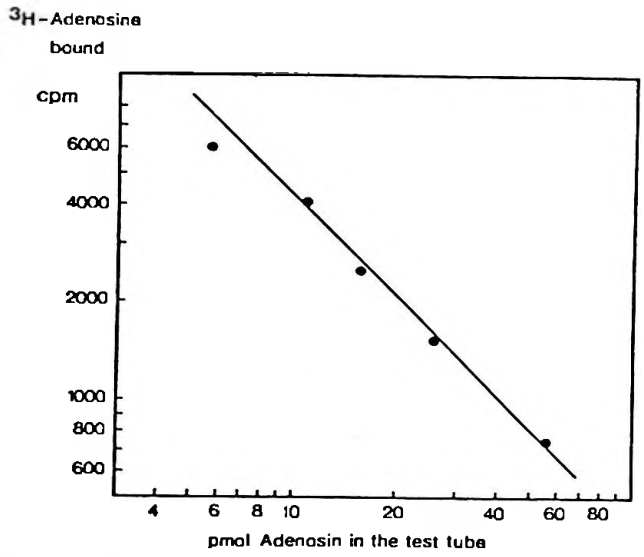

Figure 1.--Standard cruve of the adenosine assay. Ordinate: ^{3}H-adenosine recovered on cellulose filters due to binding to the adenosine-binding protein (Sacher and Osswald, 1980); Abszissa: added adenosine in the test tube, final volume 250 µl.

Table 1.--Adenosine release into the secreted duct fluid of the isolated perfused rectal gland during control conditions (2-3 periods) and following stimulation by VIP, 50 nM, (2-4 periods). Mean values ± SEM

	Control n = 5	VIP n = 7
Duct Flow Rate µl/min	12.5 ± 2.8	46.4 ±11.1
Adenosine Concentration nM	182 ± 36	423 ±207
Adenosine Release pmol/min	2.21 ± 0.21	13.5 ± 3.1

concentration in the duct fluid increased two-fold while chloride secretion increased fourfold. Adenosine release rate was enhanced from 2.2 to 13.5 pmol/min. This result clearly shows that adenosine can be released by an active NaCl transporting epithelium at an enhanced rate when the Na, K-ATPase is hydrolysing ATP at a higher rate. The data compare well with the finding of Rubio et al (MDIBL Bull. 20, 143-145, 1980) showing a fall of high energy phosphates in the gland tissue in response to stimulation of chloride secretion by saline loading the fish. Thus, active ion transport work in the rectal gland generates a metabolite which by itself at appropriate concentrations may regulate blood flow and chloride secretion. This work was supported by a Grant from the Deutsche Forschungsgemeinschaft, Os 42/6-7.

BINDING OF LIGANDS TO ADENOSINE RECEPTORS IN THE RECTAL GLAND OF SQUALUS ACANTHIAS

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Adenosine is released by the isolated perfused rectal gland into the venous effluent at an increased rate when

secretion is stimulated by VIP (Osswald et al., Bull MDIBL 23:, 1983). Adenosine when added to the perfusate can stimulate chloride secretion in the isolated gland (Erlij et al., Bull MDIBL, 18:92-93, 1978). This stimulatory effect of adenosine could be blocked by theophylline (Forrest et al., Bull. MDIBL, 20:152-155, 1980). Further studies indicated that adenosine (10^{-5}M) acts to increase chloride secretion via an increase in cAMP tissue levels (Forrest et al., in Regulatory Function of Adenosine, p. 511, Nijhoff Publishers, 1983). There is preliminary evidence that at lower concentrations adenosine analogs may inhibit chloride secretion and cyclic AMP accumulation via an inhibitory receptor that is linked to adenylate cyclase (Poeschla et al. Bull. MDIBL 22:519, 1983; Kelley et al., Bull. MDIBL 23:1983).

The present experiments were designed to characterize the assumed adenosine receptors in a membrane fraction of the rectal gland by means of a specific ligand binding assay and a rapid filtration technique.

