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1-ETHYL-4-(ISOPROPYLIDENEHYDRAZINO)-1*H*-PYRAZOLO-(3,4-b)-PYRIDINE-5-CARBOXYLIC ACID, ETHYL ESTER, HYDROCHLORIDE (SQ 20009)—A POTENT NEW INHIBITOR OF CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASES*

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Abstract—The properties of SQ 20009 [1-ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl] as a cyclic nucleotide phosphodiesterase inhibitor have been investigated. The phosphodiesterase preparations used in this study were ammonium sulfate-fractionated supernatants of homogenates of rat brain, rabbit brain, rat adrenal, rat lipocyte and cat heart; commercially available beef heart phosphodiesterase was also studied. The concentrations of SQ 20009 required to inhibit these phosphodiesterase activities 50 per cent were 2·0, 4·8, 20, 21, 27 and 60 μM, respectively, using $1{\cdot}6 \times 10^{-7}$ M cyclic AMP as substrate. SQ 20006 (the parent of SQ 20009 lacking the 4-isopropylidene moiety), theophylline and caffeine were also tested against all six enzyme preparations. Whereas SQ 20009 was more potent than SQ 20006 using the phosphodiesterase prepared from rat adrenal, the potencies were reversed when the lipocyte enzyme was used. SQ 20009 was approximately 60 and 75 times as potent an inhibitor of rat brain cyclic AMP phosphodiesterase as were theophylline and caffeine respectively. The kinetic properties of the phosphodiesterases prepared from rat brain, cat heart and beef heart were also investigated. Using the rat brain enzyme, two K_m values for cyclic AMP, $4{\cdot}0 \times 10^{-6}$ and $1{\cdot}2 \times 10^{-4}$ M and a single K_m, $2{\cdot}0 \times 10^{-5}$ M, for cyclic GMP were confirmed. The K_i of SQ 20009 against the low K_m cyclic AMP phosphodiesterase was $2{\cdot}0 \times 10^{-6}$ M and that for cyclic GMP hydrolysis was $2{\cdot}4 \times 10^{-5}$ M. The inhibition by SQ 20009 of the hydrolysis of both cyclic nucleotides by both the rat brain and beef heart phosphodiesterases was competitive. The cat heart cyclic nucleotide phosphodiesterase was inhibited non-competitively by SQ 20009; the K_i for cyclic AMP hydrolysis was $6{\cdot}4 \times 10^{-5}$ M, and the K_i for cyclic GMP hydrolysis was $3{\cdot}0 \times 10^{-5}$ M. The inhibition by SQ 20009 of cyclic AMP hydrolysis by both the rat brain and cat heart preparations was reversible.

SUTHERLAND and Rall[1] have reported that cyclic AMP† was hydrolyzed to 5′-AMP by tissue extracts from several sources and that this hydrolysis was inhibited by caffeine. These results were confirmed and extended by Butcher and Sutherland,[2] who demonstrated that the methyl xanthines inhibited cyclic 3′,5′-nucleotide phosphodiesterase; the most potent inhibitor was theophylline, with a K_i of $1{\cdot}0 \times 10^{-4}$ M.

* A preliminary report of these studies has been previously communicated: M. Chasin, *Fedn Proc.* **30,** 1268 (1971).

† The following abbreviations are used: cyclic AMP, cyclic adenosine 3′,5′-monophosphate; cyclic GMP, cyclic guanosine 3′,5′-monophosphate; SQ 20009, [1-ethyl-4-(isopropylidenehydrazino)-1*H*-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride]; SQ 20006, [1-ethyl-4-hydrazino-1*H*-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride].

Since then, theophylline has been used by a number of workers as a reference inhibitor of cyclic 3′,5′-nucleotide phosphodiesterase.

