Activities *in Vitro* of 8-Substituted Derivatives of Adenosine-3′,5′-Cyclic Monophosphate on Guinea Pig Trachea and Rat Portal Vein (35765)

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Several recent reviews have summarized the literature of the past decade on adenosine-3′,5′-cyclic monophosphate (cyclic AMP) and related nucleotides (1–5). Cyclic AMP mediates many of the actions of a great variety of hormones and drugs, and it can also regulate the release of many hormones. Adenyl cyclase, found in cell membranes, catalyzes the formation of cyclic AMP from ATP; apparently the receptors for these hormones may be very closely related to adenyl cyclase. Cyclic AMP is inactivated by phosphodiesterases (PDE), which convert it to 5′-AMP; some of these catabolic enzymes are inhibited by methylxanthines and also by certain other compounds. It has been suggested (6) that the various biological properties attributed to cyclic AMP may be mediated through the stimulation of kinases that catalyze phosphorylation of a variety of cellular proteins.

In some cases, it may be possible to mimic the effect of a hormone or of exogenous cyclic AMP by the use of more potent analogs of cyclic AMP. Such analogs either may penetrate cell membranes more readily or be more resistant to the action of PDE, or both (1, 2).

Recently, Posternak and Cehovic (7) synthesized 8-substituted analogs of cyclic nucleotides, some of which have shown biological activities (release of TSH and GH from rat pituitary tissue *in vitro* that are greater than those of cyclic AMP. Among their more potent nucleotides were the 8-thio- and 8-bromo-analogs of cyclic AMP or of N^6-2′-*O*-dibutyryl cyclic AMP (dibutyryl cyclic AMP).

Muneyama *et al.* (8) synthesized a series of 8-substituted analogs of cyclic AMP and tested their relative effectiveness: (a) as alternate activators of the cyclic AMP-dependent protein kinase isolated from bovine brain; and (b) as alternate substrates for a PDE isolated from porcine brain. Some of these analogs were highly effective as activators of the protein kinase; furthermore, several analogs also were found to be inhibitors of PDE. A number of these 8-substituted analogs also were: (a) potent activators *in vitro* of steroidogenesis in rat adrenal cells and of lipolysis in rat epididymal fat cells (9); as well as (b) potent inhibitors of rat brain PDE, cat heart PDE, or both (10).

This report describes the relaxant activities *in vitro* of a number of 8-substituted analogs of cyclic AMP on guinea pig trachea and rat portal vein. Comparisons with cyclic AMP, the dibutyryl analog of cyclic AMP, and theophylline have been included.

Materials and Methods. Twelve 8-substituted analogs of cyclic AMP were obtained from Dr. R. K. Robins of the ICN Nucleic Acid Research Institute, Irvine, California (8). Cyclic AMP and the sodium salt of its dibutyryl analog were purchased from Zellstoffefabrik Waldhof, West Germany. Theophylline was purchased as aminophylline, USP, from G. D. Searle & Co., Chicago, Illinois.

Seventy-four albino guinea pigs of either sex, weighing 200–350 g, and 56 female rats of the Sprague-Dawley strain, weighing 150–250 g, were used in these tests. The trachea was excised from each guinea pig, and then cut in a zigzag manner, as described by Piper and Vane (11). Each trachea was suspended isotonically with a 1-g load in a 10-ml isolated-tissue bath filled with Krebs solution (12), bubbled with 95% O_2–5% CO_2

and maintained at 37°. The tone of each tracheal strip was increased by the addition of histamine phosphate to the bath solution [0.5 μg of histamine (base)/ml as final bath concentration]; at the end of 10 min, either cyclic AMP, or an analog thereof, or theophylline, was added to the bath solution for another 10 min. At the end of this 20-min period, the tissue strips were "washed" with about 50 ml of drug-free Krebs solution. After a 10-min recovery period, histamine was reintroduced, and the test cycle was repeated if the typical contractile response to histamine was again elicited. β-Adrenergic stimulant amines, as well as theophylline, typically reduce the increased tone of the histamine-contracted guinea pig trachea without inhibiting, after washing, the contractile response to the next dose of histamine.

