STABLE CHOLINERGIC-MUSCARINIC INHIBITION OF RAT PAROTID ADENYLATE CYCLASE

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1. Introduction

There are numerous reports describing α-adrenergic and cholinergic inhibition of cAMP accumulation in various tissues [1–10]. The rat parotid gland provides an excellent model system for the elucidation of inter-receptor interactions. The stimulation of α-adrenergic, β-adrenergic and cholinergic-muscarinic receptors has been well characterized pharmacologically, biochemically and morphologically and has been integrated into an overall scheme in [11,12]. Cholinergic-muscarinic and α-adrenergic inhibition of cAMP accumulation, stimulated by activation of β-adrenergic receptors in the rat parotid has been reported [13–15]. Recent data from our laboratory demonstrated a different mechanism of action for the cholinergic and α-adrenergic effect and also indicated adenylate cyclase as the target enzyme [16,17]. In the present communication we report a stable inhibition of parotid adenylate cyclase in homogenates and washed membrane preparations following the exposure of tissue slices to carbamylcholine.

2. Experimental methods

Male Wistar rats (120–200 g) were used throughout. Rats were fasted overnight prior to sacrifice. The parotid slice incubation system was similar to that in [15], except that 10 mM β-hydroxybutyrate was included in the Krebs-Ringer bicarbonate medium (KRB). Rat parotid homogenate was obtained by homogenizing slices which were decanted free of KRB in sucrose 0.3 M containing 5 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N'*-tetra-acetic acid (EGTA) and 0.04 mM of ethylene diamine-*N,N'*-tetra-acetic acid (EDTA), pH 7.5 (sucrose medium) in a loose fitting Teflon–glass homogenizer (10–15 strokes at 0–2°C). The homogenate was strained through nylon mesh to remove large particles. Crude particulate fraction was obtained by centrifuging the homogenate in a Beckman Microfuge for 5 min and resuspending the pellet in the original volume of sucrose medium. Hypotonically washed membranes were obtained by a procedure similar to that in [18]. Briefly, slices were homogenized in sucrose medium (1–2 ml/gland), centrifuged at 250 × *g* for 10 min at 4°C and the pellet resuspended in (1–2 ml/gland) Tris–HCl 10 mM, EDTA 0.04 mM, $MgCl_2$ 1 mM, pH 7.5 (Tris medium) by homogenization (5 strokes at 0–2°C). The suspension was centrifuged at 1500 × *g* for 10 min at 4°C and the cycle repeated one more time. The final pellet was resuspended in sucrose medium and strained through nylon mesh. The adenylate cyclase assay system consisted of Hepes (pH 7.5) 50 mM/[α-^{32}P]ATP 0.5 mM (10–30 cpm/pmol)/GTP 0.1 mM/cAMP 1 mM/$MgCl_2$ 10 mM/EDTA 1 mM/RO 20-1724/001 (cAMP phosphodiesterase inhibitor) 0.1 mM/phosphoenolpyruvate 6 mM/pyruvate kinase 0.11 mg/ml/bovine serum albumin 0.1 mg/ml/various additions and homogenate

Abbreviations: cAMP, adenosine-3',5'-monophosphate; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N'*-tetra-acetic acid; EDTA, ethylene diamine-*N,N'*-tetra-acetic acid; PDE, phosphodiesterase

or membrane suspension in final vol. 0.1 ml. The reaction was initiated by the addition of adenylate cyclase preparation at 37°C and terminated by adding 0.8 ml solution of 0.25% sodium dodecyl sulphate–5 mM ATP (pH 7.5). The method in [19] was used for the separation of cAMP. Basal activity was assayed in the presence of 10 μM propranolol, and fluoride-stimulated activity in the presence of 10 mM KF. (–)-Isoproterenol, atropine, carbamylcholine and pyruvate kinase were obtained from Sigma, RO 20-1724/001 from Hoffman-LaRoche, [α-^{32}P]ATP from Amersham. All other chemicals were of analytical grade.

