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# INHIBITION OF ADENYLATE CYCLASE OF CATFISH AND RAT HEPATOCYTE MEMBRANES BY 9-(TETRAHYDRO-2-FURYL)ADENINE (SQ 22536)‡

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The adenosine analogue 9-(Tetrahydro-2-furyl)adenine, SQ 22536, inhibited adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity of crude membrane preparations from catfish (*Ictalurus melas*) and rat isolated hepatocytes in a non-competitive manner. The $IC_{50}$s were reduced in the presence of NaF. SQ 22536 reduced the activity of adenylate cyclase also in the presence of increasing concentrations of GTP, as well as $Mg^{++}$ and $Mn^{++}$. In the presence of catecholamines (epinephrine, norepinephrine, isoproterenol, phenylephrine) SQ 22536 reduced their activating effect on adenylate cyclase in both catfish and rat membranes. SQ 22536 also inhibited the effect of glucagon (0.1 $\mu$M) on rat membrane cyclase activity.

KEY WORDS: Hepatocyte membranes, adenylate cyclase, catfish, rat, SQ 22536, 9-(Tetrahydro-2-furyl) adenine.

## INTRODUCTION

Liver adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (AC) and its activation or inhibition by hormones and drugs has been widely studied in mammals. The processes which are based on the activation of AC by hormones have been reviewed by several Authors[1,2]. The main components of the system hormone-AC responsible for the synthesis of cAMP from the intracellular ATP are: (a) the hormone receptor, (b) the catalytic moiety, (c) the guanosine nucleotide regulatory protein, or G-protein.

In other classes of vertebrates, including fish, AC and its modulation have been scarcely studied. In particular we have not found studies concerning fish liver membranes. For this reason, and to continue our research on the mechanisms of hormonal regulation of glycogenolysis in fish liver[3,4], we have previously[5] studied some properties of AC present in hepatocyte membranes from catfish, and its response to catecholamines and glucagon. We found that both natural (epinephrine and norepinephrine) and synthetic (isoproterenol and phenylephrine) catecholamines stimulated the AC activity of catfish hepatocyte membranes, and their action was inhibited by

‡Part of this work has already been presented in abstract form (Brighenti, L. *et al.*, (1989) *Eur. J. Physiol.*, **415**, n.3, S4).

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Abbreviations: AC, adenylate cyclase, cAMP, cyclic adenosine 3′:5′-monophosphate; $IC_{50}$, concentration giving 50% inhibition.

the $\beta$-blocking agent propranolol. Glucagon, compared to catecholamines, was less effective in stimulating the AC activity of catfish membranes.

In the study of several physiological processes linked to the synthesis of cAMP, much information has been obtained by the use of specific activators or inhibitors of AC. Adenosine and some of its analogues have often been employed as AC inhibitors. 9-(Tetrahydro-2-furyl)adenine (SQ 22536), an analogue of adenosine, was found to be active in inhibiting the AC activity of many cell and tissue types, e.g. rat adipocyte[6,7] and guinea pig lung membranes[8], platelets[9–12], rat hippocampal slices[13], sympathetic ganglia[14], neutrophils[15], pituitary[16] and bone[17,18] cells. The purpose of the present study is to establish if SQ 22536 is also an inhibitor of AC from fish hepatocyte membranes, so permitting further studies in liver cells of the processes in which AC may be involved. Accordingly we have tested the effect of SQ 22536 on the AC activity of crude membranes from hepatocytes of catfish (*Ictalurus melas*), and of rat for comparison.

## MATERIALS AND METHODS

### *Materials*

Reagents for teleost-Ringer solution and buffers for cell membrane preparation and cAMP tests were obtained from Merck (Darmstadt, FRG). ATP and GTP were from Calbiochem (La Jolla, CA, USA). Collagenase and all other reagents for cell isolation and tests, catecholamines and mammalian (porcine/bovine) glucagon were from Sigma Chem. Co. (St. Louis, MO, USA). The binding protein for cAMP analysis were prepared from beef adrenals according to the method of Brown *et al.*[19]. [$^3$H]cAMP was obtained from Amersham International plc (Amersham, UK), and liquid scintillation solution (Readygel) from Beckman Instruments Inc. (Mervue, Galway, Ireland). 9-(Tetrahydro-2-furyl) adenine (SQ 22536) was a gift from the Squibb Institute (Princeton, NJ, USA).

### *Animals*

Adult catfish (*Ictalurus melas*) of both sexes, weighing 200–300 g, were purchased from local dealers. Fish were maintained in tanks containing well-aerated, dechlorinated and continuously depurated tap water at the environmental temperature (18–24°C), and fasted for 4–6 days before the experiment. In rat experiments, male adult Wistar rats, weighing about 300 g and fasted for 1 day, were used.