METHODS

Plasma membranes were prepared from freshly excised rectal glands by the method described by Eveloff et al., (Pflügers Arch. 378:87-92, 1978) with modifications. (We thank Dr. E. Kinne-Saffran for her generous help with the membrane preparation technique). In brief, dogfish caught by line in Frenchman Bay were maintained in live cars and used within 2-4 days of capture. Two to three glands (2 - 6 g) were homogenized using a Waring blender in 20 - 40 ml ice cold buffer A consisting of (mM) sucrose, 250; HEPES, 20; EDTA, 5; adjusted pH 6.8 with TRIS base. The homogenate was centrifuged for 15 min at 7500 rpm in a SS 34 rotor of a Sorvall refrigerated ultracentrifuge, type RC 5B. The supernatant was again centrifuged at 7500 rpm at 0 - 4°C. The resulting pellet was resuspended in buffer B, consisting of (mM) mannitol, 200; HEPES, 20; Mg $(NO_3)_2$, 1.2; adjusted to pH 7.6 and homogenized in a teflon tissue grinder with a hand drill at approximately 1000 rpm and 12 strokes. The membranes were collected in a last centrifugation step at 17500 rpm for 45 min. The resulting pellet was subjected to a hypoosmotic shock to disrupt the membrane vesicles by fivefold dilution in buffer C made of (mM) mannitol, 20; HEPES, 20; Mg $(NO_3)_2$ 1.2; pH 7.6. For binding studies we used the membranes on the day of preparation or after storage on dry ice for 2-6 days.

The stock solution of the membrane suspension was diluted in buffer C to give a final concentration of about 1 mg protein per ml buffer C. Membrane protein concentration was measured according to the method of Lowry (J. Biol. Chem. 193: 265-275, 1951) using bovine serum albumin as standard. Membranes were preincubated for 10 min at 25°C with adenosine deaminase (ADA) 0.5 - 1.2 U/ml (except experiments shown in Figure 1) to remove endogeneously formed adenosine from the receptors. We used 5-N-ethylcarboxamide-^{3}H-adenosine (^{3}H-NECA spec. act. 25-40 Ci/mmol) and (L)-N^6-phenylisopropyl-^{3}H adenosine (^{3}H-PIA, spec. act. 30-50 Ci/mmol) as ligands for the adenosine receptors. Membranes were incubated with ligands in a final volume of 250 µl at 0°C for 30 min. Separation of the membrane receptor-ligand complex from the incubation medium was achieved by filtering 220 µl of the incubate through GB/F (Whatman) filters followed by immediate flushing with 5 ml ice cold buffer C. The filters were flushed within 1-2 sec with suction achieved by a vacuum pump. The radioactivity retained on the filters was counted in a liquid scintillation spectrometer. Undisplacable binding (B_u) of the radioactive ligands to the membrane was defined in the presence of 5000 fold excess concentration of the unlabelled ligand. Specific (displaceable) binding (B_s) is therefore the difference of total binding (B_t) minus B_u. Each assay condition was measured in duplicate.

RESULTS

The binding of ^{3}H-NECA and ^{3}H-PIA to rectal gland (RG) membranes was rapid and reversible and was at equilibrium at 16-20 min. Half maximal binding occurred at 82 sec. The rapid dissociation of the ligand receptor complex (Figure 1) made it necessary to use a rapid filtration technique. Experiments in Figure 1 were performed

without preincubation with adenosine deaminase (ADA). The association and dissociation rate constants were essentially the same with ADA preincubation.

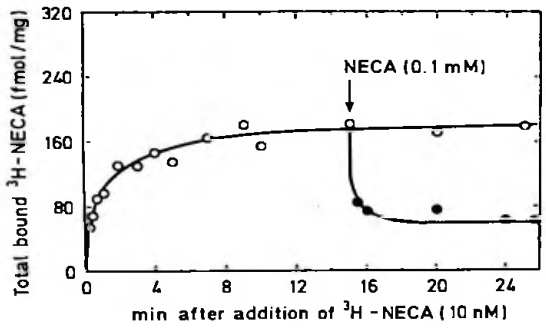


Figure 1.--Incubation of 129 μg of membrane protein in buffer C with 10 nM ^{3}H-NECA at 0°C for various time intervals. Arrow indicates addition of unlabelled NECA to give a final concentration of 0.1 mM. Data are the mean of two experiments.

The amount of specific bound ^{3}H-NECA increased linearly with increasing protein concentration (20-180 μg per tube) in the medium. Urea up to a concentration of 500 mM decreased specific binding of ^{3}H-NECA (Table 1).

