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EFFECT OF SOME PHOSPHODIESTERASE INHIBITORS ON ADENYLATE CYCLASE FROM THE LIVER FLUKE, *FASCIOLA HEPATICA*

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Abstract—The present investigation was prompted by our previous finding that in the liver fluke, *Fasciola hepatica*, some phosphodiesterase inhibitors, instead of potentiating the rise in endogenous cAMP caused by 5-hydroxytryptamine (5-HT), antagonized it. Papaverine, 1-ethyl-4-(isopropylidenehydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl (SQ 20009), 6,7-dimethyl-4 ethyl-quinazoline (Quazodine) and caffeine inhibited 5-HT-activated adenylate cyclase from particulate fractions of the liver fluke. This may explain their *in vivo* antagonism to the 5-HT-mediated rise in endogenous cAMP levels. Isobutylmethylxanthine (IBMX), which did not antagonize the 5-HT effect *in vivo*, did not inhibit 5-HT-activated adenylate cyclase in fluke particles. None of the above compounds inhibited the NaF-activated adenylate cyclase. Kinetic studies showed that inhibition of 5-HT-activated adenylate cyclase by papaverine or SQ 20009 was not competitive with the substrate, ATP, or with GTP. While high levels of 5-HT decreased the degree of inhibition by papaverine and SQ 20009, the kinetics of inhibition does not appear to be strictly competitive.

The present paper extends the investigations on the pharmacology and biochemistry of the liver fluke, *Fasciola hepatica*, which have been reported from this laboratory for many years. Our interest in studying this trematode is 2-fold. Development of a rational chemotherapy depends upon knowledge of the basic biochemistry of the parasite as well as of the biochemistry of its mammalian host. Welch, as early as 1945, called for such a rational approach to the chemotherapy of parasites rather than the indiscriminate screening of possible chemotherapeutic agents which is still common today [1]. Parasitic diseases are considered to be the leading cause of illness and debilitation in the world, and the economic consequences of human and domestic animal infestation are enormous. Furthermore, since the liver fluke is especially rich in serotonin (5-HT)* receptors, new insights into the biochemical effects of serotonin can be gained, using intact as well as cell-free extracts from flukes.

Serotonin appears to be a regulator of carbohydrate metabolism and neuromuscular activity in the liver fluke [2]. It stimulates the motility of flukes and it increases the endogenous concentration of cyclic 3′,5′-adenosine monophosphate (cAMP) by activating adenylate cyclase [3, 4]. The adenylate cyclase from *F. hepatica* is one of the most active studied and in many ways can be considered a model serotonin receptor system. Indoleamine analogs of 5-HT stimulate fluke motility and activate adenylate cyclase in direct relation to their structural similarity to 5-HT [4]. Lysergic acid diethylamide, while stimulating fluke motility, can mimic or antagonize the effects of 5-HT on adenylate cyclase by interacting with the serotonin receptor [5]. The rank order potencies of LSD derivatives in stimulating fluke motility were found to correlate with hallucinogenic potency in man [6]. Thus, a better understanding of the fluke serotonin receptor-adenylate cyclase system may contribute to our knowledge of the hallucinogenic effects of these agents in man.

In conjunction with our studies of serotonin receptor mediated adenylate cyclase, we have studied cAMP phosphodiesterase. The kinetics and apparent competitive inhibition by several widely used inhibitors of this enzyme were reported. We have found that none of the inhibitors tested, except IBMX, would potentiate the rise in endogenous cAMP mediated by 5-HT *in vivo*. SQ 20009 and papaverine had the unexplained effect of decreasing the rise in endogenous cAMP caused by 5-HT although they inhibited phosphodiesterase in cell-free extracts as well as did IBMX [7]. The present investigation was undertaken in order to find out whether the phosphodiesterase inhibitors which prevented the 5-HT-induced elevation of endogenous cAMP did so by inhibiting adenylate cyclase.

MATERIALS AND METHODS

Quazodine was a gift of Mead Johnson, Evansville, IN and SQ 20009 was given by E. R. Squibb & Sons, Inc., Princeton, NJ. Papaverine was purchased from Sigma., St. Louis, MO. All other materials were obtained as described previously [5, 7].