### *Preparation of crude membrane fraction*

Hepatocytes were isolated using the technique of Seglen[20], adapted by us[21] for fish experimentation. The isolated cells were homogenized in 3 times their volume of water to obtain crude membranes. After centrifugation (20,000 $\times$ g for 10 min at 4°C) the pellet was re-suspended in water to give a final protein concentration of 1.5 mg/ml, as determined by the method of Lowry[22].

### *Adenylate cyclase assay*

Membrane suspension (0.1 ml, about 150 $\mu$g total protein) was added to test tubes

containing 400 $\mu$l of a standard reaction mixture consisting of 1 mM ATP, 3.75 mM $Mg^{++}$, 0.1 mM EGTA (all dissolved in 0.1 M Tris-HCl buffer, pH 8.0), 0.5 mM GTP and 3 mM aminophylline. Assays were carried out in triplicate. Agonists and SQ 22536 were added as indicated in the test and Figures. After 10 min incubation, performed in a shaking bath at 30°C, the reaction was stopped by immersing the tubes in boiling water for 2 min; samples were frozen and left overnight at −20°C. After melting, samples were centrifuged (3,000 × g for 10 min at 4°C) and cAMP levels were evaluated in the supernatant according to the method of Brown *et al.*[14]. Results are expressed as pmoles cAMP formed/mg protein/10 min. The concentration of SQ 22536 giving a decrease of 50% ($IC_{50}$) in the activity found in the absence of the inhibitor was calculated from the inhibition curves.

*Statistical analysis*

Values are expressed as the mean ± SEM of at least 3 enzyme preparations from hepatocytes of different livers. Since SEMs did not exceed the 10% of mean values, the bars indicating SEM were omitted in the graphs. Statistical analysis was carried out by using the paired Student's $t$ test. Only differences having a $p < 0.05$ were considered significant.

## RESULTS

The basal activities of AC were 37.9 ± 3.5 pmoles cAMP/mg protein/10 min ($n = 12$) and 51.5 ± 2.9 pmoles cAMP/mg protein/10 min ($n = 15$) for catfish and rat liver membranes, respectively. The effect of SQ 22536 is shown in Figure 1. The lowest concentrations of SQ 22536 to obtain an inhibition of the enzyme were 1.8 and 1.4 $\mu$M for catfish and rat membranes, respectively. Under basal conditions, concentrations of 0.31 and 0.39 mM of SQ 22536 were required to obtain a decrease of 50% ($IC_{50}$) in the AC activity of catfish and rat membranes, respectively. The inhibitory effect of SQ 22536 (0.1 mM) on the AC activity of catfish hepatocyte membranes was evident during the first 5 min, and lasted up to 30 min of incubation (Figure 2).

In order to evaluate the synthesis of cAMP by intact catfish liver cells incubated in the presence of SQ 22536, some experiments were performed with the technique previously used[4]. Results (not shown) indicated that only inhibitor concentrations greater than 0.1 mM induced an appreciable decrease in cAMP synthesis. However, this decrease did not exceed a value of 30% at a concentration 1.0 mM of SQ 22536.

Lineweaver-Burk plots for the synthesis of cAMP by AC in the presence of 0.1 mM SQ 22536 are shown in Figure 3. For catfish membranes, under our experimental conditions, the $K_m$ value was 0.035 mM ATP, and the $V_{max}$ decreased in the presence of SQ 22536 from 57.2 to 46.5 pmoles cAMP/mg protein/10 min. For rat membranes the $K_m$ value was 0.089 mM ATP, and the $V_{max}$ decreased in the presence of SQ 22536 from 46.3 to 22.1 pmoles cAMP/mg protein/10 min. In a previous study[5] we reported a higher $K_m$ value for catfish AC, but in that study the total $Mg^{++}$ concentration was constant at all ATP levels. Since the ratio $Mg^{++}$/ATP is an important condition for the linearity of Lineweaver-Burk plots[8], in order to maintain the availability of free $Mg^{++}$ constant, in the present study the concentration of this ion was increased

