Cholinergic regulation of amylase gene expression in the rat parotid gland

Inhibition by two distinct post-transcriptional mechanisms

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Stimulation of the β -adrenergic or cholinergic muscarinic receptors are the principal mechanisms by which parotid salivary secretion is regulated *in vivo*. In this study we have examined the effects of cholinergic stimulation on amylase gene expression in dispersed rat parotid cells. [3 H]Leucine incorporation into amylase and total protein was inhibited by carbamylcholine. Within 5 min of its addition, 10 μ M carbamylcholine induced a 50–60% reduction in the rate of amylase synthesis which was sustained for more than 2 h. Blockade of the muscarinic receptor with atropine 8 min after addition of 10 μ M carbamylcholine reversed the carbamylcholine-induced inhibition of amylase synthesis. When cells were exposed to carbamylcholine for 2 h before addition of atropine, there was only a slight reversal of inhibition. Carbamylcholine had no significant effect on the rate of total RNA synthesis but caused a progressive loss of amylase

mRNA. After 2 h, amylase mRNA in cells treated with 10 μ M carbamylcholine was 46% of control levels. Actinomycin D (5 μ g/ml) lowered amylase mRNA by 8%; cycloheximide and phorbol 12-myristate 13-acetate had no effect. Isoprenaline (isoproterenol; at a concentration of 10 μ M), which is an inducer of amylase gene transcription, elevated the amylase mRNA content by 30% after 2 h. The calcium ionophore A23187 mimicked the effect of carbamylcholine by inhibiting [3 H]leucine incorporation into amylase and lowering amylase mRNA content. The results suggest that acute stimulation of the muscarinic cholinergic receptor inhibits amylase biosynthesis in parotid cells not only by rapid attenuation of translation but also by causing a gradual loss of amylase mRNA, apparently by a Ca^{2+} -dependent destabilization of the mRNA.

INTRODUCTION

Amylase is the most abundant protein present in rat parotid glands, accounting for up to 30% of the total protein synthesized by this gland [1]. It is secreted together with other salivary proteins in response to a variety of neurotransmitters including noradrenaline, acetylcholine, substance P and vasoactive intestinal peptide [2–4]. Extensive studies on the participation of the different receptor types in regulating parotid secretion have shown that stimulation of the β -adrenergic receptor represents the most efficient mechanism for inducing amylase or protein secretion [2,3]. However, substantial secretion of amylase together with electrolytes can also be induced by activation of the muscarinic cholinergic receptor [4–6]. Agonist binding to this receptor is coupled to the hydrolysis of phosphatidylinositol 4,5-bisphosphate and results in the activation of protein kinase C as well as the elevation of cytosolic free Ca^{2+} . Both arms of this signalling pathway are believed to play important roles in the signal transduction processes that link muscarinic receptor activation to salivary secretion [6,7]. The regulation of secretory protein biosynthesis by the different receptors present on the parotid cell membrane and the mechanisms that control the expression of genes encoding parotid secretory proteins have not been well characterized. Early investigations conducted *in vivo* showed that secretory protein synthesis in the rat parotid gland was maximally elevated 4–6 h after acute stimulation with the β -adrenergic agonist isoprenaline [8]. Grand and Gross [9,10], however, reported a more immediate stimulation of amylase and total protein synthesis in parotid slices incubated *in vitro* with adrenaline and proposed that the increase in amylase biosynthesis occurs through a cyclic AMP-dependent activation of the translational process. In more recent studies [11], we observed that activation of the β -adrenergic receptor results in rapid stimulation of RNA synthesis in parotid cells and a 2–3-fold increase in amylase mRNA content. Taken together, these studies dem-

onstrate that the β -adrenergic receptor regulates both amylase gene transcription as well as translation of the mRNA. This concerted regulation of both transcription as well as translation provides a mechanism by which amylase secretion in response to β -adrenergic receptor activation may be co-ordinated with its synthesis.

