

A1 adenosine receptors inhibit chloride transport in the shark rectal gland. Dissociation of inhibition and cyclic AMP.

G G Kelley, ... , H V Barron, J N Forrest Jr

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Research Article

In the in vitro perfused rectal gland of the dogfish shark (*Squalus acanthias*), the adenosine analogue 2-chloroadenosine (2Clado) completely and reversibly inhibited forskolin-stimulated chloride secretion with an IC₅₀ of 5 nM. Other A1 receptor agonists including cyclohexyladenosine (CHA), N-ethylcarboxamideadenosine (NECA) and R-phenylisopropyladenosine (R-PIA) also completely inhibited forskolin stimulated chloride secretion. The "S" stereoisomer of PIA (S-PIA) was a less potent inhibitor of forskolin stimulated chloride secretion, consistent with the affinity profile of PIA stereoisomers for an A1 receptor. The adenosine receptor antagonists 8-phenyltheophylline and 8-cyclopentyltheophylline completely blocked the effect of 2Clado to inhibit forskolin-stimulated chloride secretion. When chloride secretion and tissue cyclic (c)AMP content were determined simultaneously in perfused glands, 2Clado completely inhibited secretion but only inhibited forskolin stimulated cAMP accumulation by 34-40%, indicating that the mechanism of inhibition of secretion by 2Clado is at least partially cAMP independent. Consistent with these results, A1 receptor agonists only modestly inhibited (9-15%) forskolin stimulated adenylate cyclase activity and 2Clado markedly inhibited chloride secretion stimulated by a permeant cAMP analogue, 8-chlorophenylthio cAMP (8CPT cAMP). These findings provide the first evidence for a high affinity A1 adenosine receptor that inhibits hormone stimulated ion transport in a model epithelia. A major portion of this inhibition occurs by a mechanism that is independent of the cAMP messenger system.

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A₁ Adenosine Receptors Inhibit Chloride Transport in the Shark Rectal Gland

Dissociation of Inhibition and Cyclic AMP

Grant G. Kelley, Eric M. Poeschla, Harold V. Barron, and John N. Forrest, Jr.

Section of Nephrology, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510; and The Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine 04672

Abstract

In the *in vitro* perfused rectal gland of the dogfish shark (*Squalus acanthias*), the adenosine analogue 2-chloroadenosine (2Clado) completely and reversibly inhibited forskolin-stimulated chloride secretion with an IC₅₀ of 5 nM. Other A₁ receptor agonists including cyclohexyladenosine (CHA), *N*-ethylcarboxamideadenosine (NECA) and *R*-phenylisopropyladenosine (R-PIA) also completely inhibited forskolin-stimulated chloride secretion. The "S" stereoisomer of PIA (S-PIA) was a less potent inhibitor of forskolin-stimulated chloride secretion, consistent with the affinity profile of PIA stereoisomers for an A₁ receptor. The adenosine receptor antagonists 8-phenyltheophylline and 8-cyclopentyltheophylline completely blocked the effect of 2Clado to inhibit forskolin-stimulated chloride secretion. When chloride secretion and tissue cyclic (c)AMP content were determined simultaneously in perfused glands, 2Clado completely inhibited secretion but only inhibited forskolin-stimulated cAMP accumulation by 34–40%, indicating that the mechanism of inhibition of secretion by 2Clado is at least partially cAMP independent. Consistent with these results, A₁ receptor agonists only modestly inhibited (9–15%) forskolin-stimulated adenylate cyclase activity and 2Clado markedly inhibited chloride secretion stimulated by a permeant cAMP analogue, 8-chlorophenylthio cAMP (8CPT cAMP). These findings provide the first evidence for a high affinity A₁ adenosine receptor that inhibits hormone-stimulated ion transport in a model epithelia. A major portion of this inhibition occurs by a mechanism that is independent of the cAMP messenger system. (*J. Clin. Invest.* 1990. 85:1629–1636.) adenosine • adenosine receptors • chloride transport • cAMP • adenylate cyclase

Introduction

Adenosine is a potent autacoid regulator of diverse physiologic functions (1–4). These effects are mediated by two distinct extracellular adenosine receptors: a stimulatory receptor termed A₂ or R_s and an inhibitory receptor termed A₁ or R_i (5, 6). The stimulatory receptor has a low micromolar affinity for adenosine, whereas the inhibitory receptor has a 1,000-fold greater affinity with a K_d in the low nanomolar range (1). Each receptor has a unique affinity profile for specific adenosine

analogues (6, 7) and both are competitively antagonized by methylxanthines (1, 7–9). The mechanism of signal transduction of these receptors is considered to be through G-protein coupled stimulation or inhibition of adenylate cyclase (2). Inhibitory adenosine receptors, however, have also been shown to be coupled to other effectors including K⁺ (10–12) and Ca²⁺ channels (13–15).

Recently, stimulatory A₂ adenosine receptors have been widely identified in both secretory and absorptive epithelia. Adenosine has been shown to stimulate secondary active chloride transport in the amphibian cornea (16), shark rectal gland (17), rabbit ileum (18), rabbit colon (19), and canine trachea epithelium (20), and to stimulate sodium transport in the A6 toad kidney epithelial cell line (21). Dillingham and Anderson (22) observed that high concentrations of the adenosine analogue *N*-ethylcarboxamideadenosine (NECA)¹ increased hydraulic conductivity in perfused rabbit cortical collecting tubules. In primary cultures of this nephron segment, Arend et al. (23) demonstrated the presence of A₂ receptors that stimulate and A₁ receptors that inhibit cyclic (c)AMP accumulation.

In contrast to A₂ receptors, a physiologic effect of inhibitory A₁ receptors on transepithelial ion movement has not been shown previously. The isolated perfused elasmobranch rectal gland is a tubular epithelial structure that has been widely used as a model for sodium coupled secondary active chloride transport (24, 25). Previous studies have indicated striking similarities between chloride transport in this tissue and the mammalian thick ascending limb of Henle's loop (17, 24, 26). The present studies demonstrate the presence of a high affinity inhibitory A₁ adenosine receptor in the rectal gland that potently inhibits hormone-stimulated chloride secretion. A major portion of this inhibition occurs by a cAMP independent mechanism.