Potentiation of a hormonally stimulated cellular response by inhibitors of phosphodiesterase has been proposed as one of four criteria for proving that this response is mediated by intracellular increases in cyclic AMP.[3] Recently, however, theophylline has been shown to have a number of actions unrelated to phosophodiesterase inhibition. McNeill *et al.*[4] showed that theophylline had direct effects on cardiac contractility, apparently unrelated to inhibition of phosphodiesterase. Furthermore, lipolytic concentrations of both theophylline[5] and caffeine[6] could not be demonstrated to elevate the levels of cyclic AMP in isolated lipocytes. Dalton *et al.*[7] have reported that theophylline probably has several activities in lipocytes, only one of which is inhibition of phosphodiesterase. Finally, Sheppard[8] has reported that theophylline can inhibit norepinephrine-stimulated adenyl cyclase in ghosts of rat erythrocytes.

Other agents have been shown to inhibit cyclic 3′,5′-nucleotide phosphodiesterase. Cheung[9] showed that ATP, pyrophosphate and citrate inhibited the high K_m enzyme prepared from rat brain, perhaps by chelation of magnesium. Several drugs inhibit the enzyme, with K_i values greater than 1×10^{-4} M. These include ethacrynic acid,[10] 3,5,3′-triiodo-L-thyronine[11,12] and thyroxine,[12] chlorpromazine and promethazine[13] and others. Several papaverine analogs have been reported[14,15] with K_i values for the high K_m enzyme between 2 and 10×10^{-5} M. Finally, a number of xanthine derivatives[16,17] have also been shown to inhibit the high K_m cyclic 3′,5′-nucleotide phosphodiesterase, with K_i values between 10^{-5} and 10^{-3} M.

The present paper presents a new inhibitor of phosphodiesterase with a K_i of $2{\cdot}0 \times 10^{-6}$ M for the low K_m enzyme prepared from rat brain, and a K_i of $6{\cdot}4 \times 10^{-5}$ M for the low K_m enzyme prepared from cat heart.

MATERIALS AND METHODS

All cyclic 3′,5′-nucleotide phosphodiesterase assays were performed by the method of Brooker *et al.*,[18] with a cyclic AMP concentration of $1{\cdot}6 \times 10^{-7}$ M, unless otherwise indicated. Preliminary experiments with the inhibitors described in this paper were also conducted using the spectrophotometric assay for phosphodiesterase described by Drummond and Perrott-Yee,[19] except that the 5′-adenylic acid deaminase used in this assay was prepared by the method of Smiley *et al.*,[20] and generously donated by those authors. Results obtained by both methods were in close agreement.

All phosphodiesterase assays were conducted using partially purified enzyme preparations from the appropriate tissues. The preparation of the phosphodiesterase from rat brain will be described; the other phosphodiesterase preparations were prepared in a similar manner. It should be noted that the rat lipocyte phosphodiesterase was prepared from cells isolated by the method of Rodbell.[21] The beef heart phosphodiesterase was purchased from Sigma.

For a typical preparation of rat brain phosphodiesterase, the brains (1·9 g each) of five male, Sprague–Dawley rats were homogenized in 10 ml of 0·05 M Tris–HCl buffer, pH 7·4. Cacodylate and imidazole buffers have also been used. Enzymes prepared in cacodylate and Tris–HCl were of similar specific activities, whereas use of imidazole resulted in a final enzyme preparation with approximately twice the specific activity of that prepared utilizing either of the other two buffers; this result is in agreement with the demonstrated ability of imidazole to stimulate phosphodiesterase

activity.[2] The homogenate was centrifuged at 40,000 *g* for 30 min at 2–4° in a Sorvall model RC-2B refrigerated centrifuge. To the resulting supernatant solution was added sufficient solid ammonion sulfate to bring the final solution to 50 per cent saturation. After mixing at 0° for 15 min, the preparation was allowed to stand in ice for an additional hour. After centrifugation as described above, the resulting precipitate was redissolved in 0·05 M Tris–HCl, pH 7·4 and centrifuged again; the final supernatant solution was dialyzed at 4° overnight against several changes of the Tris buffer, thus providing a 5-fold purification. The freshly prepared enzyme was used immediately for all assays. The stability of the preparations from the various tissues varied; the rat brain phosphodiesterase was found to be stable for at least 12 months at 4°. All protein determinations were by the method of Lowry *et al.*[22]

Ammonium sulfate, enzyme grade, used in the phosphodiesterase preparation was purchased from Mann. The 8-^{3}H-cyclic AMP, 16·3 c/m-mole, and 8-^{3}H-cyclic GMP, 1·1 c/m-mole, were purchased from Schwarz–Mann. Theophylline, caffeine and unlabeled cyclic AMP and cyclic GMP were obtained from Calbiochem. *Ophiophagus hannah* venom was purchased from Sigma. All reagents were of the highest obtainable commercial purity.