Cell-free particles from liver flukes were prepared as reported previously [5].

Adenylate cyclase was assayed by the method of Salomon *et al.* [8]. Reactions were initiated by addition of particles (0.045 to 0.45 mg protein) to a reaction mixture, pre-warmed to 37° for 2 min, containing 100 mM sucrose, 50 mM glycylglycine (pH 7.5), 5 mM phosphocreatine, 5 units creatine phosphokinase, 2 mM $MgCl_2$, 5 mM 2,3-dimercapto-1-propanol,

* Abbreviations used: 5-HT (5-hydroxytryptamine) or serotonin, LSD (D-lysergic acid diethylamide), IBMX (isobutyl-methylxanthine), Quazodine (6,7-dimethyl-4 ethylquinazoline), and SQ 20009 [1-ethyl-4-(isopropylidene-hydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride].

Table 1. Comparison of effects of cAMP phosphodiesterase inhibitors on the basal, NaF-activated and 5-HT-activated adenylate cyclase*

Additions	Adenylate cyclase activity (pmoles/mg protein/min)		
	Basal	+ NaF (5 mM)	+ GTP (1 μM) + 5-HT (10 μM)
None	17.7 ± 2.0	2680 ± 446	337
Papaverine (1 mM)	15.5 ± 1.3	3148 ± 548	131
SQ 20009 (1 mM)	15.5 ± 1.9	2339 ± 569	213
Quazodine (1 mM)	21.3 ± 3.7	2981 ± 624	259
Caffeine (1 mM)		2826 ± 636	280
IBMX (0.5 mM)	18.8 ± 1.5	2750 ± 388	334

* Activity was assayed as described in Materials and Methods. This is a representative experiment. Basal values are quadruplicate, NaF values are triplicate assays ± S.E.M. GTP and 5-HT values are duplicates which did not vary more than 5%.

0.1 mM Na_2ATP (unless otherwise noted), 1–2 Ci [^{32}P]ATP tetra(triethylammonium) salt, and a "cold trap" of cAMP which varied from 0.5 to 1 mM non-radioactive cAMP in a total volume of 0.25 ml. Incubations were carried out at 37° for up to 10 min. The reaction was stopped by addition of a reagent containing 2% sodium dodecyl sulfate (pH 7.4), 10 mM NaEDTA, 10 mM Na_2ATP and 1 mM cAMP in a total volume of 0.25 ml. Approximately 10,000 cpm of [^{3}H]cAMP was added as a recovery standard, and the [^{32}P]cAMP formed during the reaction was separated by column chromatography and measured in a liquid scintillation spectrometer. Proteins were determined by the method of Lowry *et al.* [9].

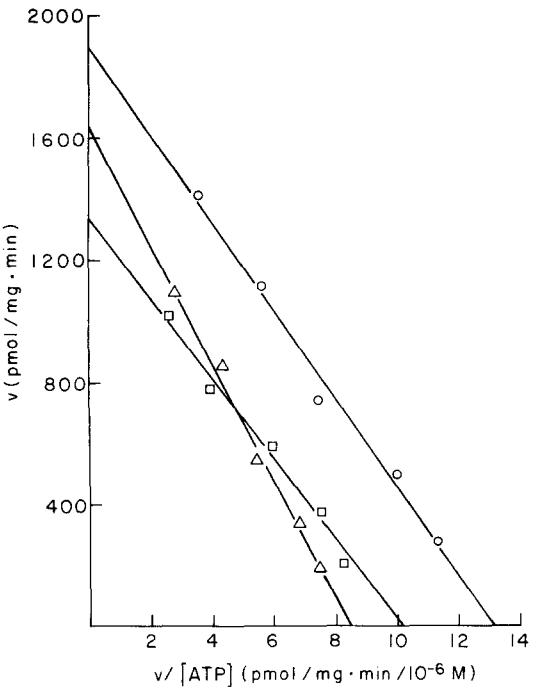

Fig. 1. Hofstee plots of velocity of serotonin-activated adenylate cyclase as a function of ATP concentration with no inhibitor (○), with 1 mM papaverine (△) or 1 mM SQ 20009 (□). Adenylate cyclase was determined as described in Materials and Methods. Reaction mixtures contained 100 μM GTP and 100 μM 5-HT; ATP concentrations varied from 25 to 400 μM. Lines were determined by linear regression ($r^2 > 0.99$).