Little is known about the role of the muscarinic cholinergic receptor in regulating amylase gene expression. The rate of protein synthesis in parotid glands stimulated *in vivo* with pilocarpine has been reported to be elevated 0.5 to 2 h after stimulation [12]. *In vitro*, however, acute stimulation of parotid slices with cholinergic or α -adrenergic agonists inhibits amylase and total protein synthesis [13,14]. The molecular mechanism by which these Ca^{2+} -mobilizing agonists inhibit translation is not known but it has been suggested that Ca^{2+} -dependent phosphorylation of eEF-2 may mediate the response [15]. For several other cell types, however, inhibition of protein synthesis induced by Ca^{2+} mobilization is thought to result from depletion of the endoplasmic reticular Ca^{2+} pool as well as phosphorylation of eIF-2 [16–18]. In view of the physiological importance of the cholinergic pathway in regulating parotid salivary secretion, we embarked on a study aimed at determining the role of this receptor in regulating the transcription and translation of the parotid amylase gene. Our results suggest that acute stimulation with carbamylcholine not only induces a rapid inhibition of translation, but also results in destabilization and loss of cellular amylase mRNA.

MATERIALS AND METHODS

Materials

[3 H]Uridine, [γ - 32 P]ATP, [α - 32 P]dCTP, a random primer (Mega-primer) labelling kit and a 5'-end labelling kit were obtained from Amersham International (Amersham, U.K.). The pancreatic amylase cDNA [19] was kindly donated by Dr. Raymond

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J. MacDonald, Southwest Medical Center, University of Texas at Dallas, Dallas, U.S.A. A 24-nucleotide oligomer complementary to 18S rRNA was synthesized by Oswel DNA Service, Edinburgh, U.K. Collagenase (type IV), trypsin (type II), trypsin inhibitor (type II-S) and other biochemical compounds were from Sigma Chemical Co. Reagents used for molecular biology were obtained from GIBCO BRL.

Preparation of parotid cells

Parotid glands were obtained from male Wistar rats (180–200 g) which had been starved overnight and dispersed acinar cells prepared according to the method of Kanagasuntheram and Randle [20], except that the trypsin and collagenase concentrations were reduced to 660 units/ml and 290 units/ml respectively. The dissociated cells were resuspended in bicarbonate-buffered medium [20] supplemented with 5 mM glucose, 5 mM β -hydroxybutyrate, 2% (w/v) BSA and a minimal essential medium (MEM) amino acid supplement and routinely stabilized by preincubation for 1 h at 37 °C before initiating any experiments. The acinar cells obtained by this procedure retained their ability to respond to cholinergic and adrenergic stimuli, as assessed by their secretory response. Parotid lobules isolated and incubated as described previously [11] were used in some preliminary experiments as indicated in the text.

RNA isolation and estimation

Parotid cells or lobules were pelleted by centrifugation for 10 s at 2000 *g* and the pellets lysed in 5 M guanidine isothiocyanate, 20 mM Tris/HCl, pH 7.4, 10 mM EDTA, 1% (w/v) sarcosine and 5% (v/v) of 2-mercaptoethanol. Total RNA was isolated after phenol–chloroform extraction [21] and estimated by A_{260} . Accurate determination of RNA concentration was carried out by the orcinol method [22].

Northern- and slot-blot analysis

Northern-blot analysis of RNA was carried out essentially as described by Sambrook et al. [23]. Approximately 1–2 μ g of formamide-treated RNA samples were resolved on a 1.2% denaturing agarose gel and transferred to nitrocellulose membranes by capillary transfer. The membranes were baked, prehybridized and then hybridized at 42 °C for 16 h with a 1179 bp pancreatic cDNA probe [19]. The cDNA was labelled with [α - 32 P]dCTP using a Megaprimer labelling kit. Amylase mRNA was determined by autoradiography and densitometry using a LKB Ultrascan laser densitometer. 18S rRNA, which was used as an internal standard, was quantified by hybridization to a 24 bp oligonucleotide fragment complementary to bases 1047 to 1070 [24]. The membranes were stripped by washing twice in $0.1 \times$ SSC (SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0, 0.1% SDS) at 98 °C for 5 min, prehybridized and then hybridized at 42 °C for 4 h with the oligonucleotide end-labelled with [γ - 32 P]ATP to a specific activity of about 1×10^8 c.p.m./ μ g. The amylase mRNA of all samples was normalized against 18S rRNA.