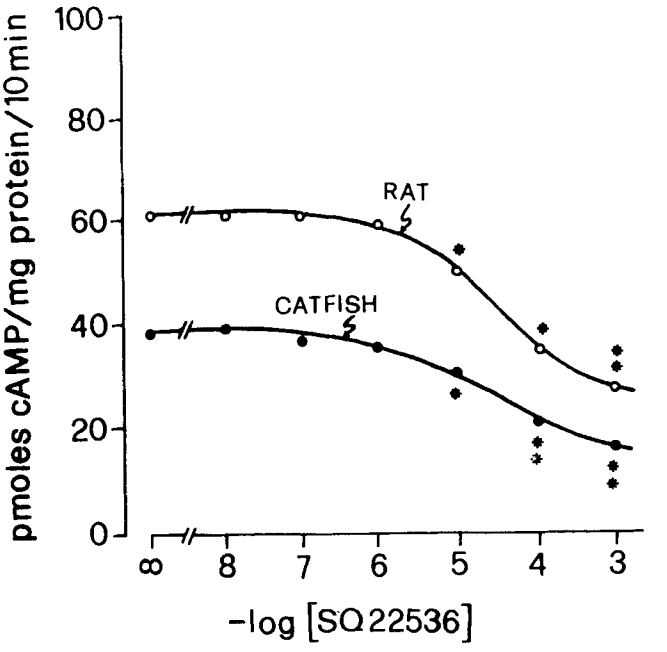


FIGURE 1 Inhibition curves of SQ 22536 on the basal activity of adenylate cyclase of catfish (●) and rat (○) hepatocyte membranes. Levels of significance (paired Student's $t$ test): (*) $p < 0.05$ and (**) $p < 0.01$ as compared to control values ($-\log$ [SQ 22536] $= \infty$).

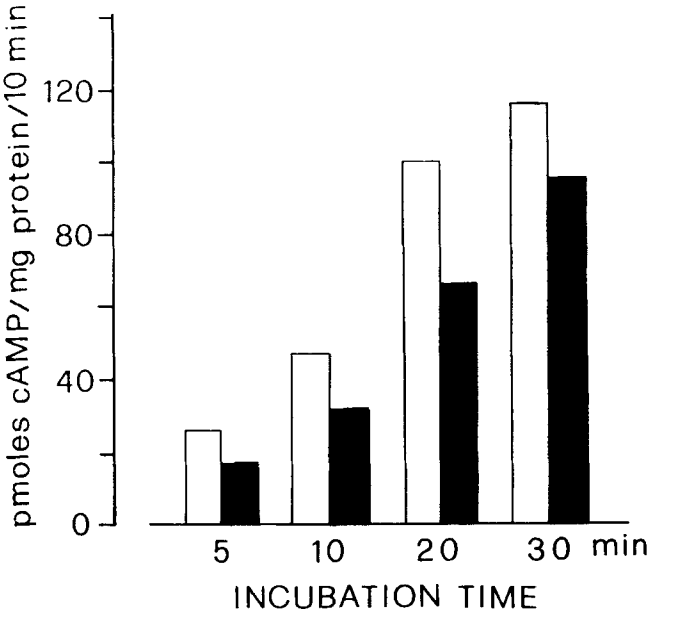


FIGURE 2 Time course of the SQ 22536 (0.1 mM) inhibition on the basal activity of adenylate cyclase of catfish hepatocyte membranes, □, controls; ■, in the presence of SQ 22536.

