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β-ADRENERGIC STIMULATION OF THE ADENOSINE 3′,5′-MONOPHOSPHATE SYSTEM REGULATED BY CHOLINERGIC STIMULI IN THE PROSTATE

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Abstract—Acetylcholine significantly inhibited isoproterenol-stimulated adenosine 3′,5′-monophosphate (cAMP) levels of rat prostatic tissue in a concentration-dependent fashion. Atropine but no hexamethonium reversed the inhibitory action of acetylcholine. Tetracaine and verapamil abolished the inhibitory effect of acetylcholine on isoproterenol-stimulated accumulation of cAMP. Exclusion of calcium also eliminated the effect of acetylcholine. Inhibitory regulation of cAMP levels was reproduced by the divalent cation ionophore A23187. These observations suggest that β-adrenergic stimulation of the cAMP system of the prostate is regulated by cholinergic stimulation involving a specific muscarinic receptor with calcium-dependent mechanism sensitive to verapamil or tetracaine.

Previous studies [1–3] have demonstrated adrenergic stimulation of the adenylate cyclase system through β-adrenoceptors in rat ventral prostatic membranes. Interactions of cholinergic agents with the adrenergic response in the cAMP system have been also reported in various tissues [4–11]. In many systems that possess muscarinic cholinergic receptors, cholinergic agonists were observed to attenuate hormone-stimulated cAMP generation.

Recent studies have shown muscarinic, calcium-dependent cholinergic receptors in the astrocytoma cell [5], the murine heart [9], and the parathyroid cell [10]. However, other studies have demonstrated a muscarinic cholinergic inhibition of the cAMP system independently of extracellular calcium in the human diploid cell [6] and the parotid gland [11].

In the present experiment, effects of cholinergic agents on an isoproterenol-stimulated cAMP system in rat ventral prostate were investigated.

MATERIALS AND METHODS

Preparation of tissue. Prostate glands from male rats, weighing 250–300 g, of the Donryu strain (Nippon Rat Co., Ltd., Tokyo, Japan) were minced and preincubated in Krebs–Ringer buffer solution (pH 7.4) that contained 1 mg/ml of glucose and bovine serum albumin (KRBG buffer) and were gassed with 95% O_2–5% CO_2 at 37° for 15 min. Subsequently, the tissue was transferred to fresh medium with or without various test agents for the final 15 min of incubation. Incubations with acetylcholine were performed in the presence of 10 μM physostigmine which alone had no effect on basal levels of cAMP (data not shown).

Assay of cAMP. After incubation, tissues were homogenized in 5% trichloroacetic acid at 0° and centrifuged. Supernatant fractions were ether-extracted and then lyophilized. cAMP levels were routinely determined by the method of Gilman [12], using a cAMP-binding protein purified from rabbit skeletal muscle through the DEAE-cellulose column step. Protein concentrations were measured by the method of Lowry *et al.* [13].

Membrane preparation. The tissues were homogenized in 20 mM Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose, 4 mM $MgCl_2$, 1 mM EDTA, and 3.25 mM 2-mercaptoethanol with a Potter–Elvhjem glass homogenizer (10–20 strokes), followed by filtration through two layers of gauze. The homogenate was centrifuged twice at 600 *g* for 10 min. The supernatant fraction was centrifuged at 10,000 *g* for 30 min. The particulate fraction that was obtained at 10,000 *g*, and washed with the Tris–HCl buffer, was referred to as membrane particles. Adenylate cyclase activity was assayed by incubation of the membrane particles in 40 mM Tris–HCl buffer (pH 7.4) that contained 4 mM $MgCl_2$, 5 mM theophylline, 2 mM ATP and an ATP regenerating system for 10 min, as described previously [1–3, 14].

Chemicals. The following drugs were donated: cAMP (Daiichi Seiyaku, Tokyo, Japan), 1-isoproterenol (Nicken Chemicals, Tokyo, Japan), verapamil (EISAI Co., Ltd., Tokyo, Japan) and tetracaine (Kyorin Pharmac., Tokyo, Japan). A23187 was a gift from Dr. R. L. Hamill, Eli Lilly Research Laboratories, Indianapolis, IN, U.S.A. Acetylcholine-chloride, atropine-sulfate, hexamethonium-chloride, theophylline, and IBMX‡ were purchased from the Wako Chemical Co., Ltd., Tokyo, Japan.

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‡ Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; and EGTA, ethyleneglycol bis (β-aminoethylether)-*N*,*N*′-tetraacetic acid.

