Table 2. Effects of 2′-deoxycoformycin (DCF) on the cytotoxicity to H. Ep. # 2 cells of some O^6-alkyl-8-azainosines and O^6-methyl-8-azaguanosine*

Compounds and concentration (μM)	Colony formation: Per cent of control (range)
DCF, 3.8	97 (93–107)
O^6-methyl-8-azainosine, 0.18	24 (18–33)
O^6-methyl-8-azainosine, 0.18, + DCF, 3.8	68 (64–72)
O^6-methyl-8-azainosine, 0.35	4 (3–6)
O^6-methyl-8-azainosine, 0.35, + DCF, 3.8	17 (8–31)
O^6-ethyl-8-azainosine, 0.17	4 (3–5)
O^6-ethyl-8-azainosine, 0.17, + DCF, 3.8	5 (3–7)
O^6-methyl-8-azaguanosine, 3.4	24 (9–33)
O^6-methyl-8-azaguanosine, 3.4, + DCF, 3.8	92 (85–99)

* One hundred cells were placed in 4 oz prescription bottles containing control cultures with 10 ml of SRI-14 medium [13], and treated cultures with 10 ml of medium to which the candidate inhibitors had been added. After the cultures had been incubated at 37° for 7–10 days, the medium was decanted and the cells adhering to the glass were washed with phosphate-buffered NaCl solution (0.85%), fixed with Bouin's fixative, and stained with Giemsa stain. The microscopic colonies present were then counted. The cloning efficiency of control cultures was 40–70 per cent. Each value in the last column represents the average of three or more experiments run in triplicate. The concentration of DCF was that determined in previous experiments to be non-toxic and to give essentially complete inhibition of ADA.

product of the action of ADA is 8-azaguanosine, which would be cytotoxic as a result of its further metabolism, via 8-azaguanine, to nucleotides of 8-azaguanine [15]. The corresponding thio compound, *S*-methyl-6-thio-8-azaguanosine, has no toxicity to H. Ep. # 2 cells [7]; this fact is in accord with the absence of kinase activity for this type of compound and with the resistance of the 6-alkylthio group to the action of ADA. The ribonucleosides of O^6-alkyl-8-azahypoxanthines and O^6-methyl-8-azaguanine are rare examples of nucleosides that are converted by ADA to compounds that retain toxicity. To our knowledge, O^6-methyl-8-azaguanosine is the only example of a compound the biological activity of which is entirely dependent upon the action of ADA.

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Methacholine-induced attenuation of methylisobutylxanthine- and isoproterenol-elevated cyclic AMP levels in isolated rat atria

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Recent studies have shown that cholinergic agents, which have no significant effects on basal cyclic AMP (cAMP) levels, markedly lower cAMP elevations induced by: (a) epinephrine in rat uterus [1] and perfused rat heart [2]; (b) isoproterenol in rat ventricular slices [3], rat lung slices [4], bovine tracheal smooth muscle [5], guinea pig ileum [6] and human astrocytoma [7]; (c) norepinephrine in rat parotid gland [8]; (d) glucagon in rat ventricular slices [3] and per-

fused rat heart [2]; (e) dopamine in bovine superior cervical ganglion [9]; (f) thyrotropin in pig [10], horse and dog [11] thyroid slices; and (g) adenosine and prostaglandin E_1 in human astrocytoma [7]. In all the above studies, the cholinergic lowering of cAMP levels that had been elevated by stimulation of cAMP formation catalyzed by adenylate cyclase was examined. In the present study, the question of whether a cholinergic agent could reduce cAMP levels that had been elevated by inhibition of cAMP breakdown rather than by stimulation of its synthesis was investigated. The effects of methacholine (MCh) were examined in isolated, superfused rat left atria in which cAMP levels were elevated by methylisobutylxanthine (MIX), a potent inhibitor of cAMP hydrolysis. For comparison, the study was extended to include the effects of MCh on cAMP levels that were elevated by isoproterenol-induced stimulation of adenylate cyclase and also the effects of the different agents on tissue levels of cyclic GMP (cGMP).