Methods

Materials. [³H]cAMP, [³²P]ATP, and Radioimmunoassay Kit for cAMP were obtained from New England Nuclear (Boston, MA). Forskolin and 8-phenyltheophylline were from Calbiochem-Behring (San Diego, CA). *R* and *S*-phenylisopropyladenosine and cyclohexyladenosine were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). 8-cyclopentyltheophylline was a generous gift from Warner-Lambert Co. (Ann Arbor, MI). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

***In vitro* perfusion.** Rectal glands were obtained from dogfish sharks, *Squalus acanthias*, of either sex weighing 2–6 kg that were sacrificed by segmental transection of the spinal cord. Cannulae were placed in the artery, vein, and duct. The rectal glands were then placed in a glass

Address reprint requests to Dr. Forrest, Section of Nephrology, Department of Internal Medicine, 87 LMP, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510.

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1. *Abbreviations used in this paper:* ADA, adenosine deaminase; 2Clado, 2-chloroadenosine; 8CPT, cyclopentyltheophylline; 8PT, 8-phenyltheophylline; mTAL, medullary thick ascending limb; NECA, *N*-ethylcarboximideadenosine; R-PIA, *R*-phenylisopropyladenosine; VIP, vasoactive intestinal polypeptide.

perfusion chamber that was kept at 15°C with running sea water and glands were perfused with an elasmobranch Ringer's solution containing 270 mM NaCl, 4 mM KCl, 3 mM MgCl₂, 2.5 mM CaCl₂, 1 mM KH₂PO₄, 8 mM NaHCO₃, 350 mM urea, 5 mM glucose, and 0.5 mM Na₂SO₄ and equilibrated to pH 7.5 by bubbling with 99% O₂ and 1% CO₂. When vasoactive intestinal peptide (VIP) was used, 0.1 mg/ml bovine serum albumin was added to an equilibrated Ringer's solution to prevent binding of VIP to the perfusion bottle. Adenosine deaminase, 0.1 to 0.5 U/ml, was added to the perfusate to remove endogenous adenosine. Measurements of duct flow were made at 10-min intervals in all experiments. All glands were first perfused for 30 min in the absence of hormones or drugs to achieve basal (unstimulated) rates of chloride secretion. All basal values given in the text and tables are the last basal measurement (20–30 min). Results are expressed as microequivalents of chloride secreted per hour per gram wet weight ($\mu\text{eq/h/g}$) \pm SEM.

Radioimmunoassay for tissue cAMP content. Rectal glands were perfused *in vitro* as described above and chloride secretion rates were determined. At various time points, the tip of the gland proximal to the artery was tied with 4-0 silk and a small piece of tissue was removed and frozen quickly in liquid nitrogen for cAMP determination. A second slice was transected from the middle of the gland at the end of the experiment. Tissue slices were homogenized with a Tekmar Tissue-mixer (Tekmar Co., Cincinnati, OH) at high speed for 20 s in 1 ml of 6% TCA. cAMP was extracted with a mixture of Freon and trioctylamine as described by Riss et al. (27). cAMP in the neutral supernatant was measured in triplicate by radioimmunoassay (28). cAMP content is expressed as picomoles cAMP/milligram protein and all values are the mean \pm SEM. cAMP content did not vary significantly in slices taken throughout the length of the gland.

Adenylate cyclase assay. Basolateral membranes of the shark rectal gland were prepared similar to the method of Eveloff et al. (26). Two to three glands were homogenized in a Waring blender for 1 min in 20–40 ml of 250 mM sucrose, 5 mM EDTA, 20 mM Hepes adjusted to pH 6.8 with Tris. This homogenate was then centrifuged in a Sorvall SS34 rotor (DuPont, Wilmington, DE) at 7,500 rpm for 15 min. The supernatant was then centrifuged at 17,500 rpm for 45 min. The resulting pellet was resuspended in 10–20 ml of 200 mM mannitol, 20 mM Hepes-Tris pH 7.6 and homogenized with 10 strokes of a Dounce teflon homogenizer driven by a hand drill at high speed. The membranes were then pelleted at 17,500 rpm for 45 min and then resuspended in 500–1,000 μ l of 20 mM mannitol, 20 mM Hepes-Tris pH 7.6 and stored in liquid nitrogen. Membrane protein concentration was measured by the method of Lowry et al. (29) using albumin at the standard.

Adenylate cyclase activity was determined in triplicate in an assay (final volume 100 μ l) that contained 25 mM Tris acetate pH 7.5, 50 U/ml creatine kinase, 20 mM creatine phosphate, 10 mg/ml bovine serum albumin, 50 U/ml myokinase, 2.5 mM Mg acetate, 100 mM NaCl, 0.5 mM EGTA, 50 μ M GTP, 0.25 mM ATP, 200 cpm/pmol [³²P]ATP, 1 mM cAMP, and 5–10 U/ml adenosine deaminase (ADA). The reaction was started with the addition of membranes (30–40 μ g protein), incubated at 30°C for 10 min and stopped with the addition of 100 μ l of 2% SDS, 1 mM ATP, 1 mM cAMP, and 10,000–15,000 cpm [³H]cAMP. The reaction product ([³²P]cAMP) was separated from the substrate by the method of Salomon (30) using sequential Dowex (AG-50W-X4) and neutral alumina (WN-3) column chromatography. Results are expressed as picomoles of cyclic AMP formed per minute per milligram protein (cAMP formed/min per mg protein).

Statistics. All results are mean \pm SEM. Data were analyzed for significance with Student's *t* test for paired or unpaired data.

Results

In vitro perfusion

Inhibition of forskolin and VIP-stimulated chloride secretion by A₁ receptor agonists. Initial studies were performed with 2-chloroadenosine (2Clado), an adenosine deaminase-resis-

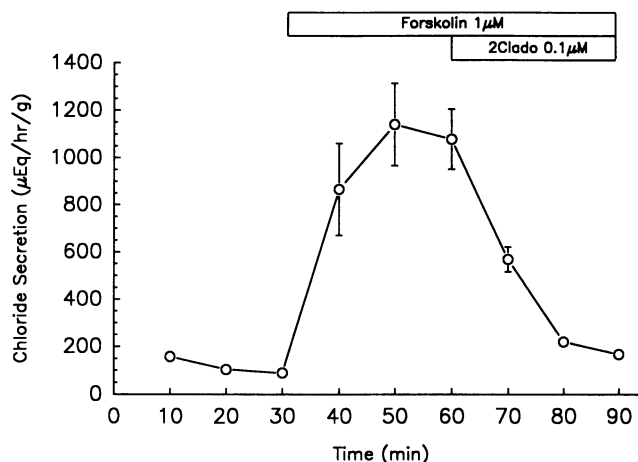


Figure 1. Effect of 2Clado (0.1 μM) on forskolin-stimulated chloride secretion in the *in vitro* perfused rectal gland. Rectal glands were perfused for 30 min to basal values then forskolin, a direct activator of adenylate cyclase, was added to the perfusate to increase the rate of chloride secretion. 2Clado was added to the perfusate at 60 min. Chloride secretion was measured at 10-min intervals and values are mean \pm SEM of four experiments.