RESULTS AND DISCUSSION

Figure 1 demonstrates the inhibition of rat brain cyclic AMP phosphodiesterase produced by various concentrations of caffeine, theophylline and SQ 20009. Using the concentration of drug that produced 50 per cent inhibition of the enzymatic activity (I_{50}) as a measure of potency, it may be seen that, when the concentration of cyclic AMP was $1{\cdot}6 \times 10^{-7}$ M, SQ 20009 was approximately 60 and 75 times more potent an inhibitor than theophylline and caffeine respectively. Using phosphodiesterase prepared from cat heart, SQ 20009 was approximately twice as potent an inhibitor as either of these methyl xanthines. The structure of SQ 20009 is shown in Fig. 2.

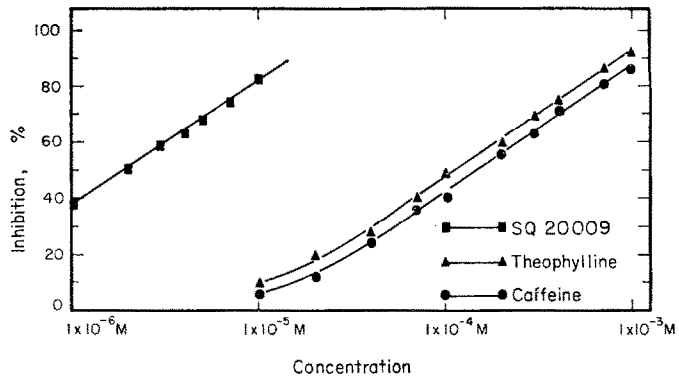

FIG. 1. Inhibition of rat brain cyclic 3′-5′-AMP phosphodiesterase by SQ 20009, theophylline and caffeine. Assays were performed as described in Methods. Each point represents a single determination.

Similar preparations of cyclic nucleotide phosphodiesterase from rat brain have demonstrated two K_m values for cyclic AMP, suggesting that they contain at least two different phosphodiesterases able to cleave cyclic AMP, or one enzyme displaying

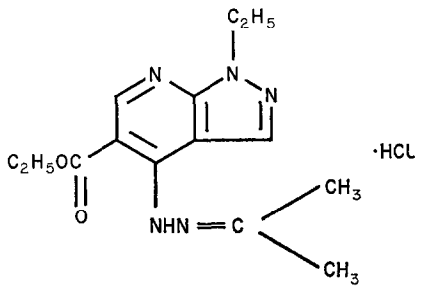

FIG. 2. Structure of SQ 20009.

negative cooperativity.[18,23] Our rat brain preparation also demonstrated two different K_m values for cyclic AMP, $4{\cdot}0 \times 10^{-6}$ M and $1{\cdot}2 \times 10^{-4}$ M. By assaying the preparation at a cyclic AMP concentration of $1{\cdot}6 \times 10^{-7}$ M, the contribution to the total activity by the high K_m enzyme can be ignored; the relative potencies of SQ 20009, theophylline and caffeine reported here refer to inhibition of the low K_m enzyme only. The enzyme prepared from cat heart also contained two cyclic nucleotide phosphodiesterases.

In an experiment to determine whether the inhibition of rat brain cyclic AMP phosphodiesterase by SQ 20009 was reversible, a 50-fold dilution of the rat brain phosphodiesterase was divided into four portions. To two of these, sufficient SQ 20009 was added to achieve a final concentration of 1·0 mM. To each of the other two samples was added an equal volume of buffer. One aliquot of both the treated and untreated enzymes was stored at 4° for 6 days, whereas the other aliquot of each pair was dialyzed, with frequent changes of buffer, for the same period. The enzyme solutions were further diluted 18-fold and 50-μl aliquots of each of them was assayed for phosphodiesterase activity. The nondialyzed, SQ 20009-treated enzyme was inhibited 88 per cent, but the corresponding dialyzed sample was inhibited only 2 per cent, thus demonstrating that the inhibition of cyclic AMP phosphodiesterase by SQ 20009 was reversible. Dialysis for this same period did not affect the untreated phosphodiesterase. Similar results were obtained using the phosphodiesterase prepared from cat heart.