3. Results

The inclusion of cAMP (1 mM) and the PDE inhibitor RO 20-1724/001 in the adenylate cyclase assay system, each improved the assay of adenylate cyclase in parotid homogenates (fig.1). The reaction

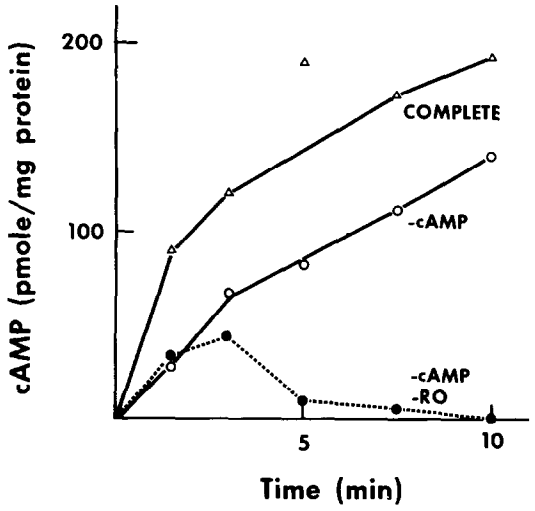

Fig.1. The requirement for cAMP and cAMP phosphodiesterase inhibitor in adenylate cyclase assay in parotid homogenates. Rat parotid glands were excised and incubated in KRB as in section 2. At the end of the incubation, slices were pelleted by rapid centrifugation (1500 × *g*, 30 min), the KRB was aspirated and the tissue homogenized in sucrose medium (0.5 ml/gland). The homogenate was strained through nylon mesh and assayed for adenylate cyclase activity in the presence of 1 mM cAMP and 0.1 mM RO 20-1724 (complete), without cAMP, or without cAMP and RO 20-1724 in the presence of (–)-isoproterenol (20 μM).

was not linear with time and short incubation times were required. GTP (0.1 mM) in the assay system dramatically improved the basal and (–)-isoproterenol-stimulated activities (4–7-fold, not shown) without affecting the fluoride-stimulated activity. This resulted in an improved ratio of (–)-isoproterenol-stimulated and fluoride-stimulated activities.

(–)-Isoproterenol-stimulated adenylate cyclase activity was inhibited in homogenates prepared from carbamylcholine-treated slices. Basal and fluoride activities were unaffected. The (–)-isoproterenol inhibition was larger in hypotonically-washed membranes. The basal and fluoride activities in washed membranes were inhibited, albeit less than the (–)-isoproterenol-stimulated activities (table 1). Although the activities in the absence of cAMP and the PDE inhibitor in the assay system were lower, their absence did not affect the inhibition of (–)-isoproterenol-stimulated activity caused by carbamylcholine in the homogenate (table 2). Atropine (20 μM) added before carbamylcholine (35 μM) abolished the inhibition of adenylate cyclase in washed membranes (table 3). Membranes stored at –20°C in sucrose medium preserved their activity and the carbamylcholine inhibition (not shown). The addition of carbamylcholine to homogenization medium, Tris medium or assay system did not result in higher inhibition (not shown). When control membranes or membranes

Table 1
Adenylate cyclase inhibition in homogenates and washed membranes

	Inhibition of adenylate cyclase activity (%)	
	Homogenate	Washed membranes
Basal	−0.5 ± 4.1	21.0 ± 3.3
(–)-Isoproterenol 20 μM	28.0 ± 4.9	50.2 ± 3.4
Fluoride 10 mM	−0.2 ± 7.1	27.7 ± 6.5

Parotid slices were incubated as in section 2 with and without carbamylcholine (50 μM, 18 min). Homogenates and washed membranes were prepared as in section 2. Reaction time was 1–3 min in homogenates and 10 min in washed membranes. The results are mean ± SEM of 6 individual experiments. (–)-Isoproterenol-stimulated and fluoride-stimulated activities were calculated as net increases after subtraction of basal activities

Table 2
Cholinergic inhibition of adenylate cyclase in homogenates in the presence and the absence of cAMP and PDE inhibitor (pmol cAMP/mg protein × min)

Additions	Control		Carbamylcholine	
	None	cAMP RO 20-1724	None	cAMP RO 20-1724
Basal	3.1	5.4	1.6	5.3
(−)-Isoproterenol 20 μM	10.8	29.6	8.3	22.1
Fluoride 10 mM	8.6	21.2	8.5	21.1

Homogenates of control and carbamylcholine-treated parotid slices (50 μM, 18 min) were prepared as in section 2. Homogenates were assayed for adenylate cyclase activity with and without cAMP (1 mM) and RO 20-1724/001 (0.1 mM) for 1.5 min. Basal activities were subtracted from (−)-isoproterenol-stimulated and fluoride-stimulated activities

derived from slices pre-exposed to carbamylcholine were challenged with increasing concentrations of (−)-isoproterenol the resulting dose–response curves were similar in shape with lower V_{max} for the carbamylcholine-treated membranes (fig.2) and indicated either negative cooperativity or two different populations of adenylate cyclase–receptor systems (fig.2, inset).