Table 1.--Specific binding (B_s) of ^{3}H-NECA (10 nM) to RG membranes incubated in buffer C for 30 min at 0°C (mean ± SEM, n = number of experiments)

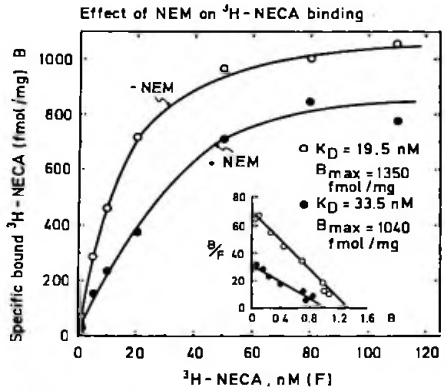
Experimental Condition		B_s of ^{3}H-NECA in f mol/mg protein
Without ADA pre-incubation n = 8		111 ± 20
With ADA pre-incubation n = 6		250 ± 24
Boiling for 10 min n = 5		35 ± 22
Urea	0	216 ± 42
(mM)	5	183 ± 42
n = 3	30	260 ± 36
	100	187 ± 8
	300	154 ± 27
	500	128 ± 22

Preincubation of the membranes with ADA, 0.5-1.5 U/ml, for 10 min at 25°C increased ^{3}H-NECA binding two-fold (Table 1) and enhanced the ratio of specific binding to total binding from 0.6 to 0.9. Heating membranes for 10 min in boiling water resulted in a complete loss of specific binding.

In 10 saturation experiments from different membrane preparations ^{3}H-NECA appeared to label one binding site with an apparent affinity (K_D) of 21.6 ± 2.8 nM (Mean ± SEM) and a maximum of binding sites (B_{max}) of 1.01 pmol per mg membrane protein. Scatchard plots gave straight lines and Hill coefficients were nearly one (see Figure 2a for an individual experiment). However, displacement experiments with 2-chloroadenosine, L-PIA, D-DIA and IBMX showed a biphasic inhibition of specific ^{3}H-NECA binding suggesting that two binding sites might be present (data not shown). We therefore examined additional factors that might separately modify ^{3}H-NECA as opposed to ^{3}H-PIA binding.

The sulfhydryl reagent N-ethylmaleimide (NEM) recently has been shown to inhibit hormone-mediated in-

hibition of platelet adenylate cyclase without affecting hormone-mediated stimulation of adenylate cyclase (Jacobs et al., J. Biol. Chem. 257:2829, 1983). Green and Yeung reported that NEM blocks binding of ^{3}H-NECA to inhibitory R_i sites in rat hippocampus without affecting binding of ^{3}H-NECA to R_a receptors in striatum. (Londos, in Regulatory Function of Adenosine, p 96, Nijhoff Publishers, 1983). The results of NEM treatment of the RG membranes on ^{3}H-NECA binding is shown in Figure 2a. The reduction of affinity by NEM treatment (1.7 fold) was greater than the reduction in number of binding sites (1.3 fold). However, the effect of NEM was much more pronounced when ^{3}H-PIA was the ligand (Figure 2b). The affinity of the RG membrane for PIA was reduced 12-fold and was nearly lost. The ability of theophylline to displace ^{3}H-NECA from its binding sites was slightly increased following NEM treatment (data not shown).



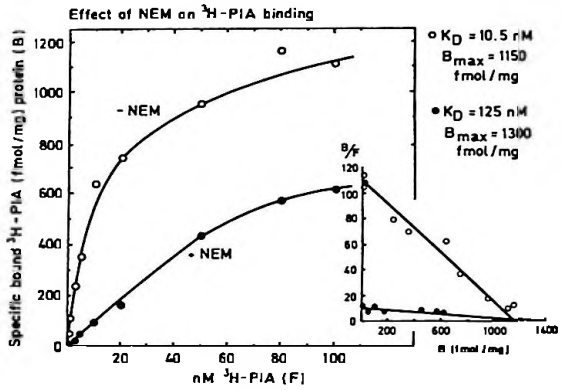


Figure 2a.--Saturation curve of ^{3}H-NECA in a paried membrane preparation: - NEM corresponds to sham treated control membranes; + NEM indicates preincubation of membrane suspension in 25 ml of buffer C in the presence of 3 mM N-ethylmaleimide and ADA (1 U/ml) for 15 min at 25°C. Following this preincubation 25 ml of ice cold buffer C was added and the suspension was centrifuged at 45000 x g for 45 min. The resulting pellet was treated thereafter as usual.

Figure 2b.--Identical experimental design as in Figure 2a except that the ligand is ^{3}H-PIA.