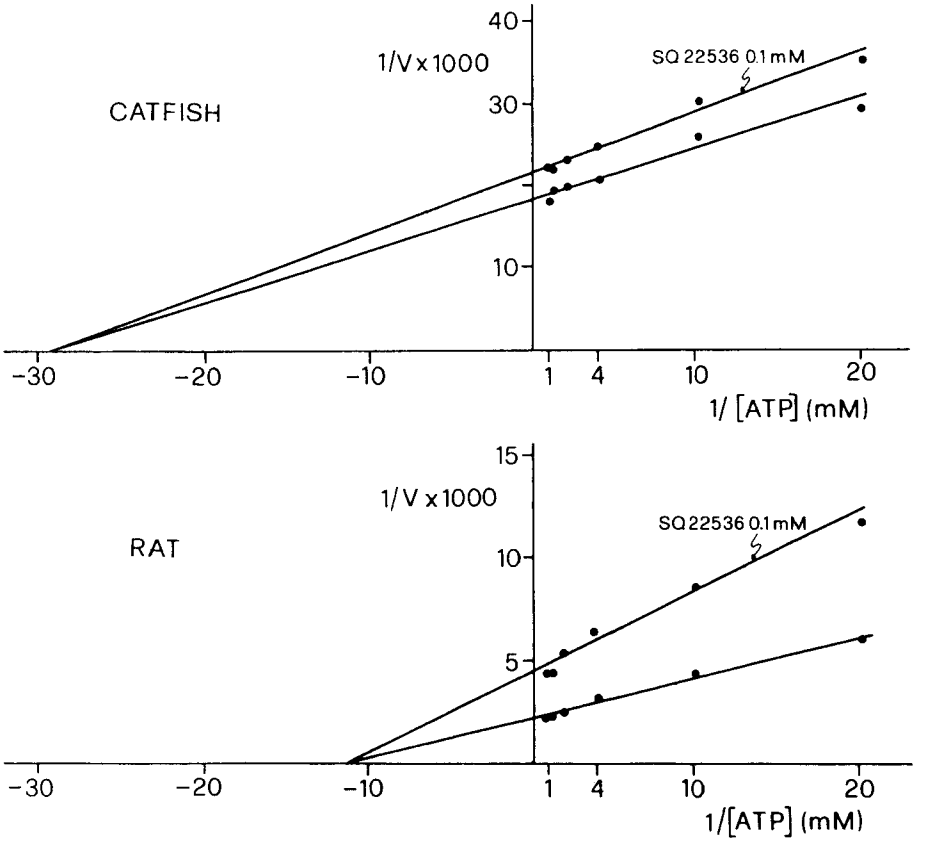


FIGURE 3 Lineweaver-Burk plots for the adenylate cyclase activity (substrate: ATP) of catfish and rat hepatocyte membranes in the presence of 0.1 mM SQ 22536.

relative to the increase in the ATP level. For both membranes the Lineweaver-Burk plots indicated non-competitive inhibition.

In the presence of 10 mM NaF (Figure 4) the AC activities, either in the absence or in the presence of SQ 22536, were about 2.5 times higher than under basal conditions (Figure 1), and the required concentrations of inhibitor for the $IC_{50}$ diminished to 0.08 and 0.27 mM for catfish and rat membranes, respectively (Table I).

The washing of catfish liver membranes pre-incubated for 10 min at 20°C in the presence of 0.1 mM SQ 22536 did not change the effect of the inhibitor on AC activity. However, if the membranes were also incubated in the presence of 10 mM NaF, the washing of membranes induced an increase in the AC activity to the control level (Table II).

The effect of GTP concentration on the AC activity of rat liver cell membranes was conducted in the presence and absence of 0.1 mM SQ 22536 (Figure 5). As we previously observed[5], in the absence of the inhibitor the curve reached a maximum value at a concentration of about 0.01 mM GTP, and remained constant at this level.

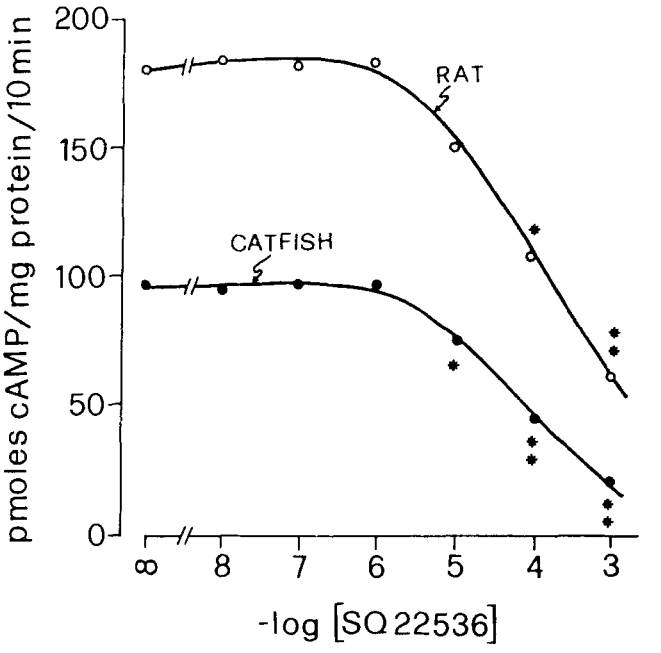


FIGURE 4 Inhibition curves of SQ 22536 on the activity of adenylate cyclase of catfish (●) and rat (○) hepatocyte membranes in the presence of 10 mM NaF. Levels of significance as in Figure 1.

TABLE I
$IC_{50}$ of SQ 22536 on adenylate cyclase of catfish and rat hepatocyte membranes.

| | catfish | | rat | |
|---|---|---|---|---|
| Agonist[a] | % increase[b,c] | $IC_{50}$(mM) | % increase | $IC_{50}$(mM) |
| None (basal | | 0.31 | | 0.39 |
| NaF | + 179.6 ± 12.0** | 0.08 | + 364.5 ± 55.9* | 0.27 |
| Epinephrine | + 38.9 ± 7.7* | 0.19 | + 45.0 ± 7.2* | 0.08 |
| Norepinephrine | + 37.8 ± 9.4* | 1.45 | + 77.0 ± 4.3* | 0.05 |
| Isoproterenol | + 53.8 ± 5.5* | 0.21 | + 94.2 ± 10.3* | 0.08 |
| Phenylephrine | + 20.4 ± 1.8* | 0.92 | + 13.0 ± 4.0 | 0.17 |
| Glucagon | ND[d] | ND | + 424.1 ± 8.4** | 0.30 |

[a]Agonist concentrations; NaF 10 mM; catecholamines 0.01 mM; glucagon 0.1 μM. [b]In the absence of SQ 22536. [c]Mean values ± SEM ($n = 4$) of percentage differences as compared to the corresponding control values. [d]Not determined. Levels of significance (paired Student's $t$ test): *) $p < 0.05$ and $p < 0.01$ as compared to control values in the absence of inhibitor.