tant adenosine analogue that has an affinity comparable to adenosine at A₁ and A₂ receptors. Fig. 1 demonstrates the effect of this analogue on forskolin-stimulated chloride secretion. After rectal glands were perfused to basal secretion rates, forskolin, a direct activator of adenylate cyclase, was added to the perfusate to increase the rate of chloride secretion. Forskolin, 1 μM , increased chloride secretion 12.8-fold above basal values from 89 ± 26 $\mu\text{eq/h per g}$ to a maximum of $1,140\pm 173$ $\mu\text{eq/h per g}$. The addition of 0.1 μM 2Clado inhibited this forskolin stimulated chloride secretion to near basal values of 167 ± 18 $\mu\text{eq/h per g}$ ($P < 0.001$; $n = 4$).

Inhibition of the forskolin response by 2Clado was reversible. Fig. 2 illustrates experiments in which the adenosine analogue was added with the initial addition of forskolin and then

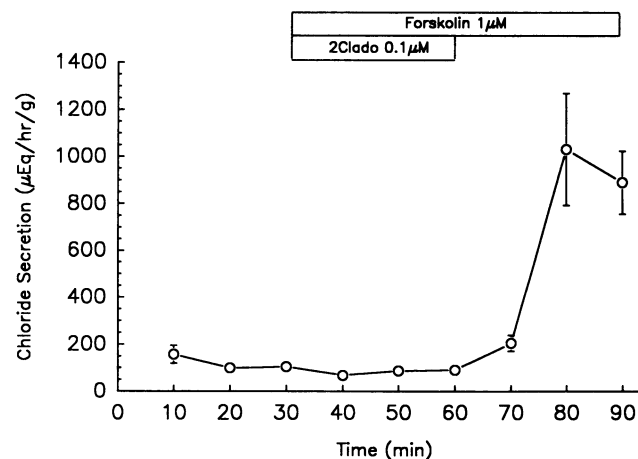


Figure 2. Reversibility of 2Clado inhibition of forskolin-stimulated chloride secretion in the *in vitro* perfused rectal gland. Rectal glands were perfused for 30 min to basal values and then forskolin and 2Clado were added to the perfusate. After 30 min the 2Clado was removed. Chloride secretion was measured at 10-min intervals and values are mean \pm SEM of six experiments.

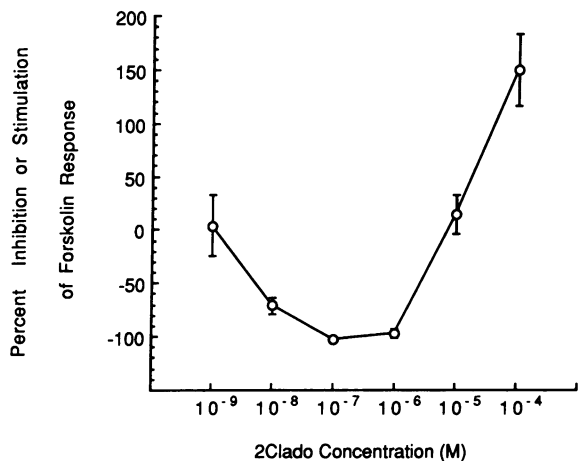


Figure 3. Dose response to 2Clado in the in vitro perfused rectal gland. Rectal glands were perfused with forskolin in the presence and absence of varying concentrations of 2Clado. The relative inhibition or stimulation of the forskolin response above basal is illustrated. Forskolin alone stimulated chloride secretion from 128 ± 21 to $1,005 \pm 106$ ($n = 26$).

the analogue was removed. In the presence of $0.1 \mu\text{M}$ 2Clado, the response to $1 \mu\text{M}$ forskolin was completely inhibited ($91 \pm 14 \mu\text{eq/h per g}$) and chloride secretion remained at basal levels ($105 \pm 23 \mu\text{eq/h per g}$). Removal of the adenosine analogue resulted in a prompt stimulation of chloride transport to maximal forskolin stimulated values ($1,032 \pm 238 \mu\text{eq/h per g}$; $P < 0.001$ compared to 2Clado + forskolin $n = 6$).

A dose response for 2Clado was determined by perfusing rectal glands with $1 \mu\text{M}$ forskolin in the presence and absence of varying concentrations of 2Clado (10^{-9} to 10^{-4} M). The response to 2Clado and forskolin relative to forskolin alone is shown in Fig. 3. 2Clado at 1 nM did not significantly inhibit the forskolin response ($P = \text{NS}$; $n = 6$). 2Clado at 10 nM inhibited the forskolin response by $71 \pm 8\%$ ($P < 0.001$; $n = 9$). 100 nM and $1 \mu\text{M}$ completely inhibited chloride secretion ($P < 0.001$; $n = 19$ and $P < 0.01$; $n = 7$, respectively). The IC_{50} for 2Clado was 5 nM .

At concentrations above $1 \mu\text{M}$, however, inhibition was not seen (Fig. 3). In the presence of $10 \mu\text{M}$ 2Clado the secretory response was not different from forskolin control values,

and at $100 \mu\text{M}$, 2Clado enhanced the response to forskolin by $150 \pm 33\%$ ($P < 0.001$; $n = 4$). At these concentrations 2Clado activates the stimulatory A_2 receptor which is present in the rectal gland (17).

2Clado also inhibited VIP-stimulated chloride secretion. VIP, 3 nM , stimulated chloride secretion 15.8 -fold above basal from 91 ± 26 to $1,438 \pm 107 \mu\text{eq/h per g}$. 2Clado, at 100 nM , inhibited this VIP stimulation by 47% to 805 ± 170 ($P < 0.02$; $n = 5$).

Other A_1 receptor agonists also inhibited forskolin-stimulated chloride secretion (Table I). At concentrations of 100 nM , R-PIA, CHA, and NECA completely inhibited the response to forskolin. Inhibition by each of these agonists was reversible (data not given). By contrast, the S stereoisomer of PIA, S-PIA, was less potent than R-PIA at inhibiting chloride secretion. S-PIA at 100 nM only inhibited the forskolin response by $70 \pm 7\%$ and secretion increased 2.7 -fold above basal ($P < 0.01$ compared to basal; $n = 8$).