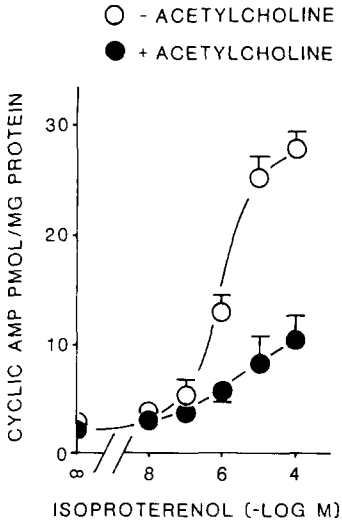

Fig. 1. Response of cAMP to isoproterenol concentration in prostatic tissues. Prostatic tissues were incubated with various concentrations of isoproterenol in the presence or absence of 100 μM acetylcholine for 15 min. Each point is the mean of quadruplicates ± S.E.

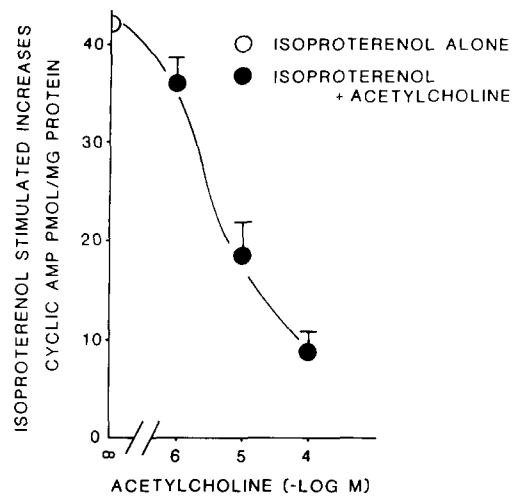

Fig. 2. Acetylcholine inhibition of isoproterenol-induced increases in intracellular cyclic AMP levels. Acetylcholine was added simultaneously with 10 μM isoproterenol for 15 min of incubation. Each point is the mean of triplicates ± S.E.

RESULTS

Effect of acetylcholine on cAMP levels. cAMP accumulation induced by isoproterenol was apparently reduced by addition of acetylcholine, with no apparent effect on basal cAMP levels (Fig. 1). Acetylcholine attenuated the isoproterenol-induced increase in cAMP levels in a concentration-dependent manner (Fig. 2). Reduction of isoproterenol-elevated cAMP levels by acetylcholine was prevented by 1 μM atropine but not by 1 μM hexamethonium (Table 1). The addition of 10 μM tetracaine or 10 μM verapamil was also effective in reversing the acetylcholine inhibition (Table 2). Atropine, hexamethonium, verapamil or tetracaine did not affect the isoproterenol-induced increases in cAMP accumulation (Tables 1 and 2). The divalent cation ionophore greatly reduced the cAMP accumulation induced by isoproterenol (Fig. 3). In calcium-free

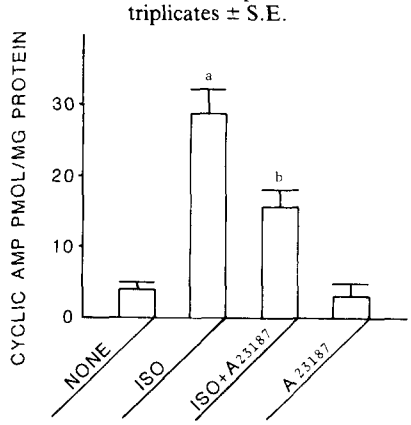

Fig. 3. Inhibitory effect of A23187 on isoproterenol-induced increases in intracellular cyclic AMP levels. A23187 (10 μM) was added simultaneously with 10 μM isoproterenol for 15 min of incubation. All values represent the means of triplicates ± S.E. Key: (a) significantly different from no addition ($P < 0.01$); and (b) significantly different from isoproterenol ($P < 0.01$).

Table 1. Effects of atropine and hexamethonium on actions of isoproterenol alone and in combination with acetylcholine*

Additions				
Isoproterenol (10 μM)	Acetylcholine (100 μM)	Atropine (1 μM)	Hexamethonium (1 μM)	Cyclic AMP (pmoles/mg protein)
−	−	−	−	2.04 ± 0.53
−	+	−	−	1.80 ± 0.60
−	−	+	−	1.57 ± 0.43
−	−	−	+	1.27 ± 0.43
+	−	−	−	24.96 ± 1.67†
+	−	+	−	22.93 ± 0.76†
+	−	−	+	26.10 ± 3.71†
+	+	−	−	8.33 ± 0.77‡
+	+	+	−	20.25 ± 2.48†§
+	+	−	+	5.93 ± 1.07‡

* Prostatic tissues were incubated for 15 min with or without various test agents. Atropine or hexamethonium was added 1 min prior to isoproterenol or acetylcholine. Values represent the means ± S.E. of four separate incubations.

† Significantly different from no addition ($P < 0.01$).