Each excised rat hepatic portal vein was slit lengthwise and suspended isometrically (2-g initial tension) in 10-ml isolated-tissue baths filled with Krebs solution, bubbled with 95% O_2–5% CO_2 and maintained at 37°. The procedure employed was similar to that described by Johansson *et al.* (13) for monitoring spontaneous contractile activity of the rat portal vein.

Each nucleotide was freshly dissolved in Krebs solution prior to each test and added in 1.0-ml volumes about 1 min after withdrawal of 1.0 ml of Krebs solution from each bath.

α-Adrenergic stimulant amines, such as norepinephrine, typically increase the tone and frequency of contractions of excised rat portal vein; whereas theophylline, as well as β-adrenergic stimulant amines such as isoproterenol, produce little or no change in resting tone, but decrease the amplitude of spontaneous contractions and may increase the contractile frequency within the first few minutes. Preliminary experiments with cyclic AMP and some of its analogs suggested that some of these nucleotides decreased amplitude of contraction of the portal vein at slower rates; hence, each nucleotide was left in the bath for 30 min before washing. At least another 10 min was allowed for recovery of control activity, at which time another test concentration was added to the bath. Generally, only one compound was tested on each tissue strip.

Recordings of contractile activity were made via transducers coupled to an ink-writing Beckmann-Offner dynograph. Nucleotides that were relaxants of smooth-muscle activity at 400 μg/ml in each of these two types of preparations *in vitro* were tested in 2–9 guinea pig tracheal strips and in a like number of rat portal veins. Furthermore, lower concentrations of the more active nucleotides, such as 100, 25, and 6.25 μg/ml, were also tested. The IC_{50} (*i.e.*, the concentration producing 50% relaxation or decrease in tone of tracheal strip or decrease in amplitude of spontaneous contraction of portal vein) was estimated graphically by plotting log concentration against the degree of relaxation (relative to prenucleotide tone or amplitude) for each tissue. The average IC_{50} (μg/ml), as well as the standard error of the mean, was calculated for each of the more active nucleotides, which were tested with 3 or more tissues. Several analogs of cyclic AMP that showed little or no relaxant activity at 400 μg/ml were tested in only 2 guinea pig tracheal strips and 2 rat portal veins.

Results. The IC_{50} values for cyclic AMP, for each of the 8-substituted analogs of cyclic AMP, as well as for dibutyryl cyclic AMP, and for theophylline, are shown in Table I. Cyclic AMP was relatively inactive at concentrations as high as 400 μg/ml. Among the 8-substituted analogs of cyclic AMP, the 8-benzylthio analog was the most potent in this series; this analog was at least 7 times more potent than cyclic AMP on the portal vein and about 20 times more potent on the trachea. Relative to dibutyryl cyclic AMP, the 8-benzylthio analog of cyclic AMP was 1 to 2 times more potent on the portal vein, but about 10 times more potent on the tracheal strip, with a more rapid onset of relaxant activity on the former preparation. Each of the other analogs was less than 1/4 as potent as the 8-benzylthio analog as a tracheal relaxant; however, the 8-azido analog was 1/2 to 1 1/3 times as potent as the 8-benzylthio analog as a relaxant of the portal vein. The development of maximal relaxant effect of the 8-azido analog within

TABLE I. Relaxant Concentrations *in vitro* of Theophylline and Analogs of Cyclic AMP.[a]