The presence of SQ 22536 in the incubation medium shifted the curve to a lower level ($p < 0.05$ at all GTP concentrations), and at concentrations greater than 0.5 mM GTP there was a decrease in AC activity below the initial level.

The effects of increasing concentrations of $Mg^{++}$ and $Mn^{++}$ in the presence and in the absence of 0.1 mM SQ 22536 were also tested on rat hepatocyte membranes (Figure 6). The influence of SQ 22536 on the enzyme activity was more evident when the $Mg^{++}$ concentration was high but was about the same at all levels of $Mn^{++}$.

TABLE II
Effect of washing on adenylate cyclase activity of catfish hepatocyte membranes pre-incubated with SQ 22536 and NaF.

| | − NaF | | | + NaF[a] | | |
|---|---|---|---|---|---|---|
| Number of Washes | Control | + SQ[b] | SQ/Control | Control | + SQ | SQ/Control |
| None | 38.5[c,d] | 32.6 | 0.85 | 141.0 | 51.2 | 0.36 |
| 1 | 40.2 | 30.8 | 0.77 | 110.2 | 119.1 | 1.08 |
| 3 | 39.0 | 29.1 | 0.75 | 60.1 | 65.2 | 1.09 |

[a]NaF 10 mM. [b]SQ 22536 0.01 mM. [c]AC activity = pmoles cAMP formed/mg protein/10 min. [d]Mean values of 2 experiments. Membranes were washed 1 or 3 times with the incubation medium, centrifuged and resuspended in the test solution.

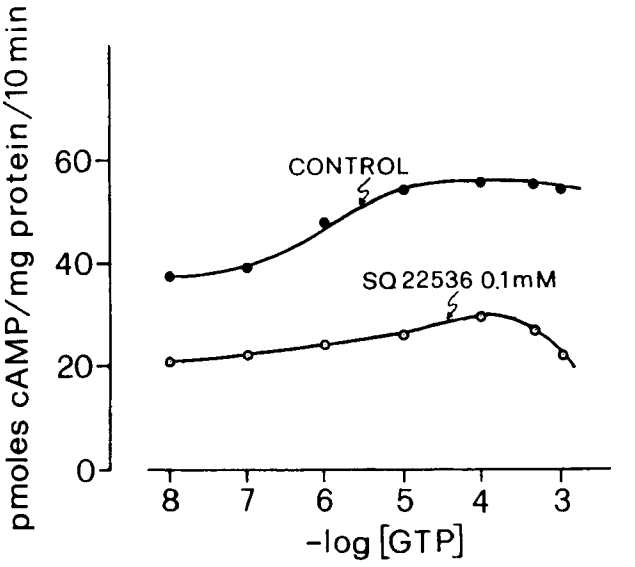


FIGURE 5 Effect of GTP on the adenylate cyclase activity in control and SQ 22536 treated (0.1 mM) rat hepatocyte membranes.

The treatment of membranes with catecholamines increased the basal AC activity by different degrees as compared to a control. Figure 7 shows the effect of increasing concentrations of SQ 22536 on the activity of catfish and rat liver membranes stimulated by 0.01 mM epinephrine and other catecholamines. The percentage inhibitory effects of SQ 22536 were of the same order of magnitude as those observed under basal conditions (compare with Figure 1). Pre-incubation (1 to 10 min) with SQ 22536 of agonist-treated membranes did not increase the effect of the compound, demonstrating that inhibition occurs rapidly (data not shown). The initial concentration required to obtain an inhibitory effect varied from about 1.5 to 3.4 $\mu$M according to the catecholamine and animal concerned. The $IC_{50}$s were higher for catfish membranes, norepinephrine and phenylephrine showing the highest values. Table I sum-