‡ Significantly different from isoproterenol ($P < 0.01$).

§ Significantly different from isoproterenol plus acetylcholine ($P < 0.01$).

Table 2. Effects of verapamil and tetracaine on actions of isoproterenol alone and in combination with acetylcholine*

Additions				
Isoproterenol (10 μM)	Acetylcholine (100 μM)	Verapamil (10 μM)	Tetracaine (10 μM)	Cyclic AMP (pmoles/mg protein)
−	−	−	−	1.91 ± 0.72
−	+	−	−	1.27 ± 0.47
−	−	+	−	1.60 ± 0.28
−	−	−	+	1.68 ± 0.42
+	−	−	−	26.53 ± 1.66†
+	−	+	−	28.31 ± 1.40†
+	+	−	−	10.12 ± 0.88‡
+	+	+	−	21.90 ± 0.75§
+	+	−	+	19.32 ± 1.21§

* Prostatic tissues were incubated for 15 min with or without various test agents. Values represent the means ± S.E. of four separate incubations.
† Significantly different from no addition ($P < 0.01$).
‡ Significantly different from isoproterenol alone ($P < 0.01$).
§ Significantly different from isoproterenol plus acetylcholine ($P < 0.01$).

Table 3. Effect of calcium-free incubation media on cyclic AMP levels*

Incubation 1	Incubation 2		Cyclic AMP
Calcium	Calcium	Test agent	(pmoles/mg protein)
+	+	None	1.86 ± 0.23
+	+	Isoproterenol	32.25 ± 2.11†
+	+	Isoproterenol + acetylcholine	6.83 ± 0.89†‡
−	−	None	1.80 ± 0.11
−	−	Isoproterenol	16.80 ± 0.90†
−	−	Isoproterenol + acetylcholine	17.47 ± 1.27†

* Prostatic tissues were initially incubated at 37° for 30 min in KRBG buffer with or without 3 mM Ca^{2+} (incubation 1) and then transferred to fresh buffer with or without 3 mM Ca^{2+} for a terminal 15 min (incubation 2). All buffers contained 0.1 mM EGTA. Where indicated, 10 μM isoproterenol and 100 μM acetylcholine were present for incubation 2. Values represent the means ± S.E. of four separate incubations.
† Significantly different from no addition ($P < 0.01$).
‡ Significantly different from isoproterenol ($P < 0.01$).

Table 4. Effects of isoproterenol and acetylcholine on cyclic AMP levels in the presence of theophylline or IBMX*

	Cyclic AMP (pmoles/mg protein)		
Additions	None	Theophylline	IBMX
None	2.02 ± 0.34	12.25 ± 1.30	17.93 ± 2.15
Isoproterenol	36.92 ± 2.89†	178.03 ± 12.36†	202.46 ± 18.77†
Isoproterenol + acetylcholine	10.71 ± 1.55†‡	40.27 ± 4.80†‡	64.75 ± 7.26†‡

* Prostatic tissues were incubated for 15 min in KRBG buffer with or without 10 mM theophylline or 0.5 mM IBMX. Concentrations of isoproterenol and acetylcholine were 10 and 100 μM respectively. Values represent the means ± S.E. of four separate incubations.
† Significantly different from no addition ($P < 0.01$).
‡ Significantly different from isoproterenol alone ($P < 0.01$).

Table 5. Effect of preincubation with acetylcholine on isoproterenol-stimulated adenylate cyclase activity*

Preincubation	Adenylate cyclase activity [pmoles cyclic AMP·(mg protein)$^{-1}$·min^{-1}]	
	Isoproterenol	
Acetylcholine	−	+
−	2.07 ± 0.17	7.67 ± 0.39†
+	2.16 ± 0.11	8.00 ± 0.41†

* Prostatic tissues were preincubated for 15 min in KRBG buffer, with or without 100 μM acetylcholine, and washed rapidly twice with KRBG buffer prior to cell-free preparation. Washed membranes were incubated in the adenylate cyclase assay system with or without 10 μM isoproterenol for 10 min. Each result is the mean ± S.E. of five separate incubations.

† Significantly different from no addition ($P < 0.01$).

medium, the cAMP response to isoproterenol was apparently lower than in the presence of calcium. Meanwhile, acetylcholine-mediated inhibition of isoproterenol stimulation was completely abolished by removal of calcium from the medium (Table 3). Theophylline and IBMX increased both basal and isoproterenol-stimulated cAMP accumulation (Table 4). Acetylcholine was found to inhibit the cAMP accumulation elicited by isoproterenol even in the presence of theophylline or IBMX (Table 4), although the inhibition was partially reversed in the presence of these phosphodiesterase inhibitors.