Reversal of inhibition with adenosine receptor antagonists. To demonstrate further that the inhibition of secretion by 2Clado was receptor mediated, the effect of the potent adenosine receptor antagonist 8-phenyltheophylline (8PT) on the 2Clado inhibition of forskolin-stimulated secretion was examined (Fig. 4). Rectal glands were first perfused with 2Clado and forskolin and complete inhibition was observed. The addition of $1 \mu\text{M}$ 8PT to the perfusate completely blocked the 2Clado inhibition and secretion increased from an inhibited value of $75 \pm 11 \mu\text{eq/h per g}$ to $944 \pm 92 \mu\text{eq/h per g}$ ($P < 0.001$; $n = 4$). 8-Cyclopentyltheophylline (8CPT), another methylxanthine A_1 receptor antagonist, at $1 \mu\text{M}$ also completely blocked the 2Clado inhibition (data not shown). These receptor antagonists at the above concentrations had no effect on soluble or particulate cAMP-dependent phosphodiesterase activity in the rectal gland (data not shown).

Measurements of tissue cAMP content during A_1 receptor-mediated inhibition of chloride secretion. Hormonal stimulation of chloride secretion in the rectal gland is considered to be mediated by an increase in tissue cAMP (31–33). Since A_1 receptors have been shown to inhibit adenylate cyclase in other tissues (2, 6), chloride secretion and tissue cAMP content were measured simultaneously to determine if the inhibitory effect of 2Clado on forskolin-stimulated chloride secretion was mediated by an inhibition of cAMP accumulation. The results of these experiments are shown in Fig. 5. Under basal conditions

Table I. Inhibition of Forskolin-stimulated Chloride Secretion by Adenosine Analogues in Isolated Perfused Rectal Glands

Adenosine analogue	Basal	Forskolin $1 \mu\text{M}$ + adenosine analogue	Percent inhibition of forskolin response above basal	n
	$\mu\text{EqCl/h per g}$	$\mu\text{Eq/h per g}$		
100 nM				
Forskolin control	128 ± 21	$1,005 \pm 106^*$		26
R-PIA	116 ± 14	$137 \pm 6^\ddagger$	98 ± 1	5
S-PIA	171 ± 36	$459 \pm 51^{\text{ }}$	70 ± 7	8
CHA	149 ± 76	$163 \pm 76^\ddagger$	99 ± 2	4
2Clado	134 ± 24	$124 \pm 17^\S$	102 ± 2	19
NECA	153 ± 41	$170 \pm 45^\S$	98 ± 5	6

* $P < 0.001$ compared to forskolin control basal. $^\ddagger P < 0.01$ compared to forskolin control. $^\S P < 0.001$ compared to forskolin control.

$^{\text{||}} P < 0.01$ compared to S-PIA basal.

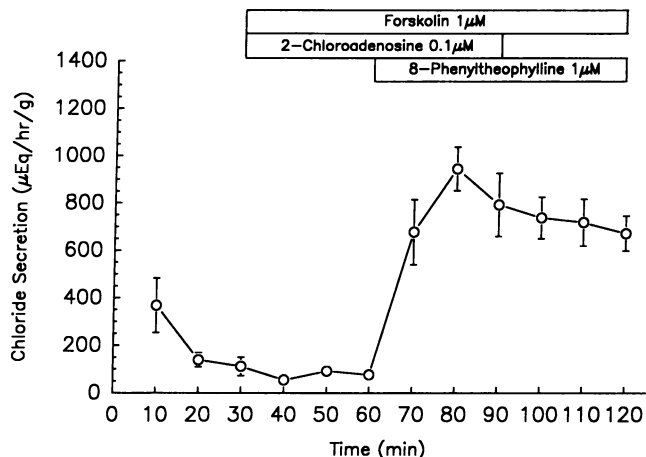


Figure 4. Effect of the A_1 receptor antagonist 8PT to reverse 2Clado inhibition of forskolin-stimulated chloride secretion. Rectal glands were perfused for 30 min to basal values and 2Clado and forskolin were then added to demonstrate the inhibitory effect of 2Clado. 8PT was then added to the perfusate. Chloride secretion was measured at 10-min intervals and values are mean \pm SEM of four experiments.

(first panel, Fig. 5), chloride secretion and cAMP content were 127 ± 28 $\mu\text{Eq/h}$ per g and 6 ± 0.8 pmol/mg protein at 50 min, respectively, and 84 ± 27 $\mu\text{Eq/h}$ per g and 9.1 ± 0.4 pmol/mg at 80 min. In glands perfused with $1 \mu\text{M}$ forskolin (second panel, Fig. 5), both chloride secretion and cAMP content increased markedly. At 50 min, chloride secretion was 7.4-fold above basal at 946 ± 149 $\mu\text{Eq/h}$ per g and tissue cAMP content was 45-fold above basal at 268 ± 29 pmol/mg. At 80 min chloride secretion decreased slightly compared to the 50-min time point but remained 7.6-fold above basal at 640 ± 64 $\mu\text{Eq/h}$ per g and cAMP content further increased to 58-fold above basal at 528 ± 73 pmol/mg. Glands were next perfused with forskolin

and 2Clado (third panel, Fig. 5). 2Clado at 100 nM completely inhibited forskolin-stimulated chloride secretion and secretion rates remained at basal levels ($P = \text{NS}$ compared to basal controls). Compared to forskolin controls, tissue cAMP content was inhibited by 40% to 161 ± 21 pmol/mg protein at 50 min ($P < 0.01$) and 34% to 350 ± 26 pmol/mg at 80 min ($P < 0.05$). However, despite complete inhibition of chloride secretion, tissue cAMP levels remained significantly greater than basal values: 27-fold greater than basal values at 50 min ($P < 0.01$) and 35-fold greater than basal values at 80 min ($P < 0.001$).

Silva et al. (34) have shown previously that somatostatin inhibits hormone-stimulated chloride secretion in the perfused rectal gland. To compare the effects of this inhibitor with 2Clado, rectal glands were perfused with forskolin and somatostatin (fourth panel, Fig. 5). Similar to 2Clado, somatostatin (100 nM) completely inhibited chloride secretion to basal levels ($P = \text{NS}$). Unlike 2Clado, however, somatostatin markedly inhibited cAMP accumulation by 86% to 37 ± 15 pmol/mg protein at 50 min ($P < 0.01$) and by 88% to 66 ± 29 pmol/mg at 80 min ($P < 0.001$). The cAMP content at 80 min, however, remained significantly ($P < 0.05$) elevated sevenfold above basal levels.

