STIMULATION AND INHIBITION OF MEMBRANE ADENYLATE CYCLASE BY ADENOSINE ANALOGS IN THE SALT GLAND OF SQUALUS ACANTHIAS

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Adenosine has been shown to inhibit (e.g. fat cells) and stimulate (e.g. platelets, brain) adenylate cyclase (AC) via external membrane receptors called A_1 or R_i and A_2 or R_a respectively. Evidence has been provided for both stimulatory A_1 and inhibitory A_2 receptors in the perfused gland (Forrest et al., Bull. MDIBL 20:152-155, 1980; Forrest et al., Kidney Int. 21:253, 1982; and Poeschla et al., Bull. MDIBL 22:S19-S23, 1982), in tissue slice experiments measuring content of cAMP (Poeschla et al., Bull. MDIBL 22:S15-S18, 1982) and in an AC assay (Kelley et al., Bull. MDIBL 23:86-88, 1983). The purpose of this work was to characterize further adenosine receptor modulation of dogfish rectal gland adenylate cyclase.

MATERIALS AND METHODS: Shark rectal glands were isolated from 3-6 male dogfish and placed in ice cold isolation buffer (IB) containing 150 mM NaCl, and 20 mM Hepes pH 7.5. All procedures were done on ice. Glands were decapsulated, weighed, minced into fine pieces with scissors, and homogenized with a Tekmar tissue homogenizer in 10 vol % IB for 4 X 15 sec with a 30 sec rest between 15 sec intervals. The homogenate was then centrifuged for 15 min at 6000 RPM. The supernatant was saved and the pellet was rehomogenized in 5 vol % IB for 4 X 5 sec with a 30 sec rest between 5 sec intervals and then centrifuged as above. The rehomogenization step was repeated. The three supernatants were combined and centrifuged for 45 min at 19000 RPM. The pellet obtained was resuspended in 5 mls of IB with a teflon pestle and pipetted onto a 12 ml bed of 25% sucrose and 150 mM NaCl and centrifuged for 40 min at 25000 RPM (50,000 G). The white plasma membrane (PM) fraction on top of the sucrose bed was removed and either used immediately or frozen in liquid (N_2) for subsequent experiments. The average yield was approximately 3 mg PM/g tissue. Protein was determined by the Lowry method using bovine serum albumin as a standard.

The AC assay was performed as previously described (Kelley et al., Bull. MDIBL 23:86-88, 1983). The assay cocktail contained CK 53 U/ml, BSA 0.1 mg/ml, $CrPO_4$ 20 mM, MgCl 2.5 mM, EGTA 0.5 mM, glycylglycine buffer pH 7.5 50 mM, cAMP I mM, ADA 5 U/ml, ATP 0.25 mM, GTP 50 μM, 10,000 cpm ^{3}(H)cAMP and α-32(P)ATP 100-175 cmp/pmole cold ATP. The reaction was carried out at 30°C for 10-15 min.

Results are presented as means ± SEM of cAMP formed (pmoles/min/mg protein) unless otherwise stated.

RESULTS AND DISCUSSION: Basal AC activity of the PM preparation was approximately 3-4 fold greater than the microsomal preparation used previously. This increased activity of the PM preparation increased the sensitivity of our assay.

N-ethylcarboxamideadenosine (NECA), 2 chloroadenosine (2ClADO), and L-phenylisopropyladenosine (PIA) significantly increased AC activity. On the average at 10^{-4} M of each agonist, NECA stimulated 2.5 fold above basal, 2ClADO stimulated 1.7 fold above basal, and PIA stimulated 1.4 fold above basal (n = 4-6 each). A dose response curve of a typical experiment is shown in Fig. 1.

The order of potency, NECA > 2ClADO > PIA, is consistent with a stimulatory A_2 adenosine receptor.

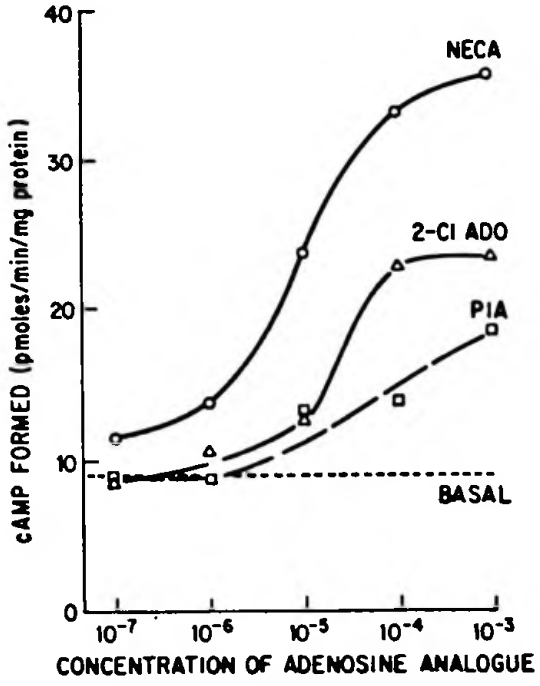

Figure 1. Dose response to NECA, 2ClADO and PIA on adenylate cyclase activity of plasma membranes from the shark rectal gland (Squalus acanthias).