For slot-blot analysis, exactly 1 or 2 μ g of denatured RNA samples were applied to nitrocellulose membranes using a Bio-Rad slot-blot manifold, fixed and hybridized [11] with the pancreatic amylase cDNA probe. Amylase mRNA was quantified by autoradiography and densitometry.

[3 H]Uridine incorporation

The rate of total RNA synthesis was determined by incubating

parotid lobules for 30 min in medium containing 10 μ M [3 H]uridine and measuring the incorporation of radioactivity into the trichloroacetic acid-insoluble fraction [11].

[3 H]Leucine incorporation into amylase and total protein

Parotid cells were pulse-labelled for 5 min in supplemented bicarbonate-buffered medium containing 10 μ M [3 H]leucine (2 μ Ci/ml) and then chase incubated for another 25 min in medium containing 1 mM leucine. The cells were pelleted by centrifugation at 13000 *g* for 10 s, lysed in unlabelled 1 mM leucine, and separate aliquots of the cell lysate were used for the parallel determination of [3 H]leucine incorporation into amylase and total protein. Radioactivity incorporated into amylase was determined by the glycogen precipitation method of Schramm and Loyter [25]. About 93–95% of the total amylase present in lysed cell homogenates was precipitated by this procedure in an essentially pure form. The overall rate of [3 H]leucine incorporation into parotid proteins (total protein synthesis) was quantified after precipitation of proteins from cell lysates with ice-cold 5% (w/v) trichloroacetic acid [14]. Less than 4% of the newly synthesized protein was recovered from the incubation medium after 30 min of incubation in either control or carbamylcholine-stimulated cells. All determinations were done in duplicate and the experiments repeated a minimum of three times.

Other assays

DNA was assayed according to the method of Burton [26].

RESULTS

The rates of amylase and total protein synthesis in dispersed parotid cells were determined after incubation of cells in medium supplemented with 10 μ M [3 H]leucine. Stable rates of [3 H]leucine incorporation were usually achieved after 1 h of preincubation and maintained for at least another 3 h. The average rate of [3 H]leucine incorporation into amylase and total protein were 0.20 ± 0.02 nmol/min per mg of DNA and 0.76 ± 0.04 nmol/min per mg of DNA respectively (means $\pm$ S.E.M., $n = 15$). For different cell preparations, incorporation into amylase accounted for 15 to 33% of total protein synthesis corroborating earlier findings [1,14] that amylase is the predominant protein synthesized by parotid acinar cells. Addition of 10 μ M carbamylcholine caused a rapid and sustained inhibition of amino acid incorporation into amylase (Figure 1). A 50–60% decrease in the rate of amylase synthesis was observed within 2 min of addition of carbamylcholine with slight recovery of translational activity occurring after more prolonged incubation. The rates of amylase synthesis, however, remained depressed by about 40–50% even after 3 h of incubation (results not shown). In order to determine the specificity of this response to carbamylcholine, we also made parallel measurements of total protein synthesis. As shown in Figure 1, carbamylcholine also decreased [3 H]leucine incorporation into total protein. The magnitude of the inhibition, as well as the temporal changes, was similar to that seen for amylase.