Adenylate cyclase activity in basolateral membranes. Consistent with the modest inhibition of cAMP accumulation by 2Clado, substantial inhibition of basolateral membrane adenylate cyclase with various adenosine analogues was difficult to demonstrate despite efforts to maximize the assay conditions for inhibition as described by Londos et al. (2). Table II demonstrates the effects of 2Clado (10^{-10} M to 10^{-4} M) on basal, $1 \mu\text{M}$ forskolin and $100 \mu\text{M}$ forskolin-stimulated adenylate cyclase activity in basolateral membranes of the rectal gland. 2Clado did not significantly inhibit basal or $1 \mu\text{M}$ forskolin-stimulated adenylate cyclase activity. However, 2Clado significantly inhibited $100 \mu\text{M}$ forskolin-stimulated adenylate cyclase activity in a dose-dependent manner: $9 \pm 3\%$ at $1 \mu\text{M}$ (P

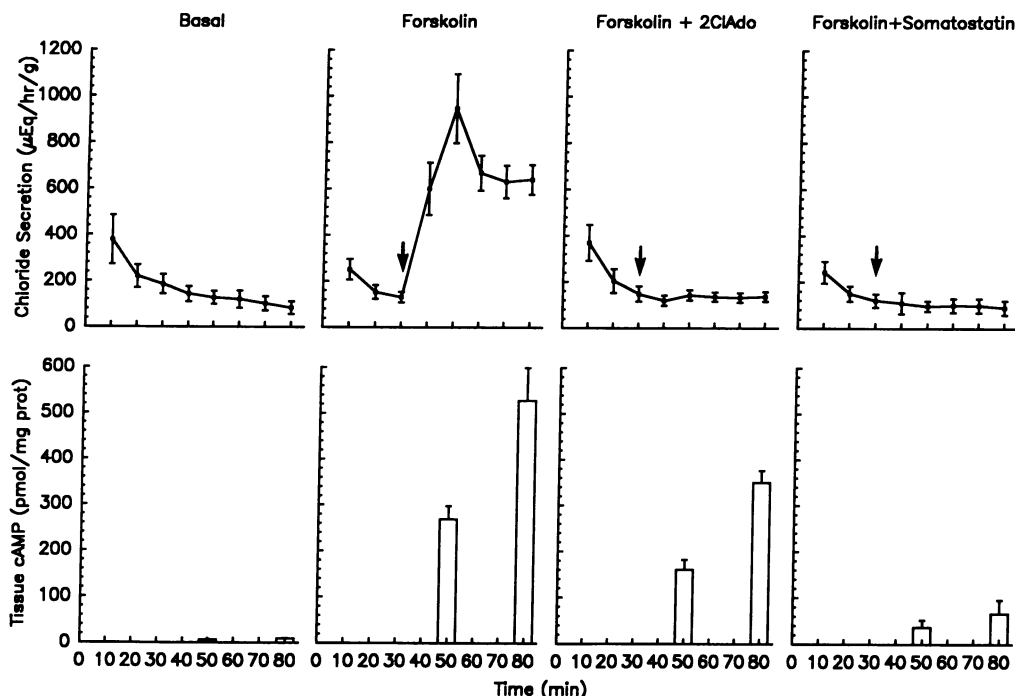


Figure 5. Simultaneous measurements of the effect of 2Clado and somatostatin on forskolin-stimulated chloride secretion and tissue cAMP content in in vitro perfused rectal glands. Four sets of experiments were performed. After 30 min of perfusion to basal values, rectal glands were perfused with basal Ringer's solution only ($n = 4$), $1 \mu\text{M}$ forskolin ($n = 14$), $1 \mu\text{M}$ forskolin and $0.1 \mu\text{M}$ 2Clado ($n = 13$), and $1 \mu\text{M}$ forskolin and somatostatin ($n = 5$). Tissue was transected from the gland for cAMP determination (at 50 and 80 min). The upper panels illustrate the chloride secretory values and the lower panels demonstrate the corresponding cAMP values.

Table II. Effect of 2Clado on Basal and Forskolin-stimulated Adenylate Cyclase Activity in Basolateral Membranes of the Shark Rectal Gland

Concentration of 2Clado (M)	Adenylate cyclase activity							
	Control	10 ⁻¹⁰	10 ⁻⁹	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
	<i>pmol of cAMP formed/min per mg protein</i>							
Basal (% change) (n = 6 exp)	7.1±0.9	7.0±0.9 (0±3)	7.1±0.9 (0±3)	7.2±1.1 (2±4)	6.9±0.6 (0±6)	7.4±0.7 (8±5)	9.7±1.5* (35±8)	11.7±1.6‡ (64±9)
+ Forskolin 1 μM (% change) (n = 4 exp)	13.4±1.3	13.7±1.8 (4±7)	13.1±1.2 (-1±7)	14.2±1.3 (8±9)	13.5±1.7 (0±6)	13.1±0.9 (-1±6)	15.8±0.7 (20±9)	16.7±1.5 (26±11)
+ Forskolin 100 μM (% change) (n = 5 exp)	49.4±7.5	50.4±7.2 (2±2)	50.3±6.8 (3±2)	46.9±7.4 (-5±4)	47.8±8.3 (-4±3)	45.1±8.0§ (-9±3)	42.8±6.9¶ (-14±2)	41.6±5.7 (-15±2)

* $P < 0.05$ compared to basal control. ‡ $P < 0.01$ compared to basal control. § $P < 0.05$ compared to forskolin 100 μM control. || $P < 0.02$ compared to forskolin 100 μM control. ¶ $P < 0.01$ compared to forskolin 100 μM control.

< 0.05), 14±2% at 10 μM ($P < 0.01$), and 15±2% at 100 μM ($P < 0.02$). R-PIA and NECA also did not inhibit basal or 1 μM forskolin-stimulated adenylate cyclase activity (data not shown), but, similar to 2Clado, R-PIA, 10 μM, significantly inhibited 100 μM forskolin-stimulated adenylate cyclase activity by 13±4% (108±13 to 94±12 pmol cAMP formed/min per mg protein; $P < 0.05$; $n = 4$).

Under these conditions, somatostatin, 100 nM, inhibited basal adenylate cyclase activity by 37% (10.9±1.1 to 6.9±0.7 pmol cAMP formed/min per mg protein; $P < 0.01$; $n = 5$), and 100 μM forskolin-stimulated activity by 25% (60±9 to 45±9 pmol cAMP formed/min per mg protein; $P < 0.02$; $n = 2$). These effects on adenylate cyclase activity are consistent with the marked decrease in forskolin-stimulated cAMP accumulation observed with somatostatin in the perfused gland.