RESULTS

Preliminary experiments showed an apparent increase in adenylate cyclase activity when either IBMX, SQ 20009 or papaverine was added to the reaction mixture. This was true whether we measured basal, NaF-activated or 5-HT-activated enzyme. The increase in adenylate cyclase activity was due presumably to the inhibition of phosphodiesterase activity by these agents. In all the experiments reported below, it was necessary to use 0.5 mM non-radioactive cAMP (cold trap) so that the phosphodiesterase present with the adenylate cyclase in the particulate enzyme preparation did not hydrolyze a significant portion of the [^{32}P]cAMP formed during the reaction.

When the cold trap was used in the adenylate cyclase reaction mixture, basal activity was not affected significantly by the phosphodiesterase inhibitors: IBMX, SQ 20009, papaverine or Quazodine (Table 1). The same compounds, as well as caffeine, were tested on the NaF-activated cyclase and the activity was not changed significantly. Papaverine, SQ 20009, Quazodine and caffeine, however, all inhibited the serotonin-activated adenylate cyclase activity. IBMX, on the other hand, did not inhibit the 5-HT-activated cyclase. It either had no effect or it slightly stimulated the enzyme. Table 1 contains representative experiments of the effects of these agents on each type of adenylate cyclase activity.

A series of experiments was done to determine if papaverine or SQ 20009, the most active inhibitors, compete with the substrate (ATP) to inhibit 5-HT-activated adenylate cyclase. A representative experiment is shown in Fig. 1. The data are presented as a Hofstee plot. The inhibition by SQ 20009 is apparently noncompetitive, while papaverine appears to have mixed kinetics of inhibition [10].

An attempt was made to determine whether papaverine or SQ 20009 was competing for the "GTP site" [11]. Figure 2 shows that inhibition by papaverine or SQ 20009 could not be overcome in the presence of higher concentrations of GTP. In fact, the percentage of inhibition is greater at 100 μM GTP than at 1 μM GTP.

Experiments were carried out to determine whether papaverine or SQ 20009 was competing for the serotonin receptor. We tested two concentrations of papaverine at varying concentrations of 5-HT. Preliminary data showed that the degree of inhibition by the phosphodiesterase inhibitors was reduced at high 5-HT levels. Careful kinetic experiments, however, did not

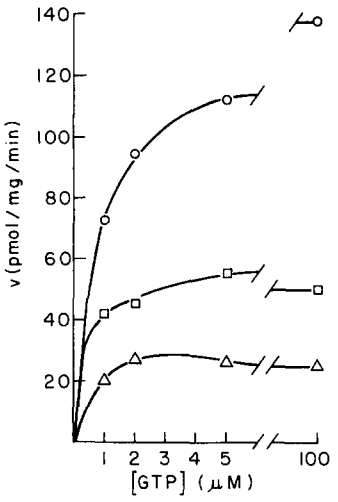

Fig. 2. Plots of velocity of serotonin-activated adenylate cyclase as a function of GTP concentration with no inhibitor (○), with 1 mM papaverine (△) or 1 mM SQ 20009 (□). The concentration of GTP varied from 1 to 100 μM. Adenylate cyclase was determined as described in Materials and Methods. Reaction mixtures contained 2 μM 5-HT and 0.1 mM ATP.