Sprague–Dawley female rats (150–200 g) were killed by a blow to the head. Following rapid excision of the hearts, the left atria were separated and superfused with a 37° oxygenated (100% O_2) HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered salt solution containing (m-moles/l): NaCl, 140.0; KCl, 4.0; $MgCl_2$, 1.0; $CaCl_2$, 2.5; EDTA (ethylenedinitrilotetra-acetic acid, disodium salt), 0.05; dextrose, 5.56; and HEPES, 3.0; the pH was adjusted to 7.3–7.4 with 3 N NaOH. All drugs were purchased from the Sigma Chemical Co., St. Louis, MO. Each atrium was electrically paced at 1 Hz with a stimulus of 1 V, 2 msec in duration. An initial diastolic tension of 500 mg was placed on each muscle. After a 15-min equilibration, the atria were given either no drug, acetyl-β-methyl choline chloride (methacholine), *l*-isoproterenol-*d*-bitartrate (ISO), 1-methyl-3-isobutylxanthine, ISO plus MCh, or MIX plus MCh. In all experiments, MCh was administered for 60 sec, ISO for 105 sec and MIX for 150 sec, since separate experiments demonstrated that the plateau of the inotropic response occurred at these times. When given in combination with ISO or MIX, MCh was administered during the last 60 sec of a continuous superfusion with the cAMP elevating agent. For β-receptor blockade experiments, propranolol was present during both the equilibration period and the subsequent MIX administration. At the appropriate time, the atria were rapidly frozen with Wollenberger-type clamps [12] precooled in liquid nitrogen. Each atrium was weighed in a Harris cryostat (−40°), homogenized in Broeck tissue grinders containing 0.5 ml of 0.77 M perchloric acid at −4°, and centrifuged at 1000 *g* for 30 min. The supernatant fraction was neutralized with 2 M K_2CO_3 and recentrifuged at 1000 *g* for 30 min. The neutralized supernatant fraction was stored at −85° until cyclic nucleotide determination. Cyclic nucleotides were assayed in triplicate by the radioimmunoassay of Steiner *et al.* [13], as modified by acetylation methodology [14, 15], without further purification, since their levels were unaltered in preliminary experiments using Bio-Rad AG 1-X8 formate anion exchange column separation.

Significant variations in cyclic nucleotide levels have often been observed between different groups of animals and at different times of the year. The reason for these variations is not known. However, to rule out any possible influence of these variations on the conclusions derived from this study, all atria in each protocol were obtained from a single shipment of animals from the supplier, randomly divided among control and drug treatment groups, run at the same time and

Table 1. Effects of MCh, ISO, MIX and propranolol, alone and in combination, on cyclic nucleotide levels in superfused, electrically paced rat left atria*

Treatment	Cyclic AMP (pm/g tissue)	Cyclic GMP (pm/g tissue)
Protocol 1		
No drug	879.8 ± 72.8 (7)	79.7 ± 9.3 (3)
MCh (250 nM)†	795.8 ± 27.8‡ (6)	67.2 ± 6.0‡ (3)
ISO (100 nM)	1768.9 ± 138.4§ (7)	51.0 ± 2.2‖ (3)
ISO (100 nM) plus MCh (250 nM)	1269.5 ± 86.4¶ (7)	72.6 ± 10.4‡,** (3)
Protocol 2		
No drug	341.4 ± 22.2 (5)	33.1 ± 3.9 (5)
Propranolol (1 μM)	329.3 ± 34.7‡ (6)	31.6 ± 2.8‡ (6)
MIX (100 μM)	926.5 ± 79.5§ (7)	45.7 ± 3.0‖ (7)
MIX (100 μM) plus propranolol (1 μM)	955.3 ± 139.2§,†† (7)	47.6 ± 6.5‖,†† (7)
Protocol 3		
No drug	786.1 ± 58.3 (7)	56.1 ± 6.3 (6)
MCh (250 nM)	712.2 ± 41.0‡ (7)	59.1 ± 7.9‡ (7)
MIX (100 μM)	1676.9 ± 141.6§ (6)	105.5 ± 7.1§ (7)
MIX (100 μM) plus MCh (250 nM)	1230.7 ± 131.6‡‡ (7)	121.9 ± 15.4††,§§ (7)

* Each value represents the mean ± S. E. M. of the number of atria in parentheses.

† Drug concentrations in the superfusion fluid. Times for exposure are 60 sec for MCh, 105 sec for ISO, 150 sec for MIX ,and 17.5 min for propranolol.

‡ Not significantly different from no drug.

§ $P < 0.001$, compared to no drug.

‖ $P < 0.05$, compared to no drug.

¶ $P < 0.01$, compared to ISO alone.

** Not significantly different from ISO alone.

†† Not significantly different from MIX alone.

‡‡ $P < 0.05$, compared to MIX alone.

§§ $P < 0.01$, compared to no drug.

assayed together. All statistical comparisons were between control and drug treatment groups within a single experiment (protocol). Means were compared by Student's *t*-test for unpaired data and were considered significantly different when P values were less than 0.05.

The first experiment was designed to demonstrate the effect of MCh on the ISO-induced cAMP increases in the superfused isolated rat atrial preparation. The data in protocol 1 of Table 1 demonstrate that MCh (250 nM, 60 sec) alone did not alter significantly basal cAMP levels, but significantly lowered ISO-induced elevations in cAMP. Furthermore, MCh, whether in the presence or absence of ISO, produced no detectable change in cGMP levels compared to controls. In this set of experiments, ISO alone lowered cGMP significantly, but this lowering effect was not found in three other experiments (each with $n = 8$) at the same or higher ISO concentrations (data not shown in table). The reason for this variation is not clear.