In further experiments we studied the effects of GTP and chloride ion on NECA and PIA binding. Both GTP and chloride (as tetramethyl-ammonium salt, TMACl), stimulated ^{3}H-NECA binding in a concentration dependent manner. When ^{3}H-NECA concentration was 10 nM, binding was enhanced maximally 2.3 fold by GTP and 1.8 fold by chloride (Table 2). Half maximal stimulation of specific ^{3}H-NECA binding was observed at 0.1 μM GTP and at about 65 mM chloride, respectively. Both, GTP and chloride increased the number of binding sites (B_{max}) but did not affect affinity (K_D) of ^{3}H-NECA binding (data not shown). In contrast, the affinity of ^{3}H-PIA to adenosine receptors of the RG membranes was markedly reduced in the presence of GTP or chloride (data not shown).

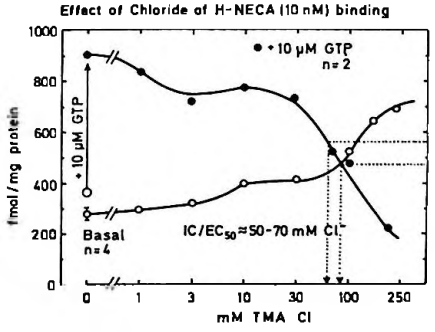


Figure 3.--Effect of tetramethylammoniumchloride (TMACl) in the absence and presence of GTP on specific ^{3}H-NECA (10 nM) binding to RG membranes (mean of 4 experiments). Note that both effects, stimulation and inhibition of NECA binding by chloride, occur at a half maximal concentration of 60-70 mM TMACl.

Table 2.--Effect of GTP and chloride on ^{3}H-NECA (10 nM) binding to RG membranes (B_s)

	B_s (fmol/mg protein)
Control n = 7	269 ± 63
GTP (2-10 μM) n = 7	652 ±110
Control n = 5	311 ± 78
Chloride (250 mM) n = 5	530 ± 69

The effect of GTP on NECA binding could be reversed by adding chloride to the incubation medium as shown in Figure 3. The half maximal inhibition of the GTP effect by chloride was observed at a chloride concentration of 60 mM. When a saturation experiment was done with ^{3}H-NECA as ligand in the presence of 10 μM GTP and 250 mM c chloride, binding affinity was greatly reduced (data not shown). The effects of chloride could also be demonstrated when the accompanying cation was lithium. Bumetanide up to 10^{-4}M did not inhibit the effect of chloride. Sodium ions, as sodium isethionate up to 200 mM, potassium ions up to 5 mM, calcium up to 5 mM did not significantly affect ^{3}H-NECA (10 mM) binding to RG membranes.

DISCUSSION

These experiments are the first attempts to characterize specific binding to adenosine receptors in a transporting epithelium. The association and dissociation rates of ^{3}H-NECA binding to RG membranes are in the range of 1-3 minutes and compare well with the rapid onset and short duration of action of adenosine and NECA in the isolated perfused gland. Adenosine derivatives and methylxanthine displace ^{3}H-NECA from its binding sites in a concentration dependent manner as predicted from previous experiments with isolated perfused glands (Poeschla et al., Bull. MDIBL 22:515, 1982). The apparent affinity of ^{3}H-NECA (K_d = 22nM) is one-half of ^{3}H-PIA affinity (K_d = 11 nM). Previous work in the perfused gland and tissue slices suggested that both R_a (or adenylate cyclase stimulating) adenosine receptors and R_i (or adenylate cyclase inhibiting) receptors were present in this tissue (Forrest et al., Kid. Intern. 21:253, 1982 and Poeschla et al., Bull. MDIBL 22, 1982). However, in the present study Scatchard analysis from ten saturation experiments with ^{3}H-NECA showed straight lines with Hill coefficients clost to unity. This result suggests either that one receptor population is dominant in the membrane preparation or that the affinities for adenosine at the two binding sites are sufficiently close (differing only by a factor of 2-4 fold) that they may not be distinguished in saturation kinetic experiments.

The experiments with NEM support the above hypothesis of separate low and high affinity adenosine receptors since high affinity binding of PIA (presumably labeling primarily R_i receptors) is destroyed by NEM whereas the affinity of ^{3}H-NECA is reduced only by a factor of 1.7 (Figures 2a and 2b).