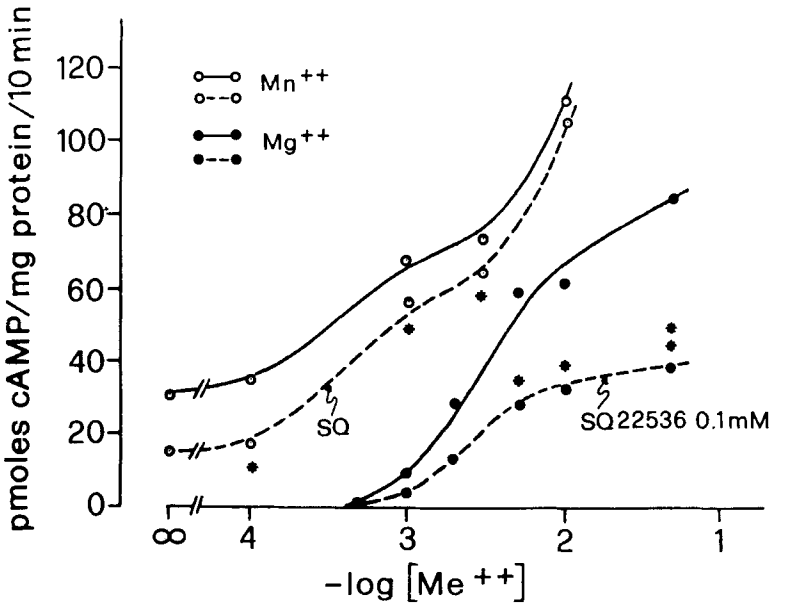


FIGURE 6 Effect of SQ 22536 on the adenylate cyclase activity of rat hepatocyte membranes in the presence of increasing concentrations of $Mg^{++}$ or $Mn^{++}$. Levels of significance (paired Student's $t$ test). (*) $p < 0.05$ as compared to values in the absence of SQ 22536 at the same $Me^{++}$ concentration.

marizes the increases of AC activity due to the agonists and the corresponding $IC_{50}$s of SQ 22536.

SQ 22536 was also tested on glucagon (0.1 $\mu$M)-treated rat membranes. Only rat membranes were used since in our previous study[5] this hormone showed no effect on catfish membranes at this concentration. The AC activity was increased by glucagon more than by catecholamines (Figure 8). SQ 22536 inhibited AC activity stimulated by glucagon, but its $IC_{50}$ value was higher than in the presence of catecholamines (Table I).

## DISCUSSION

SQ 22536 [9-(Tetrahydro-2-furyl)adenine] has already been studied as an inhibitor of AC in several cell types, e.g. neutrophils, platelets, bone and pituitary cells, and others[6-18]. In general, researches utilizing SQ 22536, as well as other adenosine analogues, were performed on intact cells, in which the change of cAMP level was determined as an index of AC activity. In our experiments intact cells were found to be less sensitive to SQ 22536 compared to cell membranes (data not shown). This reduced effect of SQ 22536 could possibly be due to inability to penetrate the cell in sufficient amounts to have a substantial effect.

In this study we have demonstrated that SQ 22536 is an inhibitor of AC present in crude cell membranes obtained from catfish and rat isolated hepatocytes. The concentrations of SQ 22536 necessary to obtain a significant effect were similar to those found by other authors[6-18]. The effect on AC activity has also been shown in the