For the study involving phosphodiesterase inhibition, we chose the most potent of the currently available methylxanthines, methylisobutyl xanthine [16]. However, since methylxanthines may release catecholamines and, therefore, have contractile [17, 18] and cyclic nucleotide-elevating [19] effects, due to activation of adenylate cyclase, that would be reduced or abolished by β-blockade, we determined first whether β-blockade would alter the cyclic nucleotide response to MIX in our preparation. It should be noted that in protocol 2 the MIX-elevated cAMP levels were unaffected by 1 μM propanolol. In other experiments (not shown), this concentration of propranolol blocked the cAMP rise (112 per cent increase vs 5 per cent increase) induced by 100 nM ISO. We concluded, therefore, that MIX raised atrial cAMP by a mechanism other than adenylate cyclase activation by β-receptors and most likely through its inhibitory action on phosphodiesterase. This was supported by the finding that MIX also elevated cGMP levels significantly, as would be expected with phosphodiesterase inhibition.

The results of protocol 3 show that MCh lowered MIX-elevated cAMP significantly but did not affect basal cAMP, basal cGMP or MIX-elevated cGMP levels. This effect of MCh on MIX-elevated cAMP was approximately the same as on ISO-elevated cAMP, i.e. MCh lowered ISO-induced elevations from 101 to 44 per cent above basal levels (57 per cent difference) and MIX-induced elevations from 113 to 57 per cent above basal levels (56 per cent difference).

The data presented in this communication clearly show that MCh lowers the cAMP elevations produced by a known inhibitor of cyclic nucleotide phosphodiesterase (MIX). It also appears that this effect of MCh is comparable to its lowering of the cAMP elevations that result from adenylate cyclase stimulation (ISO).

Since previously published data on cholinergic lowering of cAMP elevations deal exclusively with elevations resulting from stimulation of adenylate cyclases, some of the explanations for this cholinergic action are based on antagonism of stimulated, as distinguished from basal, cyclase activity. Hypothetical mechanisms have been proposed which involve the possible cholinergic reduction of endogenous levels of GTP [2], or interference with the action of GTP [20], a nucleotide required for maximal stimulation of adenylate cyclase by adrenergic and other activators. However, the data presented here show that MCh lowered elevated cAMP levels in the absence of any apparent cyclase stimulation, i.e. in experiments in which cAMP levels were elevated by MIX-induced inhibition of cAMP breakdown. This finding tends to minimize the importance of stimulated cyclase as distinguished from non-stimulated cyclase in the mechanism of cholinergic lowering of elevated levels of cAMP in atria.

Another hypothesis has been presented by Erneux *et al.* [11] who found that carbamoylcholine decreased cAMP levels that had been elevated by thyrotropin (TSH) in horse or canine thyroid gland. This effect appeared to be due to activation of phosphodiesterase-catalyzed cAMP disappearance and mediated by rising endogenous cGMP levels. This mechanism is probably not operative in rat atria, since our experiments did not detect any elevation of cGMP levels by a cAMP-lowering concentration (250 nM) of MCh. It should be noted, however, that the possibility of cGMP elevations at other durations of superfusion with MCh than the one tested (60 sec) cannot yet be ruled out. Erneux *et al.* [11] also found that MIX (or theophylline) blocked the cholinergic lowering of TSH-elevated cAMP levels in the thyroid gland, which is in contrast to our finding in rat atria in which MCh clearly lowered cAMP in the presence of MIX (Table 1, protocol 3).

Two other hypothetical mechanisms have been proposed by Triner *et al.* [1] to explain cholinergic lowering of elevated cAMP levels in rat uterine tissue: (1) acetylcholine raises intracellular concentrations of calcium which decrease adenylate cyclase activity, and (2) acetylcholine increases cellular demand for ATP and, therefore, reduces ATP availability to the cyclase. Since it is conceivable that basal as well as stimulated cyclase activity could be lowered by these mechanisms, our data do not rule out either of these possibilities.

In summary, MCh lowers elevated levels of cAMP in isolated, electrically driven rat atria, but basal cAMP levels are unchanged by the drug. The lowering of elevated cAMP levels was found, regardless of whether the cAMP elevations had been produced by stimulation of cAMP synthesis (adenylate cyclase) or inhibition of its hydrolysis (phosphodiesterase). This finding shows that activation of adenylate cyclase is not a requirement for the cholinergic lowering of elevated atrial levels of cAMP. Furthermore, the mechanism of this cholinergic effect in atrium may not be identical with the mechanism that has been proposed for thyroid gland, in which cholinergically elevated cGMP levels appear to accelerate the breakdown of cAMP. No cholinergically induced rise in cGMP was detected in the atria, and the cholinergically induced lowering of cAMP was found in the presence of MIX in contrast to findings in the thyroid gland.