In vitro perfusion

8CPT cAMP-stimulated chloride secretion is inhibited by 2Clado. To determine if the inhibitory effect of 2Clado could also be demonstrated in the presence of a permeant cyclic AMP analogue, additional perfusion experiments were performed with 8CPT cAMP. As shown in Fig. 6, the addition of 8CPT cAMP (20 μM) alone caused a sustained increase in the chloride secretion rate, rising from a basal value of 275±67 μEq/h per g to a maximum rate of 1,704±372 μEq/h per g. The addition of 0.1 μM 2Clado in the presence of 8CPT cAMP resulted in marked inhibition of chloride secretion to near basal values.

Discussion

The present studies demonstrate the presence of an A₁ adenosine receptor in the rectal gland of the dogfish shark *Squalus acanthias* that inhibits hormone stimulated chloride transport. This receptor has several characteristics that are consistent with external inhibitory A₁ adenosine receptors described by others in nonepithelial tissues (1, 2, 5, 6). First, activation of this receptor with A₁ receptor agonists including 2Clado, R-PIA, CHA, and NECA reversibly inhibited forskolin-stimu-

lated chloride secretion, and 2Clado inhibited VIP-stimulated chloride secretion. Second, this is a high affinity receptor since low concentrations, 100 nM, of these agonists completely inhibited chloride secretion, and the IC₅₀ for 2Clado was 5 nM. Third, activation of this receptor with the S stereoisomer of PIA, S-PIA, was far less potent than R-PIA in inhibiting chloride secretion. Fourth, inhibition of forskolin-stimulated chloride secretion by 2Clado was completely blocked by the high affinity methylxanthine A₁ receptor antagonists 8PT and 8CPT.

This high affinity receptor is distinct from the low affinity stimulatory A₂ receptor also present in the rectal gland (17). The chloride secretory response to varying concentrations of

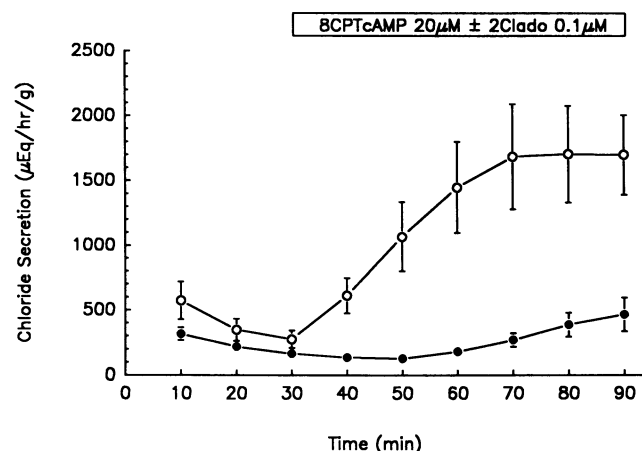


Figure 6. Effect of 2Clado on 8CPT cAMP stimulated chloride secretion in the in vitro perfused rectal gland. Rectal glands were perfused for 30 min to basal values, then 8CPT cAMP was added to the perfusate in the absence (open circles) or presence (closed circles) of 2Clado. Chloride secretion was measured at 10-min intervals and values are mean±SEM of eight experiments with 8CPT cAMP alone and seven experiments with 8CPT cAMP + 2Clado. (P values < 0.005 for 40 min and < 0.001 for 60–90-min values.)

2Clado was distinctly biphasic (Fig. 3). At low concentrations 2Clado inhibited chloride secretion but at concentrations above 1 μM 2Clado enhanced chloride secretion, demonstrating dual regulation of chloride transport by A_1 and A_2 adenosine receptors in this tissue.

The chloride secretory response to VIP was also inhibited by 2Clado, although this inhibition (47%) was less than the complete inhibition observed in the presence of forskolin. The reason for this difference in inhibition is not apparent from our studies but may be related to the unique property of forskolin observed in other tissues to potentiate receptor-mediated pathways (35, 36), or to the greater stimulation observed with VIP compared to forskolin.

The mechanism by which A_1 receptors inhibit hormone-stimulated chloride transport in the rectal gland is at least partially cAMP independent. Three sets of experiments support this conclusion. First, despite complete inhibition of chloride secretion, 2Clado inhibited forskolin-stimulated tissue cAMP content by only 38 to 48% with cyclic AMP concentrations remaining 27- to 38-fold above basal values. By contrast, somatostatin potently inhibited cAMP accumulation by 86–88%, indicating that substantial inhibition of cAMP is possible under these experimental conditions.

Second, 2Clado and other adenosine analogues did not markedly inhibit adenylate cyclase activity. 2Clado, R-PIA, and NECA did not inhibit basal or 1 μM forskolin-stimulated adenylate cyclase activity. This is in contrast to somatostatin, which inhibited basal adenylate cyclase by 37%. High concentrations of 2Clado and R-PIA, however, did inhibit 100 μM forskolin-stimulated adenylate cyclase activity by 9–15%. Under these conditions, A_1 receptor-mediated inhibition was uncovered because 100 μM forskolin maximally stimulates adenylate cyclase and prevents stimulation by the stimulatory A_2 receptor. This lesser degree of inhibition of adenylate cyclase activity compared to inhibition of cyclic AMP accumulation has also been observed in the cardiac myocyte (37) and the rat adipocyte (38) and may be due, in part, to components of the reaction mixture that generate adenosine, as emphasized by Londos et al. (2). In several nonepithelial tissues, A_1 agonists also activate a membrane-bound, low K_m cAMP phosphodiesterase (39, 40); this effect could also contribute to the greater inhibition of cAMP accumulation compared to adenylate cyclase that was observed in our study.

Third, chloride secretion stimulated by 8CPT cAMP was profoundly inhibited by 2Clado, demonstrating directly an inhibition of the action of cyclic AMP. Taken together, these results demonstrate that whereas 2Clado modestly inhibits cAMP accumulation, a mechanism independent of cAMP is responsible for complete inhibition of secretion.

Our studies do not define the cAMP independent effector system(s) by which A_1 receptors inhibit chloride secretion in the rectal gland. In other tissues, additional inhibitory signaling mechanisms have been identified for adenosine receptors and other receptors linked to inhibition of adenylate cyclase including activation of K^+ channels (10–12), inhibition of voltage sensitive Ca^{2+} channels (13–15), and acceleration of Na^+/H^+ exchange (41); (for reviews see 15, 42). Arend et al. (43) have provided evidence for adenosine receptor mediated mobilization of intracellular calcium in cultured cortical collecting tubule cells. These or other effector mechanisms may be involved in the A_1 receptor-mediated inhibition of chloride transport in the rectal gland.