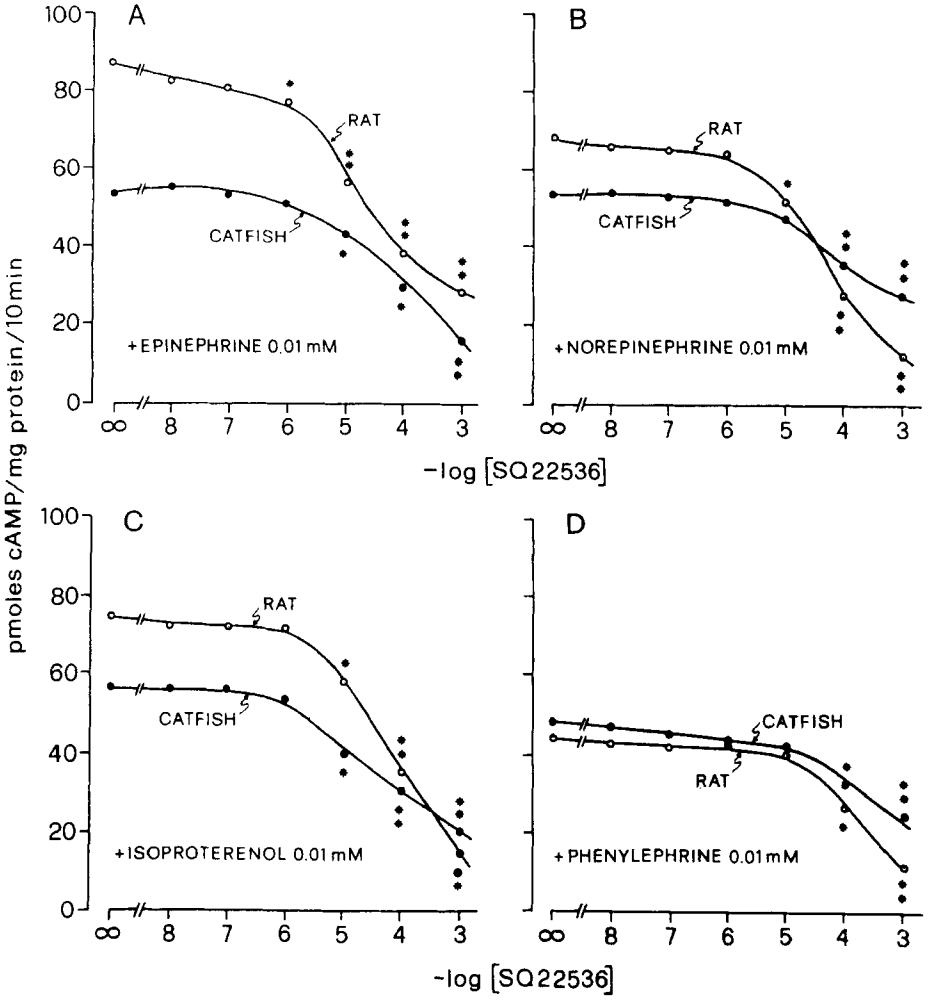


FIGURE 7 Inhibition curves of SQ 22536 on the activity of adenylate cyclase of catfish (●) and rat (○) hepatocyte membranes in the presence of 0.01 mM catecholamines (*A*: epinephrine; *B*: norepinephrine; *C*: isoproterenol; *D*:phenylephrine). Levels of significance as in Figure 1.

presence of activators (NaF, $Mg^{++}$, $Mn^{++}$) or specific agonists (catecholamines, glucagon).

Rat membranes showed higher basal AC activity compared to catfish membranes (Figure 1). The effect of SQ 22536 on the basal activity began for the AC of both animals at a concentration of about 1–2 $\mu$M although higher concentrations were required to obtain a significant effect. We found that the concentration of SQ 22536 which reduced the AC activity to half of the control level ($IC_{50}$) was about 300 and 400 $\mu$M for catfish and rat membranes, respectively (see Table I). The time-course for the pre-incubation, in which SQ 22536 was in contact with the membranes before adding the agonist, and the time-course for incubation showed that SQ 22536 was

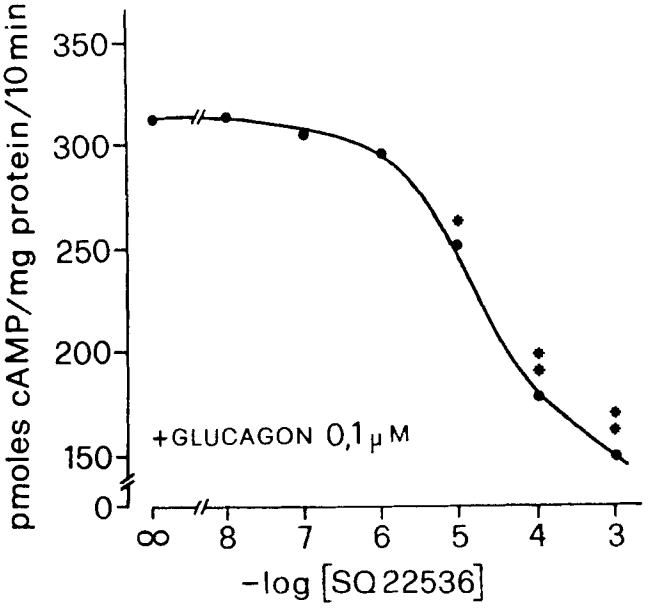


FIGURE 8 Inhibition curves of SQ 22536 on the activity of adenylate cyclase of rat hepatocyte membranes in the presence of 0.01 mM mammalian (porcine/bovine) glucagon. Levels of significance as in Figure 1.

bound immediately to the enzyme, and that the binding was not spontaneously reversible (Figure 2).

Lineweaver-Burk plots (Figure 3) for the synthesis of cAMP from ATP by AC in the absence or presence of SQ 22536 showed that in both catfish and rat membranes the inhibition of AC activity was of the non-competitive type. This fact indicated that, as in other cyclase systems[8,23], SQ 22536 interacts with the enzyme on a site (or sites) different to that for attachment of the substrate (ATP).

Most ACs are activated by fluoride through a direct action on the regulatory component of the AC system[24]. Here, it was observed that SQ 22536 had an inhibitory effect also on fluoride-stimulated AC activity (Figure 4). In both membrane preparations in the presence of 10 mM NaF the $IC_{50}$ was reduced, and a difference between catfish and rat membranes was evident (see Table I). The washing of catfish liver membranes after treatment with SQ 22536 (Table II) did not influence its inhibitory effects. On the contrary, in the presence of NaF, the washing of the membrane preparation removed the effect of SQ 22536. This indicated that SQ 22536 bound to the membrane was sufficient, after washing, to inhibit the AC under basal conditions, but when NaF was present, the concentration of the bound inhibitor was no longer sufficient to counteract the agonistic effect of NaF.