GTP increases the number of binding sites for ^{3}H-NECA without affecting affinity. To our knowledge, this is the first reported effect of GTP to increase the specific binding of an agonist. PIA affinity to RG membranes was greatly reduced by GTP. A similar observation of reduced affinity by GTP was reported by Goodman et al., Molec. Pharmacol. 21:321-335, 1982, using cyclohexyladenosine as a ligand.

Chloride ions appear to have an effect on NECA and PIA binding similar to that of GTP: the number of binding sites for NECA are increased without a significant change in affinity, whereas the membranes exhibit a

substantial loss of the high affinity to PIA in the presence of 250 mM Cl. Surprisingly, chloride ions reverse the stimulatory effect of GTP on NECA binding. Both effects of chloride occur at a concentration that is close to the intracellular chloride activity of intact transporting cells in isolated tubules of the salt gland (Greger et al., this Bulletin). We assume therefore, that the site from where chloride exerts its effects on NECA binding is the inner surface of the membrane.

As a result of these studies we propose a hypothesis for the role of adenosine in the regulation of transepithelial chloride transport. As shown in Figure 4, adenosine can stimulate two types of receptors, one R_i

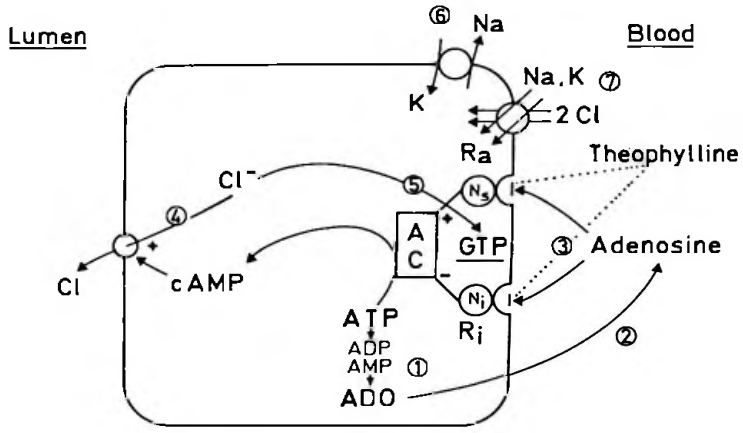


Figure 4.--Model of homocellular regulation of chloride transport in the rectal gland of Squalus acanthias. (1) ATP as the energy source for the Na-K-ATPase (6) is dephosphorylated to adenosine (ADO), the first metabolite that can leave the cell by diffusion (2); extracellular adenosine can bind to two different receptors (3); this binding can be antagonized by theophylline. (4) Intracellular cAMP generated from the adenylate cyclase (AC) which is controlled in part by R_i and R_a receptors (3) increases the chloride conductance at the luminal cell membrane. (5) By modifying the affinities of the R_i and R_a receptors for adenosine, intracellular chloride can regulate cAMP production and consequently its luminal conductance (4) and intracellular concentration.

(or A_1) which is coupled to adenylate cyclase via an inhibitory nucleotide regulatory unit (N_i) containing a GTPase and which induces inhibition of adenylate cyclase. The other receptor, R_a (or A_2), is coupled to adenylate cyclase via a stimulatory nucleotide regulatory protein (N_s) which contain a GTPase and stimulates the catalytic activity of the adenylate cyclase. This simplified scheme of dual receptor-adenylate cyclase signal transduction is the result of the work of several groups including Rodbell et al., 1971; Bourne et al., 1975; Ross and Gilman, 1977; Nostrup et al., 1980; Londos et al., 1981; and Jakobs et al., 1981.

Our data suggest that chloride ion modifies the affinities of the signal transduction components. We propose that when the intracellular chloride concentration rises, the number of binding sites for extracellular adenosine is increased and the affinity is shifted from the inhibiting high affinity receptor (R_i) towards the low affinity receptor (R_a) that stimulates the adenylate cyclase. The result is an increased rate of cAMP generation and a cAMP mediated increase in the conductance for chloride at the luminal membrane. By this mechanism chloride ion could serve to adjust its intracellular concentration. Experimental data of Greger et al., (this Bulletin) show that intact tubular cells of the rectal gland keep their intracellular chloride concentration (60-70 mM) remarkably constant over a wide range of transport rates. Consistent with this assumption of self-adjustment is that the concentration of chloride at the steepest slope of the concentration effect in Figure 3 is 60-70 mM, the intracellular chloride concentration in transporting cells.