Although we observed that somatostatin was more potent than adenosine in inhibiting cAMP accumulation and adenylate cyclase in the gland, cAMP content remained significantly greater than basal values at 80 min indicating that a cAMP-independent mechanism is also involved in the inhibition of forskolin-stimulated chloride secretion by somatostatin. Silva et al. (34) previously suggested that inhibition of chloride secretion in the perfused rectal gland by somatostatin was partially cAMP independent because it inhibited dibutyryl cAMP-stimulated chloride secretion. Because somatostatin has been shown to affect ion channels in a manner similar to adenosine in other tissues (44, 45), it is possible that the cAMP-independent actions of both inhibitors are mediated by G-protein coupled effects on ion channels (46) or other transport proteins.

We have recently observed that during hormonal stimulation of chloride transport in the perfused rectal gland, endogenous adenosine is released at inhibitory concentrations and acts as feedback inhibitor of chloride transport via this A_1 adenosine receptor (47). We propose that in the rectal gland, A_1 receptors provide an autacoid feedback mechanism linking cellular work and adenosine release to receptor-mediated inhibition of chloride transport.

Previous studies have underscored the similarities between chloride secretion in tubules of the rectal gland and chloride reabsorption in the mammalian medullary thick ascending limb of Henle's loop (mTAL) (17, 24, 26). Our laboratory recently has demonstrated a high density of A_1 receptor binding sites in rat mTAL using high resolution in vitro autoradiography (48). In preliminary studies, Burnatowska-Hledin and Spielman (49) have reported that the A_1 agonist cyclohexyladenosine inhibits both basal and AVP-stimulated cyclic AMP production in primary cultures of rabbit mTAL cells, and Epstein et al. (50) have reported that R-PIA protects against injury to the mTAL in isolated perfused rat kidneys. In the mTAL, adenosine may serve a similar autacoid role as in the rectal gland.

In summary, we have presented evidence for an inhibitory A_1 adenosine receptor in the rectal gland that potently inhibits chloride transport. This receptor-mediated inhibition is accompanied by an inhibition of cAMP accumulation and adenylate cyclase; however, a major portion of this inhibition occurs by a mechanism that is independent of the adenylate cyclase-cAMP system.

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References

1. Daly, J. W. 1982. Adenosine receptors: targets for future drugs. *J. Med. Chem.* 25:197–207.
2. Londos, C., J. Wolff, and D. M. F. Cooper. 1981. Adenosine as a regulator of adenylate cyclase. *In Purinergic Receptors. (Receptors and recognition series B)* Vol. 12. G. Burnstock, editor. Chapman and Hall, London/New York. 2287–2323.