Agonist binding to the muscarinic receptor activates a bifurcating signalling pathway resulting in the elevation of cytosolic free Ca^{2+} and activation of protein kinase C. The participation of these signalling pathways in mediating the effects of carbamylcholine on amylase biosynthesis was studied using pharmacologically active compounds that modulate the activity of the two pathways. During a 30 min incubation, addition of 10 μ M A23187, a Ca^{2+} ionophore, inhibited [3 H]leucine in-

Figure 1 Time course of the effect of carbamylcholine on $[^3\text{H}]$ leucine incorporation into amylase and total protein

Parotid cells were pulse-labelled for 5 min with $10\ \mu\text{M}$ of $[^3\text{H}]$ leucine at various times after addition of $10\ \mu\text{M}$ carbamylcholine. The cells were then chase incubated for a further 25 min in medium containing 1 mM leucine and radioactivity incorporated into amylase and total cellular proteins determined as described in the Materials and methods section. For the first time point (2 min), pulse labelling was limited to 2 min and the chase incubation continued for 28 min. $[^3\text{H}]$ Leucine incorporated into amylase (○) or total protein (●) in carbamylcholine-stimulated cells was expressed as a percentage of the incorporation into control cells. Results shown are the means $\pm$ S.E.M. of at least three separate experiments.

corporation into amylase and total protein by $53.8 \pm 5.4\%$ and $50.7 \pm 4.3\%$ respectively (means $\pm$ S.E.M., $n = 3$); however, addition of $100\ \text{nM}$ phorbol 12-myristate 13-acetate, an activator of protein kinase C, stimulated leucine incorporation into amylase and total protein by $25.2 \pm 5.5\%$ and $27.4 \pm 4.8\%$ respectively (means $\pm$ S.E.M., $n = 5$).

The ability of atropine, a muscarinic receptor antagonist, to reverse the inhibition of amylase synthesis caused by carbamylcholine is shown in Table 1. Blockade of the receptor 8 min after addition of carbamylcholine, rapidly restored the rates of amylase synthesis to values similar to those seen in control cells. If, however, the parotid cells were continuously stimulated with carbamylcholine for 2 h before addition of atropine, there was only a slight reversal of agonist-induced inhibition. Total protein synthesis also displayed similar sensitivity to reversal of inhibition by atropine, except that at 2 h there was slightly greater recovery of activity than that obtained for amylase alone.

The inability of atropine to completely reverse the effects of carbamylcholine after 2 h suggested that different regulatory mechanisms may operate in modulating the biosynthesis of amylase and other cellular proteins in parotid cells exposed to carbamylcholine depending on the duration of exposure to the agonist. In general, the biosynthesis of cellular proteins may be influenced not only by the rate of translation but also by mechanisms controlling gene transcription, post-transcriptional processing and stabilization of the gene transcript. Two different approaches were therefore adopted to establish that the initial rapid and reversible attenuation of amylase and total protein synthesis is most likely a consequence of translational rather than transcriptional regulation. First, leucine incorporation into amylase and total protein was determined in the presence of $5\ \mu\text{g/ml}$ of actinomycin D, an inhibitor of gene transcription. Previous studies have shown that at this concentration, actino-

Table 1 Effect of atropine on carbamylcholine-induced inhibition of $[^3\text{H}]$ leucine incorporation into amylase and total protein

Parotid cells were incubated with $10\ \mu\text{M}$ carbamylcholine for exactly 10 min (A) or 2 h (B) before being pulse labelled with $10\ \mu\text{M}$ $[^3\text{H}]$ leucine ($2\ \mu\text{Ci/ml}$) for 5 min. Incubation was then continued for a further 25 min in the presence of $10\ \mu\text{M}$ carbamylcholine and 1 mM of leucine. Radioactivity incorporated into amylase and total protein was determined and expressed as a percentage of corresponding control values. Atropine, when present, was added exactly 2 min prior to addition of radioactive leucine. Results shown are the means $\pm$ S.E.M. of three experiments.

