetration rate or resistance to degradation could also play a role in relaxation potency. This could explain why 8-I-1,N^2-PET-cGMP (compound **28**) was less potent than 8-Br-1,N^2-PET-cGMP in activating the two cGMP kinase isozymes but was 2-fold more potent in relaxing pig coronary arteries. Thus, the judicious determination of susceptibilities to phosphodiesterases and hydrophobicities of these cyclic nucleotides might allow a better prediction of their potencies in relaxing pig coronary arteries.

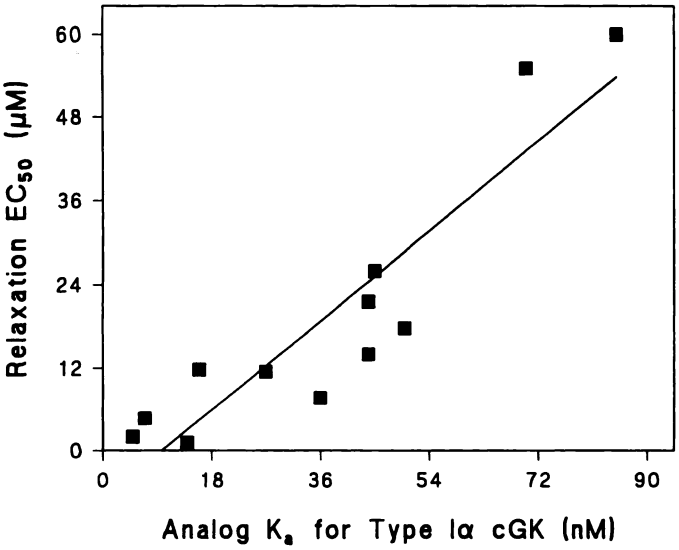

Fig. 3. Correlation between cGMP kinase K_a and EC_{50} for smooth muscle relaxation. Potencies of various phenylthio derivatives of cGMP in relaxing pig coronary arteries were correlated with K_a of these analogs in activating cGMP kinase (*cGK*) Iα. The curve was generated by a linear regression analysis program. Specific analogs used, including the K_a and EC_{50}, respectively, were 8-(2,4-dihydroxyphenylthio)-cGMP (5 nM, 2 μM), 8-(2-aminophenylthio)-cGMP (7 nM, 4.7 μM), 8-(4-hydroxyphenylthio)-cGMP (14 nM, 1.05 μM), 8-(4-aminophenylthio)-cGMP (16 nM, 11.8 μM), 8-(β-naphthylthio)-cGMP (27 nM, 11.5 μM), 8-(3-bromophenylthio)-cGMP (36 nM, 7.7 μM), 8-(4-methoxyphenylthio)-cGMP (44 nM, 14 μM), 8-(2,6-dichlorophenylthio)-cGMP (44 nM, 21.6 μM), 8-(2-bromophenylthio)-cGMP (45 nM, 26 μM), 8-(4-chlorophenylthio)-cGMP (50 nM, 17.8 μM), 8-(4-fluorophenylthio)-cGMP (70 nM, 55 μM), and 8-benzylthio-cGMP (85 nM, 60 μM).

Presence of both type Iα and Iβ isozymes in pig coronary artery and human aorta. Because the correlative studies were done using the bovine kinases and porcine tissues, the presence of the two cGMP kinase isoforms in crude pig coronary artery extracts was determined by DEAE-Sephacel chromatography, and the enzymes were characterized. The type Iα and Iβ isozymes were present in nearly equal quantities in this tissue (47% and 53%, respectively; four experiments). The relative K_a values of these crude isozymes for cGMP, cAMP, 8-Br-cGMP, and 8-(4-chlorophenylthio)-cGMP were similar to those found for the purified bovine enzymes (data not shown). Based on these observations, it is likely that the isoforms of cGMP kinase in pig coronary arteries are highly similar to the purified bovine enzymes.

Compounds such as the analogs described herein may ultimately have therapeutic use; therefore, it was important to establish the presence of the target enzymes in human tissue. Such studies might also provide an indication of species variation in isozyme distribution, which has been shown for cAMP kinase isoforms (26). Studies of crude human aortic extracts by DEAE-Sephacel chromatography revealed the presence of both isoforms of cGMP kinase. Because the resolution of the two isoforms in this chromatographic step was incomplete, the disparity in the affinities of the two isoforms for 8-(2,4-dihydroxyphenylthio)-cGMP (Table 1) was exploited, in order to differentiate more clearly the type Iα peak from the type Iβ peak. With 50 nM 8-(2,4-dihydroxyphenylthio)-cGMP, purified bovine type Iα or human type Iα (fraction 27) was fully active (Fig. 1), whereas bovine type Iβ or human type Iβ was approximately 20% active. Column fractions were initially assayed for cGMP kinase activity in the presence and absence of 10 μM cGMP, to determine the total cGMP kinase profile. Fractions were then assayed in the presence of 50 nM 8-(2,4-dihydroxyphenylthio)-cGMP, whereupon one major peak of activity, corresponding to the region indicated as Iα, was observed. The areas under these activity curves were then used to estimate the relative abundance of the type Iα and Iβ isozymes as 61% and 39% (two experiments), respectively.

The similar distinctions in the specificities of the human and bovine type Iα and Iβ isoforms for 8-(2,4-dihydroxyphenylthio)-cGMP demonstrated in this study suggested that results of analog studies with the purified bovine enzymes were applicable to the human cGMP kinases. This possibility was further supported by the fact that the amino acid sequences of either type Iα or Iβ differ by only two amino acids between the respective bovine and human isoforms (18).

The present study yielded several new potent activators of cGMP kinase type Iα and Iβ, which are also efficacious relaxants of pig coronary arteries. The most potent activator of cGMP kinase type Iα was 8-(2,4-dihydroxyphenylthio)-cGMP (compound **2**), which was 22-fold more potent than cGMP and 5-fold more potent than the most potent commercial cGMP analog, 8-Br-cGMP. 8-(2-Aminophenylthio)-cGMP exhibited the highest type Iα/type Iβ selectivity, being 195-fold more

specific for type Iα. The most selective analog synthesized and studied in this laboratory previously (5) was 8-(4-chlorophenylthio)-cGMP (compound **13**), which had an 8.8-fold higher selectivity for type Iα. This analog is now available from BioLog Life Science Institute (Bremen, Germany). Although analogs strongly selective for type Iβ were not found, 8-Br-1,N^2-PET-cGMP (compound **24**) exhibited a 28-fold higher affinity for this enzyme than did cGMP. The best artery relaxant tested was 8-I-1,N^2-PET-cGMP (compound **28**), which was 28-fold more potent than the best relaxant reported earlier, 1,N^2-PET-cGMP (5), and ~50-fold more potent than the best available commercial analogs, 8-Br-cGMP and 8-(4-chlorophenylthio)-cGMP.

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