The kinetics of the hydrolysis of cyclic AMP by the low K_m rat brain cyclic AMP phosphodiesterase and the inhibition of this hydrolysis by SQ 20009 were investigated by Lineweaver–Burk analysis and are presented in Fig. 3. As can be seen, the inhibition was competitive; the K_i was calculated to be $2{\cdot}0 \times 10^{-6}$ M. SQ 20009 is, therefore, the most potent inhibitor of the low K_m rat brain cyclic AMP phosphodiesterase yet reported. The K_i of SQ 20009 for the high K_m phosphodiesterase from rat brain was not determined. This preparation of phosphodiesterase was also able to cleave cyclic guanosine-3′,5′-monophosphate. Calculations based on Fig. 4 demonstrated that the single K_m for hydrolysis of cyclic GMP was $2{\cdot}0 \times 10^{-5}$ M, and the K_i of SQ 20009 for this activity of the enzyme preparation was calculated to be $2{\cdot}4 \times 10^{-5}$ M. Hydrolysis of cyclic GMP by the rat brain enzyme was also inhibited competitively by SQ 20009.

The kinetics of the inhibition of the hydrolysis of cyclic AMP and cyclic GMP by the low K_m cat heart cyclic nucleotide phosphodiesterase were also examined. In

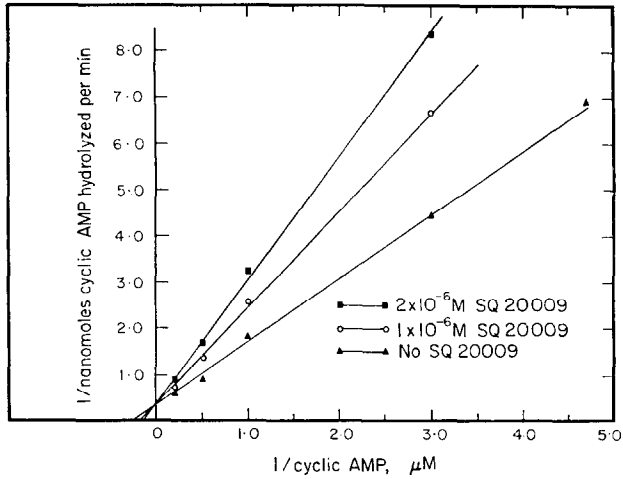

FIG. 3. Double-reciprocal plot of the hydrolysis of cyclic AMP by the low K_m rat brain cyclic AMP phosphodiesterase and the inhibition of this hydrolysis by SQ 20009. Assays were preformed as described in Methods. Each point represents the average of six determinations. Due to limitations of space, a number of points on each line have been omitted from the figure. The equations of the lines were calculated by computer for all experimental points.

contrast to the enzyme prepared from rat brain, the cat heart cyclic nucleotide phosphodiesterase was inhibited noncompetitively by SQ 20009, as can be seen in Fig. 5. The K_i of SQ 20009 for the hydrolysis of cyclic AMP by the low K_m cat heart cyclic nucleotide phosphodiesterase was $6{\cdot}4 \times 10^{-5}$ M, and the K_m for cyclic AMP was found to be $2{\cdot}3 \times 10^{-6}$ M. The noncompetitive nature of the inhibition was confirmed by analyzing the data by the method of Dixon.[24] Furthermore, SQ 20009 was a noncompetitive inhibitor of the hydrolysis of cyclic GMP, as can be seen in