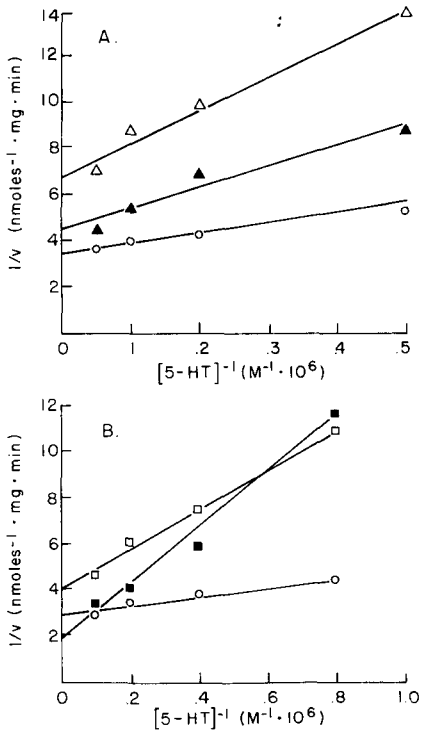

Fig. 3. Lineweaver–Burk plots of adenylate cyclase as a function of 5-HT concentration with two concentrations of papaverine (panel A) and SQ 20009 (panel B). Reaction mixtures contained 1 μM GTP and 0.1 mM ATP. In panel A, the 5-HT concentration varied from 2 to 20 μM and adenylate cyclase was assayed with no inhibitor (○), with 0.5 mM (▲) or 1 mM (△) papaverine. In panel B, the concentration of 5-HT varied from 1.25 to 10 μM and adenylate cyclase was assayed with no inhibitor (○), with 0.5 mM (■) or 1 mM (□) SQ 20009. All lines were drawn by linear regression ($r^2 > 0.96$).

support the view that such an antagonism is competitive. The results (Fig. 3A), plotted according to Lineweaver–Burk, indicate that at low 5-HT concentrations the inhibition by papaverine appears to have mixed kinetics, not clearly competitive or noncompetitive. The same type of experiment was done using SQ 20009, and the results (Fig. 3B) indicate that the kinetics of inhibition by SQ 20009 are also mixed.

DISCUSSION

The level of cAMP in the cell is determined by the activity of adenylate cyclase in relation to the activity of cAMP phosphodiesterase. Attempts to show an increase in cAMP levels in intact tissues following treatment with phosphodiesterase inhibitors have not always been successful [12]. In a previous study [7], incubation of liver fluke heads with 5-HT *in vivo* produced a 30-fold increase in endogenous cAMP levels. IBMX, a potent phosphodiesterase inhibitor, potentiated this effect of serotonin [7]. This correlates well with our present study which shows that IBMX does not inhibit adenylate cyclase. However, SQ 20009 and papaverine antagonized the *in vivo* increase in cAMP mediated by 5-HT [7]. The present results indicate that these compounds inhibit adenylate cyclase *in vitro*. We conclude that the *in vivo* antagonism of cAMP accumulation may be accounted for by inhibition of adenylate cyclase.

Papaverine and SQ 20009 do not appear to inhibit the serotonin-activated adenylate cyclase by competing for the enzyme substrate, ATP. This is indicated by the kinetic experiments as well as by the fact that they do not inhibit significantly either the basal or NaF-activated enzyme. Our results are also inconsisent with the supposition that these compounds are competing for GTP, which is essential for maximal 5-HT activation of the cyclase.

In attempting to determine if SQ 20009 or papaverine was competing with 5-HT, several types of experiments were done. Although the percentage of inhibition decreased as 5-HT levels were raised from 1 to 100 μM (results not shown), kinetic studies at low serotonin levels indicate that the inhibition by SQ 20009 or papaverine is not strictly competitive. Our present understanding of the hormone regulation of adenylate cyclases is that they are multi-component systems [11, 13, 14]. Further understanding of the mechanism of inhibition by SQ 20009 and papaverine will have to await isolation and identification of these components.

It is common practice among investigators working on adenylate cyclase to use a phosphodiesterase inhibitor to prevent the hydrolysis of the cAMP which they are trying to assay. Although Sheppard [15] reported that theopylline inhibits the basal and norepinephrine-stimulated adenylate cyclase from rat erythrocyte ghosts, he did not specify the mechanism of the inhibition. This investigation clearly shows that phosphodiesterase inhibitors must be chosen with care to insure that their effects are limited to inhibition of the phosphodiesterase and that they do not interfere with any of the components of the hormone-stimulated adenylate cyclase system.

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