Effect of acetylcholine on adenylate cyclase. The inhibition by preincubation with acetylcholine was reversed by washing and by the process of cell-free preparation (Table 5). Addition of acetylcholine to prostatic membranes also failed to inhibit isoproterenol stimulation of the adenylate cyclase (Table 6). Addition to guanosine 5′-triphosphate (GTP) exclusively enhanced isoproterenol-stimulated adenylate cyclase activity regardless of the presence or absence of acetylcholine (Table 6). These results indicate that cholinergic inhibition of the prostatic cAMP system required cell integrity.

DISCUSSION

cAMP inhibition produced by acetylcholine was apparently non-competitive with respect to the concentration of isoproterenol, suggesting that acetylcholine did not compete with isoproterenol on the β-adrenergic receptor level. The failure of acetylcholine to affect [^{3}H]dihydroalprenolol binding to the membrane (data not shown) indicated that there was no direct action of acetylcholine at the β-adrenergic receptor. Like most systems [4–8] that possess cholinergic muscarinic receptors, the cholinergic action on the prostatic cAMP system appears to have been mediated through specific muscarinic receptors. Addition of tetracaine or verapamil to the incubation medium completely abolished decreases in cAMP mediated by acetylcholine. Both cholinergic agents and A23187 are known to increase intracellular calcium [15–17]. Similar effects of acetylcholine and A23187 on inhibition of hormone-induced cAMP accumulation suggest that both agents modulate cAMP by a common calcium-dependent mechanism. In contrast to the cholinergic action on tissue calcium metabolism, tetracaine and verapamil have been shown to block calcium binding to, or transport across, cellular or subcellular membranes [18–20] and to inhibit the late channels for calcium influx [21–23]. The key role of calcium in the cholinergic inhibition of prostatic cAMP is supported by the results of these studies with the calcium antagonists, tetracaine and verapamil. Furthermore, the absence of calcium in the incubation medium specifically abolished the effect of acetylcholine on the isoproterenol-induced accumulation of cAMP, indicating that extracellular calcium is a major component in the cholinergic action on the prostate.

Table 6. Effects of acetylcholine on isoproterenol-stimulated adenylate cyclase activity*

Additions	Adenylate cyclase activity [pmoles cyclic AMP·(mg protein)$^{-1}$·min^{-1}]
None	1.67 ± 0.34
Isoproterenol	7.93 ± 0.24†
Isoproterenol + acetylcholine	8.55 ± 1.35†
Isoproterenol + GTP	15.11 ± 1.00†‡
Isoproterenol + acetylcholine + GTP	13.22 ± 0.54†‡
GTP	2.20 ± 0.17
Acetylcholine	1.89 ± 0.22

* Membranes were incubated in the adenylate cyclase assay system with 10 μM isoproterenol, 100 μM acetylcholine or 50 μM GTP. Each result is the mean ± S.E. of five separate incubations.

† Significantly different from no addition ($P < 0.01$).

‡ Significantly different from isoproterenol alone ($P < 0.01$).

The facts that cholinergic inhibition was removed by successive washing and was not directly on the prostatic adenylate cyclase system are, apparently, different from the cholinergic mechanisms in the parotid gland [7, 8] and cardiac preparations [4]. Recent studies [24–29] have demonstrated a requirement of guanine nucleotides for cholinergic or α-adrenergic inhibition of adenylate cyclase activities. GTP was without effect in inducing cholinergic inhibition of isoproterenol-stimulated adenylate cyclase in the prostatic membrane, indicating that cholinergic receptors are not directly associated with the adenylate cyclase system.

Calcium itself has been reported to inhibit the cAMP-adenylate cyclase system in several other preparations [30–33]. The presence of a crucial calcium pool at the inhibitory sites of the adenylate cyclase complex has been suggested [30, 31]. The cholinergic inhibition of prostatic cAMP would be involved in a common calcium channel sensitive to verapamil or tetracaine. Cholinergic agents, histamine, vasopressin, serotonin and α-adrenergic agents all increase cytosolic calcium and increase phosphatidylinositol turnover, which may gate extracellular calcium [34, 35]. Isoproterenol stimulation of cAMP accumulation was apparently reduced by exclusion of calcium from the medium. The decrease in response to isoproterenol after exposure of the tissue to calcium-free medium (plus EGTA) may reflect general deterioration of the tissue.

The increased concentration of intracellular calcium could cause a decrease in cAMP levels by inhibition of adenylate cyclase, by stimulation of phosphodiesterase, or by a combination of these mechanisms. The present experiments with theophylline or IBMX demonstrated a reduction in cAMP levels by acetylcholine during inhibition of phosphodiesterase. These results indicate a prominent role of calcium in cholinergic inhibition of the cAMP-generating system in the prostate.

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