	$[^3\text{H}]$ Leucine incorporation (% of control)	
	Amylase	Total protein
(A) (10 min)		
Carbamylcholine ($10\ \mu\text{M}$)	40.2 ± 1.2	38.6 ± 2.6
Carbamylcholine ($10\ \mu\text{M}$) + atropine ($10\ \mu\text{M}$)	91.2 ± 3.6	83.8 ± 5.3
Atropine ($10\ \mu\text{M}$)	99.3 ± 1.7	98.5 ± 1.2
(B) (2 h)		
Carbamylcholine ($10\ \mu\text{M}$)	43.9 ± 3.6	39.0 ± 3.9
Carbamylcholine ($10\ \mu\text{M}$) + atropine ($10\ \mu\text{M}$)	49.5 ± 2.7	53.1 ± 1.0
Atropine ($10\ \mu\text{M}$)	97.8 ± 2.9	93.8 ± 0.5

Table 2 $[^3\text{H}]$ Uridine incorporation into total RNA in parotid lobules stimulated with carbamylcholine or isoprenaline

Parotid lobules were incubated in supplemented Hanks' medium containing additions as indicated. $0.4\ \mu\text{M}$ $[^3\text{H}]$ uridine ($2\ \mu\text{Ci/ml}$) was added 0 and 1 h after addition of agonists and the radioactivity incorporated into total RNA determined after 1 h. Results were expressed as a percentage of control values and are the means $\pm$ S.E.M. of three experiments. * $P < 0.001$ for difference from control.

	$[^3\text{H}]$ Uridine incorporation (% of control)	
	1st h	2nd h
Carbamylcholine ($10\ \mu\text{M}$)	$119 \pm 10^*$	99 ± 6
Isoprenaline ($10\ \mu\text{M}$)	200 ± 11	$248 \pm 18^*$

mycin D lowers the rate of RNA synthesis in parotid cells by more than 92% [11]. When cells were preincubated with actinomycin D for 1 h before determination of amino acid incorporation, $[^3\text{H}]$ leucine incorporation into amylase and total protein was decreased by 18% and 24% respectively (means of three determinations). In addition, we also determined the effect of carbamylcholine on the rate of RNA synthesis since gross changes in the rate of transcription of the amylase gene or indeed any gene coding for a major parotid protein would result in measurable changes in total RNA synthesis. As shown in Table 2, there was no significant difference in the rate of RNA synthesis in cells exposed to $10\ \mu\text{M}$ carbamylcholine for up to 2 h. Activation of the β -adrenergic receptor with $10\ \mu\text{M}$ isoprenaline, however, caused a marked increase in $[^3\text{H}]$ uridine incorporation into RNA. During the first and second hour of incubation, RNA synthesis in the isoprenaline-stimulated cells proceeded at rates that were 2 to 2.5 times those of control cells.

Cholinergic regulation of amylase mRNA levels in parotid acinar cells was initially studied by dot-blot analysis of total RNA. Over a 4 h period, incubation with $10\ \mu\text{M}$ carbamylcholine resulted in a sharp decline in amylase mRNA relative to

Figure 2 Time course of the carbamylcholine-induced decline in amylase mRNA levels

Total RNA was isolated from parotid lobules which had been incubated with 10 μ M carbamylcholine for the times indicated. Exactly 1 or 2 μ g of the RNA was transferred on to nitrocellulose membranes using a slot-blot manifold and amylase mRNA quantified after hybridization to a 32 P-labelled pancreatic amylase cDNA probe. Results were expressed as a percentage of paired controls and are the means $\pm$ S.E.M. of six experiments.

Table 3 Amylase mRNA content of parotid cells treated with carbamylcholine, isoprenaline, actinomycin D and cycloheximide

Parotid cells were incubated for 2 h in medium containing additions as indicated. Total RNA was extracted and about 1 to 2 μ g was fractionated on a formaldehyde-agarose gel. After hybridization, amylase mRNA was quantified by densitometry and standardized against 18S ribosomal RNA. Results are expressed as a percentage of paired controls and are the means $\pm$ S.E.M. of the number of determinations shown in parentheses. * $P < 0.01$ for difference from paired control.