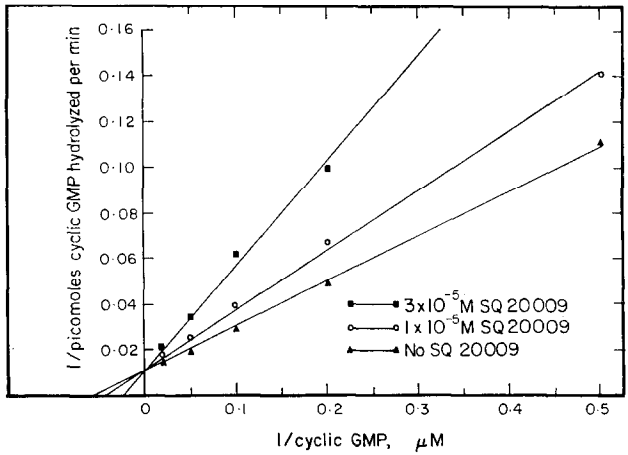

FIG. 4. Double-reciprocal plot of the hydrolysis of cyclic GMP by the rat brain cyclic GMP phosphodiesterase and the inhibition of this hydrolysis by SQ 20009. Assays were performed as described in Methods. Each point represents the average of four determinations. Details as in Fig. 3.

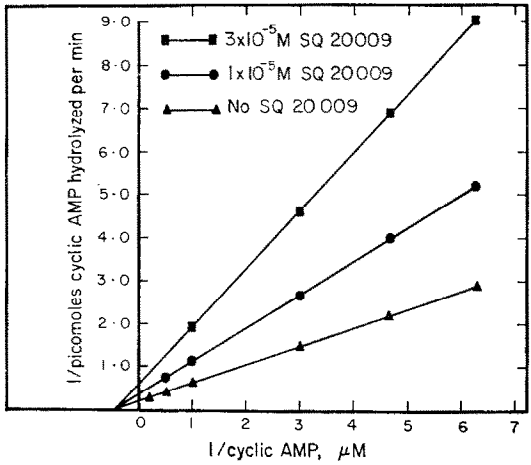

FIG. 5. Double-reciprocal plot of the hydrolysis of cyclic AMP by the cat heart cyclic nucleotide phosphodiesterase and the inhibition of this hydrolysis by SQ 20009. Assays were performed as described in Methods. Each point represents the average of three determinations.

Fig. 6; the K_i of SQ 20009 was calculated to be 3×10^{-5} M and the K_m for cyclic GMP was $5{\cdot}3 \times 10^{-6}$ M. To determine whether the noncompetitive inhibition found with the cat heart phosphodiesterase was peculiar to enzymes prepared from hearts, the cyclic AMP phosphodiesterase from beef heart was examined; two K_m values

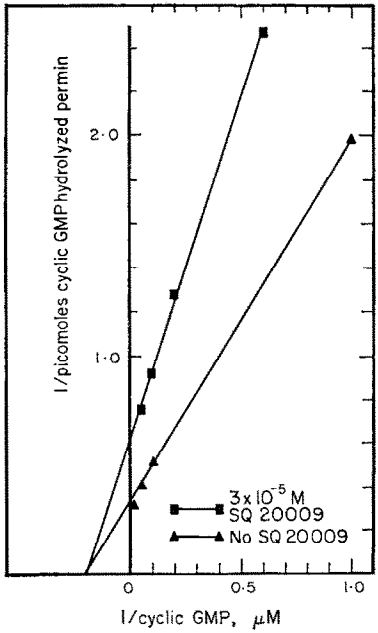

FIG. 6. Double-reciprocal plot of the hydrolysis of cyclic GMP by the cat heart cyclic nucleotide phosphodiesterase and the inhibition of this hydrolysis by SQ 20009. Assays were performed as described in Methods. Each point represents the average of three determinations.

were obtained, $3{\cdot}6 \times 10^{-5}$ M and $2{\cdot}6 \times 10^{-3}$ M. SQ 20009 inhibited the low K_m enzyme competitively; the K_i was $9{\cdot}5 \times 10^{-5}$ M. Of the three phosphodiesterases studied, it appeared that the cat heart cyclic AMP phosphodiesterase was unique, since it alone appeared to be inhibited noncompetitively by SQ 20009.

It is interesting to note that, although caffeine and theophylline are competitive inhibititors of the beef heart cyclic AMP phosphodiesterase,[2] Nair[25] reported that caffeine inhibited the dog heart enzyme noncompetitively, suggesting that the dog heart and cat heart enzymes may be similar.