3. Osswald, H., H. H. Hermes, and G. Nabakowski. 1982. Role of adenosine in signal transmission of tubuloglomerular feedback. *Kidney Int.* 22(12):136-142.
4. Spielman, W. S., and C. I. Thompson. 1982. A proposed role for adenosine in the regulation of renal hemodynamics and renin release. *Am. J. Physiol.* 242:F423-F435.
5. Van Calker, D., M. Muller, and B. Hamprecht. 1979. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* 33:999-1005.
6. Londos, C., D. M. F. Cooper, and J. Wolff. 1980. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA.* 77:2551-2554.
7. Bruns, R. F., G. H. Lu, and T. A. Pugsley. 1986. Adenosine receptor subtypes: binding studies. In *Topics and Perspectives in Adenosine Research*. E. Gerlach and B. F. Becker, editors. Springer-Verlag, Berlin/Heidelberg. 59-73.
8. Sattin, A., and T. W. Rall. 1970. The effect of adenosine and adenosine nucleotides on the cyclic adenosine 3',5'-phosphate content of guinea pig cerebral cortex slices. *Mol. Pharmacol.* 6:13-23.
9. Smellie, F. W., C. W. Davis, J. W. Daly, and J. N. Wells. 1979. Alkylxanthines: inhibition of adenosine-elicited accumulation of cyclic AMP in brain slices and of brain phosphodiesterase activity. *Life Sci.* 24:2475-2482.
10. Belardinelli, L., and G. Isenberg. 1983. Isolated atrial myocytes: adenosine and acetylcholine increase potassium conductance. *Am. J. Physiol.* 244 (*Heart Circ. Physiol.* 13):H734-H737.
11. Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986. On the mechanism of activation of muscarinic K⁺ channels by adenosine in isolated atrial cells: Involvement of GTP-binding proteins. *Pfluegers Arch. Eur. J. Physiol.* 407:264-274.
12. Trussell, L. O., and M. B. Jackson. 1987. Dependence of an adenosine-activated potassium current on a GTP-binding protein in mammalian central neurons. *J. Neurosci.* 7:3306-3316.
13. Dolphin, A. C., S. R. Forda, and R. H. Scott. 1986. Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. *J. Physiol.* 373:47-61.
14. MacDonald, R. L., J. H. Skerritt, and M. A. Werz. 1986. Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. *J. Physiol.* 370:75-90.
15. Rosenthal, W., J. Hescheler, W. Trautwein, and G. Schultz. 1988. Control of voltage-dependent Ca²⁺ channels by G protein-coupled receptors. *FASEB (Fed. Am. Soc. Eur. Biol.) J.* 2:2784-2790.
16. Spinowitz, B. S., and J. A. Zadunaisky. 1979. Action of adenosine on chloride active transport of isolated frog cornea. *Am. J. Physiol.* 237:F121-F127.
17. Forrest, J. N., Jr., F. Wang, and K. W. Beyenbach. 1983. Perfusion of isolated tubules of the shark rectal gland. Electrical characteristics and responses to hormones. *J. Clin. Invest.* 72:1163-1167.
18. Dobbins, J. W., J. P. Laursen, and J. N. Forrest, Jr. 1984. Adenosine and adenosine analogs stimulate adenosine cyclic 3',5'-monophosphate-dependent chloride secretion in the mammalian ileum. *J. Clin. Invest.* 74:929-935.
19. Grasl, M., and K. Turnheim. 1984. Stimulation of electrolyte secretion in rabbit colon by adenosine. *J. Physiol.* 346:93-110.
20. Pratt, A. D., G. Clancy, and M. J. Welsh. 1986. Mucosal adenosine stimulates chloride secretion in canine tracheal epithelium. *Am. J. Physiol.* 251(20):C167-C174.
21. Lang, M. A., A. S. Preston, J. S. Handler, and J. N. Forrest, Jr. 1985. Adenosine stimulated sodium transport in kidney A6 epithelia in culture. *Am. J. Physiol.* 249:C330-C336.
22. Dillingham, M. A., and R. J. Anderson. 1985. Purinergic regulation of basal and arginine vasopressin-stimulated hydraulic conductivity in rabbit cortical collecting tubule. *J. Membr. Biol.* 88:277-211.
23. Arend, L. J., W. K. Sonnenburg, W. L. Smith, and W. S. Spielman. 1987. A₁ and A₂ adenosine receptors in rabbit cortical collecting tubule cells. *J. Clin. Invest.* 79:710-714.
24. Silva, P. J., J. Stoff, M. Field, L. Fine, J. N. Forrest, and F. H. Epstein. 1977. Mechanism of active chloride secretion by shark rectal gland: role of Na-K-ATPase in chloride transport. *Am. J. Physiol.* 233:F298-F306.
25. Stoff, J. S., R. Rosa, R. Hallac, P. Silva, and F. H. Epstein. 1979. Hormonal regulation of active chloride transport in the dogfish rectal gland. *Am. J. Physiol.* 237:F138-1144.
26. Eveloff, J., R. Kinne, E. Kinne-Saffran, H. Murer, P. Silva, F. H. Epstein, J. Stoff, and W. B. Kinter. 1978. Coupled sodium and chloride transport into plasma membrane vesicles. *Pfluegers Arch. Eur. J. Physiol.* 378:87-92.
27. Riss, T. L., N. L. Zorich, M. D. Williams, and A. Richardson. 1980. A comparison of the efficiency of nucleotide extraction by several procedures and the analysis of nucleotides from extracts of liver and isolated hepatocytes by HPLC. *J. Liq. Chromatogr.* 3:133-158.
28. Steiner, A. L., C. W. Parker, and D. M. Kipnis. 1972. Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. *J. Biol. Chem.* 247:1106-1113.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
30. Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* 58:541-548.
31. Stoff, J., P. Silva, M. Field, J. Forrest, A. Stevens, and F. Epstein. 1977. Cyclic AMP regulation of active chloride transport in the rectal gland of marine elasmobranchs. *J. Exp. Zool.* 199:443-448.
32. Greger, R., E. Schlatter, F. Wang, and J. N. Forrest, Jr. 1984. Mechanism of NaCl secretion in rectal gland tubules of spiny dogfish (*Squalus acanthias*). III Effects of stimulation of secretion by cyclic AMP. *Pfluegers Arch. Eur. J. Physiol.* 402:376-384.
33. Greger, R., E. Schlatter, and H. Gogelein. 1985. Cl⁻-channels in the apical cell membrane of the rectal gland "induced" by cAMP. *Pfluegers Arch. Eur. J. Physiol.* 403:446-448.
34. Silva, P., J. S. Stoff, D. R. Leone, and F. H. Epstein. 1985. Mode of action of somatostatin to inhibit secretion by shark rectal gland. *Am. J. Physiol.* 249(18):R329-R334.
35. Daly, J. W., W. Padgett, and K. B. Seamon. 1988. Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: augmentation of receptor-mediated responses. *J. Neurochem.* 38:532-544.
36. Daly, J. W. 1984. Forskolin, adenylate cyclase and cell physiology: An overview. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* 17:81-89.
37. Dobson, J. G., and R. A. Fenton. 1983. Antiadrenergic effects of adenosine in the heart. In *Regulatory Function of Adenosine*. R. M. Berne, T. W. Rall, and R. Rubio, editors. Martinus Nijhoff, Boston. 363-376.
38. Trost, T., and K. Stock. 1979. Adenosine effects in adipose tissue: Effect of adenosine on the cyclic adenosine monophosphate system and lipolysis of fat cells. In *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides*. H. P. Baer and G. I. Drummond, editors. Raven Press, New York. 379-393.
39. deMazancourt, P., and Y. Giudicelli. 1984. Guanine nucleotides and GTP-dependent N6 PIA stimulation of the membrane-bound cAMP high affinity phosphodiesterases in brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 167:142-145.
40. Elks, M., M. Jackson, V. Mong, and M. Vaughan. 1987. Effect of N6-PIA and insulin on cAMP metabolism in 3T3-L1 adipocytes. *Am. J. Physiol.* 252:C342-348.
41. Isom, L. L., E. J. Cragoe, Jr., and L. E. Limbird. 1987. Multiple receptors linked to inhibition of adenylate cyclase accelerate Na⁺/H⁺ exchange in NG 108-15 cells by a mechanism other than decreased production of cAMP. *J. Biol. Chem.* 262:17504-17509.
42. Limbird, L. E. 1988. Receptors linked to inhibition of adenylate cyclase: Additional signaling mechanisms. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:2686-2695.
43. Arend, L. J., M. A. Burnatowska-Hledin, and W. S. Spielman. 1988. Adenosine signal transduction in cortical collecting tubule cells: receptor mediated calcium mobilization. *Am. J. Physiol.* 255:C581-588.

44. Lewis, D. L., F. F. Weight, and A. Luini. 1986. A guanine nucleotide-binding protein mediates the inhibition of voltage-dependent calcium current by somatostatin in a pituitary cell line. *Proc. Natl. Acad. Sci. USA.* 83:9035-9039.
45. Yatani, A., J. Codina, R. D. Sekura, L. Birnbaumer, and A. M. Brown. 1987. Reconstitution of somatostatin and muscarinic receptor mediated stimulation of K⁺ channels by isolated G_k protein in clonal rat anterior pituitary cell membranes. *Mol. Endocrinol.* 1:283-289.
46. Brown, A. M., and L. Birnbaumer. 1988. Direct G protein gating of ion channels. *Am. J. Physiol.* 254(23):H401-H410.
47. Kelley, G. G., O. S. Aassar, and J. N. Forrest, Jr. 1988. Adenosine released during hormonal stimulation is a feedback inhibitor of chloride transport in the shark rectal gland. *Kidney Int.* 33:270.
48. Brines, M. L., and J. N. Forrest, Jr. 1988. Autoradiographic localization of A₁ adenosine receptors to tubules in the red medulla and papilla of the rat kidney. *Kidney Int.* 33:256. (Abstr.)
49. Burnatowska-Hledin, M. A., and W. S. Spielman. 1988. Adenosine signal transduction in the thick ascending limb of Henle's loop. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:927a. (Abstr.)
50. Epstein, F. H., S. Rosen, G. Galicka-Piskorska, K. Spokes, S. Pear, M. Brezio, and P. Silva. 1987. Modulation by adenosine of medullary injury in isolated perfused kidneys. *Kidney Int.* 31:367a. (Abstr.)