Compound	Guinea pig trachea		Rat Portal Vein			
	IC_{50} (μg/ml ± SE)	N	IC_{50} (μg/ml ± SE)			N
(min):	10		10	20	30	
Theophylline	4.4 ± 0.4	15	68 ± 20	54 ± 12	57 ± 17	4
Cyclic AMP	≧400	9	>400	>400	>400	9
N^6,2'-*O*-Dibutyryl cyclic AMP	247 ± 13	6	136 ± 10	94 ± 10	68 ± 8	8
8-substituted analogs of cyclic AMP						
8-SCH_2-⟨O⟩	23 ± 2	4	73 ± 13	62 ± 8	77 ± 16	4
8-SCH_3	93 ± 7	4	218 ± 24	193 ± 39	186 ± 49	4
8-N_3	96 ± 7	4	132 ± 42	69 ± 16	56 ± 13	4
8=S	121 ± 11	4	230; 350	180; 210	160; 210	2
8=O	145 ± 16	4	≧400	260; 400	250; 400	2
8-SCH_2CH_3	160 ± 10	4	≧400	≧400	≧400	2
8-OCH_3	209 ± 12	4	≧400	≧400	250; 410	2
8-$N(CH_3)_2$	224 ± 33	4	≧380	≧313	≧313	3
8-Br	236 ± 67	4	≧213	100 ± 21	102 ± 26	4
8-NH_2	315 ± 29	4	>400	>400	≧400	2
8-$NHCH_3$	>400	2	>400	>400	>400	2
8-SCH_2CH_2OH	>400	2	>400	>400	>400	2

[a] IC_{50} = average concentration (μg/ml) producing 50% relaxation, relative to control response; N = number of tracheas or veins tested.

the 30-min test period was slower than that of the 8-benzylthio-cyclic AMP on the portal vein.

Theophylline and most of the 8-substituted analogs of cyclic AMP were more potent on the guinea pig tracheal strip than on the rat portal vein; two exceptions were the 8-azido and 8-bromo analogs of cyclic AMP (as well as dibutyryl cyclic AMP).

The 8-benzylthio-, 8-methylthio-, and 8-thionucleotides were among the most potent compounds in this series; the 8-ethylthio and 8-β-hydroxyethylthio analogs were among the least potent. The 8-oxo and 8-methoxy analogs were only weakly active on one or both test preparations. Among the 8-nitrogen substituted analogs, only the 8-azido analog was among the more potent relaxants. The 8-dimethylamino analog, the 8-methylamino analog, and the 8-amino analog were either inactive or among the least active, with IC_{50} values equal to or greater than 400 μg/ml. The 8-bromo analog, the only 8-halogenated compound in this series, was roughly equipotent to dibutyryl cyclic AMP as a relaxant on the guinea pig trachea, and slightly less potent as a relaxant on the rat portal vein.

Discussion. The effects of cyclic AMP on vascular smooth muscle are variable on different tissues and species (14). Bartelstone *et al.* (15) found that low concentrations of cyclic AMP potentiated the contractions of rat aortic strips in reponse to norepinephrine, but high concentrations of cyclic AMP sometimes depressed contractions. Somlyo *et al.* (16) reported that dibutyryl cyclic AMP, like theophylline, hyperpolarized smooth muscle of rabbit pulmonary artery in low concentrations of K^+. Walter and Bassenge (17) reported that cyclic AMP, as well as ATP and adenosine, caused relaxation of helical strips from portal veins, and renal or coronary arteries of the dog. Triner (18) found that increasing levels of cyclic AMP in the cell, induced by stimulation of adenyl cyclase or inhibition of PDE, or mimicking these increases by exogenous dibutyryl cyclic AMP, were accompanied by relaxation and decreased contractility in arterial smooth muscle. Berti *et al.* (19) reported that cyclic

AMP and its dibutyryl derivative, as well as theophylline, relaxed the rat portal vein *in vitro*; a negative inotropic effect accompanied by a positive chronotropic effect was observed in each instance.