In an effort to determine what specificities, if any, were demonstrated by SQ 20009, caffeine and theophylline as inhibitors of phosphodiesterase preparations from a variety of tissues, the enzyme was prepared from brain, adrenal and lipocytes of the rat, rabbit brain and cat heart; the beef heart enzyme was purchased. The I_{50} values for SQ 20009, caffeine and theophylline were determined by the method used for Fig. 1. These values are plotted in Fig. 7 for each of the six tissues. Although both caffeine and theophylline were relatively equipotent as inhibitors of all preparations of phosphodiesterase except beef heart, SQ 20009 was considerably more potent as an inhibitor of brain cyclic AMP phosphodiesterase than as an inhibitor of heart, lipocyte or adrenal phosphodiesterase. These data suggested that the phosphodiesterases prepared from different tissues differed considerably. The potency of SQ 20009 as an inhibitor of phosphodiesterase of brain was reflected in its marked anti-anxiety activity reported elsewhere.[26]

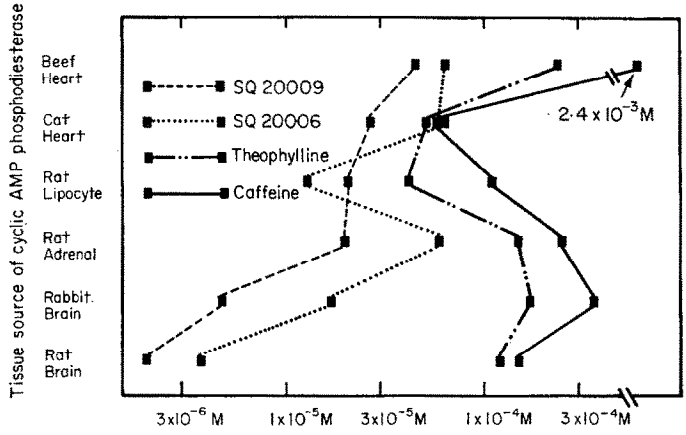

FIG. 7. Inhibition profiles of SQ 20009, SQ 20006, theophylline and caffeine as inhibitors of six cyclic AMP phosphodiesterase preparations. The heavy vertical bars represent the concentration of each drug that inhibited each particular enzyme 50 per cent, determined as shown in Fig. 1. Each heavy vertical bar represents an interpolated value from a concentration–inhibition graph having at least five concentrations of each compound for each enzyme preparation tested.

The hypothesis that the phosphodiesterases from different tissues were different was supported by the inhibition profile shown in Fig. 7 of SQ 20006, a close structural analog of SQ 20009. Although the profiles of SQ 20009 and SQ 20006 generally parallel each other, a notable exception was seen with the phosphodiesterase prepared from rat lipocytes. For this preparation, SQ 20006 was more potent than SQ 20009,

but in all other preparations, SQ 20009 was the more potent of the two compounds suggesting that the lipocyte enzyme might be different from the others. In isolated lipocytes, SQ 20006 was found to be more potent than SQ 20009 in potentiating epinephrine-stimulated lipolysis, but in isolated adrenal cells, SQ 20009 was more potent in potentiating ACTH-stimulated steroidogenesis.[27] Thus, the relative order of activities of the two phosphodiesterase inhibitors in preparations of whole cells is the same as the order of their activities in cell-free preparations.

In conclusion, SQ 20009 has been shown to be a potent competitive inhibitor of cyclic 3′,5′-nucleotide phosphodiesterase prepared from rat brain and beef heart, and a noncompetitive inhibitor of the enzyme prepared from cat heart. Further, the inhibition by SQ 20009 of the enzymes prepared from rat brain and cat heart was reversible. Evidence has been presented to suggest that cyclic 3′,5′-nucleotide phosphodiesterase differs considerably in different mammalian tissues, with respect to its inhibition by a number of inhibitors. Other workers, notably Vernikos-Danellis and Harris,[28] Smith and Mills,[29] Kukovetz and Poch,[15] and Amer and McKinney,[30] have reached similar conclusions based on differential inhibition of a variety of phosphodiesterases by several agents.

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