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A pre-synaptic inhibitory effect of 5-hydroxytryptamine on the electrically induced twitch response of the rat vas deferens

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There is now substantial evidence for the existence of presynaptic α-adrenoceptors located at noradrenergic varicosities both in the peripheral and central nervous system [1, 2]. Stimulation of these pre-synaptic α-adrenoceptors by α-adrenoceptor agonists results in a decreased release of noradrenaline whilst their blockade facilitates noradrenaline release by nerve impulses [3]. In addition to pre-synaptic α-adrenoceptors Stjärne and Brundin [4] have reported the existence of pre-synaptic β-adrenoceptors on noradrenergic nerve endings. Other pre-synaptic sites have been reported to be involved in a modulatory role on the noradrenergic nerve terminal and these include the receptors for dopamine [5], opiates [6], prostaglandins [7] and histamine [8].

It has recently been reported [9] that 5-hydroxytryptamine (5-HT) can induce an increase in the outflow of noradrenaline from sympathetic nerve terminals and that this effect may be mediated via pre-synaptic tryptamine receptors. The present study was therefore undertaken to investigate the effects of 5-HT on the electrically induced contractions or 'twitches' [10] of the rat isolated vas deferens, a preparation which has been used extensively to characterize pre-synaptic α-adrenoceptors [11–14] and presynaptic histamine receptors [8], to establish whether or not 5-HT has a modulatory role on transmitter release via a presynaptic site.

Vasa deferentia from male Wistar rats (200–250 g) were set up in organ baths and bathed in a Mg-free Krebs solution of the following composition (mM): NaCl 119, $CaCl_2$ 2.6, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2 and glucose 11.1, which was maintained at 37° and aerated with a mixture of 5% CO_2 in O_2. Silver electrodes were placed near the top and bottom of the tissue and the intramural nerves of the vas deferens stimulated according to Birmingham and Wilson [15] by square wave pulses of 3 msec duration, 20–25 V at a frequency of 0.1 Hz, provided by a Grass S4 stimulator. Isometric contractions were recorded using Devices transducers and two channel recorders.

5-HT produced a concentration dependent inhibition of the twitch response with an ID_{50} of 0.6 μM; this action was rapid in onset and readily reversed by washing with Krebs solution. A cumulative dose–response curve is shown in Fig. 1. The effect of the 5-HT antagonists cyproheptadine and methysergide on the inhibitory effect of 5-HT on the twitch response was investigated. Methysergide proved to be an effective competitive antagonist, producing a dose-dependent parallel shift of the 5-HT dose–response curve to the right (Fig. 1). Cyproheptadine, though producing antagonism of the 5-HT inhibitory response was not as effective as methysergide and at concentrations above 10 μM itself produced inhibition of the twitch response of the electrically stimulated vas deferens.

An inverse relationship between the inhibitory effect of 5-HT (2 μM), added 30 sec before stimulation, and stimulation frequency was observed; the lower the frequency the greater was the inhibitory effect of 5-HT (Fig. 2); this observation is indicative of a pre-synaptic site of action and correlates well with similar observations made for other agonists such as clonidine [11], noradrenaline [16], histamine [8] and ergometrine [17] acting on pre-synaptic receptor sites.

It has been reported [18–20] that a single pulse stimulation

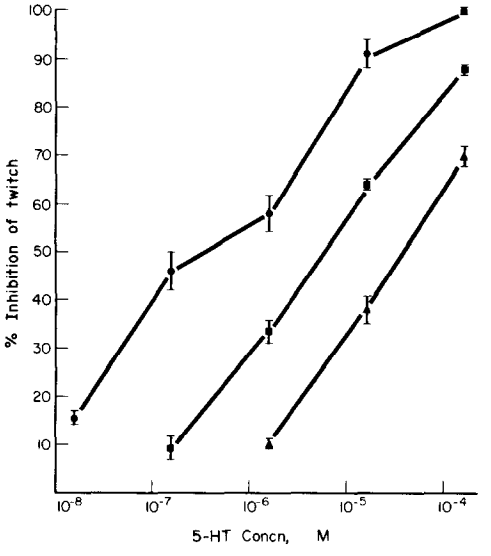

Fig. 1. The inhibition by 5-hydroxytryptamine (5-HT) of the electrically induced twitch response of the rat vas deferens and its antagonism by methysergide. ●——● Control 5-HT inhibitory response (9). ■——■ 5-HT response following a 10 min exposure to 20 μM methysergide (4). ▲——▲ 5-HT response following a 10 min exposure to 0.2 mM methysergide (4). Each point is the mean ± standard error of the mean. Figures in parentheses indicate the numbers of experiments carried out for each curve.