Table 3
Muscarinic blocking of cholinergic inhibition of adenylate cyclase

	Control	Carbamylcholine	Carbamylcholine + atropine
Basal	55.5	41.8	50.4
(−)-Isoproterenol 20 μM	229.1	98.4	193.4
Fluoride 10 mM	319.6	188.6	295.4

Parotid slices were incubated for 19 min in KRB. Atropine (20 μM) was added to one system at zero time followed 1 min later by carbamylcholine (35 μM). Washed membranes were prepared as in section 2 and assayed for adenylate cyclase activity for 10 min. Basal activities were subtracted from (−)-isoproterenol-stimulated and fluoride-stimulated activities

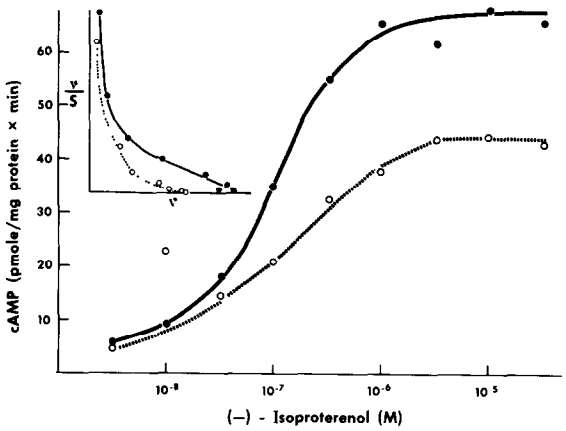

Fig.2. Dose response to (−)-isoproterenol in washed membranes. Parotid slices were treated with carbamylcholine (50 μM) for 18 min. The incubation was terminated by pelleting the tissue and aspirating the medium. Washed membranes were prepared as in section 2. The membrane protein (0.16 mg) was incubated in the adenylate cyclase assay system for 10 min. Basal activities in the presence of 10 μM propranolol were subtracted from (−)-isoproterenol-stimulated activities (30.9 pmol/mg × min for control membranes, 22.2 pmol/mg × min for carbamylcholine-treated membranes). (●——●) Control; (○---○) carbamylcholine.

4. Discussion

The rat parotid provides a model system for the interaction between different neurotransmitter receptors in the same cell. Until recently those interactions could be analyzed by indirect evidence derived from the modification of cell physiology or changes in concentrations of various compounds in the whole cell (e.g., cAMP concentration changes in rat parotid). Obviously, this precludes a direct approach to the elucidation of the molecular mechanisms involved.

We have recently reported that α-adrenergic and cholinergic-muscarinic stimulation modulate cAMP concentrations in the rat parotid [16,17]. Indirect evidence obtained from tissue-slice experiments suggest the adenylate cyclase as the target enzyme. The present report supports this conclusion in the case of the cholinergic-muscarinic receptor.

Tissue slices exposed to carbamylcholine exhibited inhibition of adenylate cyclase activity in homogenates and washed membranes. The inhibition was

unaffected by the omission of cAMP and PDE inhibitor from the assay system. Moreover, basal and fluoride-stimulated activities were inhibited to a smaller extent than the (−)-isoproterenol-stimulated activity. All of these eliminate the PDE as the target enzyme. It is possible that the exposure of parotid slices to carbamylcholine affects the stability of adenylate cyclase in addition to the inhibition of the (−)-isoproterenol-stimulated activity. The inhibition is expressed in washed membrane preparations either due to the effects of the isolation procedure, or due to the removal of a protecting agent(s) present in the homogenate. We are currently investigating these possibilities.

Dose–response curves to (−)-isoproterenol indicate that similar concentrations of (−)-isoproterenol will produce half-maximal stimulation in both control and carbamylcholine-treated membranes. The dose–response curves were non-hyperbolic and indicated negative cooperativity or, alternatively, two subpopulations of the receptor-linked adenylate cyclase. We have recently reported a similar phenomenon in the stimulation of cAMP concentrations in response to (−)-isoproterenol in parotid slices [17]. The inhibition appeared to be cholinergic-muscarinic, since the addition of atropine to the slices abolished the effect of carbamylcholine. The cholinergic inhibition was stable to both purification procedures and storage at −20°C over an extended period of time.

The development of a system exhibiting a stable inhibition of a relatively well-characterized enzyme opens the way for a systematic study of the molecular mechanisms involved. A cholinergic inhibition of cardiac adenylate cyclase was reported in 1962 [20]. A cholinergic-muscarinic inhibition of adenylate cyclase in cardiac sarcolemma has recently been reported [21]. It appears that the guanine-nucleotide regulatory site is affected in their preparation. We are currently investigating the influence of cholinergic stimulation on the kinetic and binding properties of the β-adrenergic receptor in rat parotid.

Acknowledgements

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