Middleton and Fink (*20*) reported that cyclic AMP (4×10^{-5} *M*) had no effect on isolated guinea pig tracheal muscle, whereas theophylline (10^{-5} *M*) relaxed the muscle. Moore *et al.* (*21*) found that dibutyryl cyclic AMP, at 500 μg/ml, relaxed the guinea pig tracheal chain, but that an equimolar concentration of cyclic AMP was inactive. Guirgis (*22*) also reported that cyclic AMP, at 1 mg/ml, was inactive, but dibutyryl cyclic AMP, at 250–500 μg/ml, relaxed the isolated tracheal tube of the guinea pig. Collier *et al.* (*23*) found that intravenous ATP as well as cyclic AMP, but not AMP, increased air overflow volume in Konzett-Rossler preparations of guinea pig lungs *in vivo*.

The results reported herein, which indicate that dibutyryl cyclic AMP is a more potent relaxant than cyclic AMP on excised tracheal or vascular smooth muscle, are in general agreement with those of others (*19–22*).

The relaxant activities of these several analogs *in vitro* indicated that 8-thio- and 8-methylthio-cyclic AMP are more potent than the 8-oxo and 8-methoxy analogs, which in turn are more potent than the 8-amino and 8-methylamino derivatives. A similar order of activity was found by Muneyama *et al.* (8) in assessing the activities of these compounds as alternate activators of bovine brain protein kinase. There is a general trend in both reports indicating that the respective activities of the compounds decrease as the substituent is changed from 8-thio to 8-oxo to 8-amino.

Two of the three 8-thio-substituted derivatives, 8-benzylthio- and 8-methylthio-cyclic AMP, which were among the most potent relaxants on these 2 smooth muscle preparations, were also among the most potent inhibitors of porcine brain PDE (8). The 8-benzylthio- and 8-methylthio-cyclic AMP, as well as the 8-bromo, 8-amino- and 8-ethylthio analogs were also among the most potent inhibitors of cat heart PDE, rat brain PDE, or of both (10). Muneyama *et al.* (8) have found that nearly all of their 8-substituted analogs of cyclic AMP were resistant to hydrolysis by porcine brain PDE; indeed, only 8-amino-cyclic AMP was not resistant to enzymatic hydrolysis.

It is unknown to what extent the potent relaxant activities of some of these 8-substituted derivatives of cyclic AMP are due to: (a) protein kinase activation; (b) inhibition of a specific PDE; (c) resistance to hydrolysis by PDE; (d) ease of penetration through cell membranes; or (e) some combination of these and other as yet unidentified factors. Furthermore, the relative specificity of the relaxant effects *in vitro* of some of these 8-substituted analogs of cyclic AMP remains to be ascertained with related substances, such as adenine, adenosine, AMP, ADP, ATP, etc. Thus far there is a suggestion of relative specificity, in that several 8-substituted analogs of cyclic AMP, each at 400 μg/ml, were inactive in either or both of the 2 types of smooth-muscle preparations (Table I). Levine (14) has found, however, that the inhibitory response of (intestinal) smooth muscle to cyclic AMP is nonspecific; thus, "adenosine may represent the active relaxant component which acts on a common receptor in the smooth muscle cell membrane."

Summary. Twelve 8-substituted analogs of cyclic AMP were tested on excised guinea pig trachea and rat portal vein; cyclic AMP, dibutyryl cyclic AMP, and theophylline were similarly tested. The 8-benzylthio, 8-methylthio, 8-azido, and 8-thio derivatives of cyclic AMP were among the most potent relaxants of both tracheal and venous smooth muscle. Each of these four analogs was: (a) more potent than dibutyryl cyclic AMP, but less potent than theophylline, on excised trachea; and (b) roughly equipotent to or less potent than either dibutyryl cyclic AMP or theophylline on excised vein. Several other 8-substituted derivatives of cyclic AMP, as well as cyclic AMP itself, were inactive as relaxants at concentrations of 400 μg/ml.

In general, the decreasing order of relaxant activity among the groups of 8-substituted analogs of cyclic AMP was 8-thio>8-oxo>8-amino.

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