Cells may be sensitive in different ways to adenosine analogues. Londos and Wolff[25] classified adenosine derivatives into four classes according to their ability to stimulate AC activity in Leydig or liver cell membranes. They indicated two sites where adenosine analogues could attack, (1) those with a strict structural requirement for the purine moiety (P sites), located on the internal surface of the cell membrane, and (2) those that have a strict requirement for the ribose moiety of the molecule (R sites),

present on the external surface of the cell membrane. Since liver AC does not tolerate alterations in the purine moiety, the system contains the P type of the adenosine sites. The modifications of the ribose moiety of adenosine are, on the contrary, well tolerated. SQ 22536, which contains a modified sugar but unaltered purine moiety, acts on the P site. The interaction of SQ 22536 with the AC system occurs independently of the receptor coupled with AC. According to others[7] the binding site of SQ 22536 could be at or near the catalytic subunit.

GTP increased AC activity in rat hepatocyte membranes (Figure 5) by binding to the regulatory G protein[26]. According to Gilman[27] and others, the structure of G protein occurs as $G_s$ (stimulating) and $G_i$ (inhibiting) protein. Some adenosine analogues react with $G_i$ protein inducing its activation, and, as a consequence, cause the inhibition of AC activity and the reduction of cAMP formation. Under our experimental conditions SQ 22536 decreased the GTP-stimulated AC activity, and the inhibition was greater at the high GTP concentrations (0.1 and 1 mM). This fact may indicate that SQ 22536 also activated $G_i$ protein in rat liver cell membrane.

AC is a complex enzyme, whose substrate is a metal-ATP complex[28] where the metals may be $Mg^{++}$ or $Mn^{++}$. These ions increase AC activity and its sensitivity to adenosine inhibition[29,30]. The inhibitory potency of adenosine appears to be dependent on $Mg^{++}$ concentration, and this dependence has been observed for some adenosine analogues, which are very potent inhibitors of lung AC[8]. A greater inhibitory potency of SQ 22536 in the presence of higher concentrations of $Mg^{++}$ was also observed[8]. The results of our experiments on rat hepatocyte membranes (Figure 6) agree with those of Weinryb and Michel[8]. In fact the major effect of SQ 22536 was attained starting from 10 mM $Mg^{++}$. The effect of SQ 22536 on $Mn^{++}$-stimulated AC activity was lower than that on $Mg^{++}$-increased enzyme activity. In fact the control and SQ 22536-treated curves are parallel up to 1.0 mM $Mn^{++}$, and at higher concentration the curves converged, indicating that high concentrations of $Mn^{++}$ lowered the effect of SQ 22536.

AC present in liver membranes is stimulated by many agonists, among which are some hormones that bind to specific receptors. We studied the inhibiting action of SQ 22536 on the effects of epinephrine, norepinephrine, isoproterenol, phenylephrine and glucagon on AC activity of both catfish and rat membranes (Figures 7 and 8). In rat and catfish, epinephrine, norepinephrine and isoproterenol enhanced the enzyme activity; phenylephrine had a small effect in catfish only, as shown in a previous work[5]. We also found[5] that the catecholamine-induced AC activity in catfish was blocked by propanolol and not by phentolamine. Propanolol alone did not influence the enzyme activity of controls. As propanolol is a general $\beta$-antagonist and phentolamine an $\alpha$-antagonist, we concluded[5] that catecholamines act in catfish liver via activation of $\beta$-receptors. The effect of propanolol depends on its binding to the $\beta$-receptor instead of the agonist. A different behaviour was shown by SQ 22536, which inhibited either the basal or the catecholamine-stimulated AC activity. These results demonstrate the different site of action of propanolol and SQ 22536.

Noteworthy is the observation that the $IC_{50}$ of SQ 22536 under basal condition of rat membranes was greater than the $IC_{50}$ of catfish membranes. On the contrary in catecholamine-treated membranes the $IC_{50}$s for SQ 22536 were higher in catfish membranes than the correspondent values for rat membranes. This indicated a greater sensitivity to SQ 22536 induced by catecholamines on rat membranes as compared to catfish membranes under the same conditions.

Glucagon was examined in rat membrane only, since in catfish its effect was

almost absent[5]. In the presence of glucagon SQ 22536 reduced AC activity commencing from a concentration of 1 $\mu$M, which, together with the $IC_{50}$, is of the same order as that necessary to counteract the catecholamine effect.

Present results confirm our previous findings[5] on the properties of AC of catfish hepatocyte membranes, and its sensitivity to catecholamines. As regards SQ 22536, our studies indicate that this substance is an inhibitor of AC activity of liver cell membranes suggesting that it may be useful in investigating the synthesis and the role of cAMP in the many physiological responses of these cells.



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