This stimulation was GTP dependent and competitively antagonized by theophylline (THEO), 8-phenyltheophylline (8PT), and IBMX which is also consistent with an A_2 stimulatory receptor. In two experiments, all concentrations of NECA (10^{-7}M to 10^{-4} M) stimulated more above basal in the presence of 10 μM GTP than in the absence of GTP (data not shown).

THEO and 8PT (10^{-4}M) completely blocked the stimulation from low concentrations of NECA (10^{-9} to 10^{-6} M) and partially blocked the stimulation from higher concentrations of NECA (10^{-5}M and 10^{-4} stimulation above basal by 40% and 19% respectively while 8PT 10^{-5}M inhibited NECA 10^{-5}M and 10^{-4}M stimulation above basal

by 46% and 29% respectively. IBMX also inhibited NECA stimulated AC in a dose dependent manner. Of the three xanthines, 8PT was the most potent NECA antagonist which is consistent with the findings of Smellie et al. (Life Sci. 24: 2475, 1979).

THEO 10^{-3} M also decreased basal AC activity to the same extent as adenosine deaminase (ADA) 5 U/ml (an enzyme which deaminates adenosine to inosine which is inactive at adenosine receptors). A combination of the two agents did not further decrease AC activity. In a typical experiment basal AC activity (26.9 $\pm$ 1.1 pmoles cAMP/min/Mg protein) was reduced 25% by THEO 10^{-3}M, (to 14.0 $\pm$ 0.8) by 33% by ADA 5 U/ml (14 $\pm$ 0.8) and 28% by THEO 10^{-3}M and ADA 5 U/ml (15.1 $\pm$ 1). This decrease in AC activity likely occurs because THEO and ADA antagonize endogenous adenosine stimulation via A_2 receptors. The endogenous adenosine is formed from the metabolism of high ATP and cAMP concentrations in the assay. Since a combination of the two agents did not further decrease basal AC activity, it is assumed that each drug separately was able to completely block the endogenous A_2 stimulation.

Besides the major stimulation from micromolar NECA concentrations, we consistently observed a slight stimulation from low nanomolar concentrations. In 4 experiments, 10^{-9}M NECA significantly ($p<0.01$) stimulated 28% above basal (data not shown). This stimulation may be explained by a wide affinity range for the A_2 receptor or a second higher affinity stimulatory receptor. Although a subdivision of the A_2 stimulatory receptor is possible, this finding has not been previously shown in the literature.

We also observed that 10^{-7}M NECA decreased AC activity to basal values suggesting the presence of an inhibitory A_1 adenosine receptor. This inhibition was dependent on the concentration of GTP in the assay. When 10 μM GTP was used instead of 50 μM GTP, NECA still stimulated at low nanomolar concentrations, but did not inhibit at high nonomolar concentrations (data not shown). Thus as in most systems, adenosine receptor mediated A_1 inhibition of AC requires higher GTP concentrations than receptor mediated stimulation (Londos et al., from Receptors and Recognition, Series B, Vol. 12, ed. G. Burnstock, 1981, p. 289-319).

The inhibition discussed above is minimal when compared to the dramatic adenosine inhibition of fat cell AC. This discrepancy may occur because the rectal gland has potent A_2 stimulatory receptors that may mask A_1 inhibition while the fat cell does not have an A_2 stimulatory receptor, and therefore, inhibition is maximal. Thus, comparable inhibition of rectal gland AC may be impossible to achieve until specific A_1 and A_2 agonists are available.

In summary, stimulation of rectal gland membrane adenylate cyclase from adenosine analogues with an order of potency of NECA>2ClADO>PIA, which is GTP dependent, and blocked by THEO, 8PT and IBMX is strong evidence for an A_2 stimulatory adenosine receptor. A subdivision or wide affinity range for this receptor remains to be clarified. Also, NECA inhibition, which is GTP dependent, is suggestive of an A_1 inhibitory adenosine receptor but further manipulation of assay conditions to accentuate this inhibition is required.