Additions	Amylase mRNA content (% of control)
Carbamylcholine (10 μ M)	45.3 $\pm$ 8.9* (5)
Isoprenaline (10 μ M)	130.1 $\pm$ 14.7 (5)
Actinomycin D (5 μ g/ml)	91.7 $\pm$ 20.3 (5)
Cycloheximide (10 μ g/ml)	100.8 $\pm$ 20.0 (4)

total RNA (Figure 2). A decrease of about 15% was detectable after 1.5 h. This was followed by a more rapid rate of decline to about 45% of control levels after 2.5 h. Further incubation for up to 4 h did not significantly increase the loss of amylase mRNA. Northern-blot analysis of amylase mRNA using 18S ribosomal RNA as an internal control was used to characterize further the changes induced by carbamylcholine. As shown in Table 3, 10 μ M carbamylcholine caused a 54% drop in amylase

Table 4 Effect of staurosporine, phorbol 12-myristate 13-acetate and A23187 on amylase mRNA content of parotid cells

Amylase mRNA content of cells incubated for 2 h in medium containing additions as indicated was determined as described in the legend to Table 3. Results are expressed as percentages of paired controls and are the means $\pm$ S.E.M. of six determinations. * $P < 0.01$ for difference from control.

Additions	Amylase mRNA content (% of control)
Staurosporine (50 nM)	103.0 $\pm$ 11.0
Carbamylcholine (10 μ M)	49.9 $\pm$ 5.2*
Carbamylcholine (10 μ M) + staurosporine (50 nM)	40.4 $\pm$ 15.3*
Phorbol 12-myristate 13-acetate (100 nM)	104.7 $\pm$ 14.7
A23187 (1 μ M)	45.5 $\pm$ 14.0*

mRNA relative to the 18S RNA after 2 h. During the same period, amylase mRNA of cells treated with 10 μ M isoprenaline increased by 30%. Neither carbamylcholine nor isoprenaline caused any detectable change in the molecular size of the amylase mRNA. To determine whether the decline in the amylase mRNA was due to accelerated decay of existing RNA, we compared the amylase mRNA content with that of cells in which transcription was arrested with actinomycin D. After a 2 h incubation with actinomycin D the amylase mRNA content decreased by about 8%. Inhibition of protein synthesis with 10 μ g/ml of cycloheximide, which decreased [3 H]leucine incorporation into total protein by 80 $\pm$ 7% (mean $\pm$ S.E.M., $n = 3$), had no effect on the mRNA level.

The importance of protein kinase C and Ca^{2+} ions in regulating amylase mRNA levels was also evaluated. As shown in Table 4, the amylase mRNA content of parotid cells, in which protein kinase C activity was stimulated with phorbol 12-myristate 13-acetate or inhibited with staurosporine, were similar to those of control cells. Staurosporine also failed to reverse the decline in amylase mRNA caused by carbamylcholine. Amylase mRNA in parotid cells incubated for 2 h in medium containing 10 μ M A23187 was, however, 55% lower than that of controls.

DISCUSSION

We have previously demonstrated that Ca^{2+} -mobilizing agonists such as adrenaline and carbamylcholine inhibit protein synthesis in the rat parotid gland [14]. The inhibition could not be correlated with changes in cellular ATP content or amino acid pool size, indicating that depletion of cellular ATP or inhibition of amino acid transport are therefore unlikely to be important in mediating the agonist-induced inhibition. By immunoprecipitating the newly synthesized protein, McPherson and Hales [13] identified amylase as one of the principal proteins which is synthesized at substantially reduced rates in carbamylcholine- or adrenaline-treated cells. In the present study, we have further characterized this phenomenon, focusing on cholinergic regulation of amylase biosynthesis and amylase mRNA levels. Our results confirm the earlier reports that acute stimulation of parotid cells with carbamylcholine results in a rapid attenuation of the rate of amylase biosynthesis. Moreover, our observations that the changes in amylase biosynthesis are paralleled by similar changes in total protein synthesis under the various conditions studied suggest that the regulatory mechanisms involved are not unique to amylase alone and are likely to modulate the bio-

synthesis of all, or at least a majority, of the newly synthesized parotid proteins. In the rat submandibular gland, Takuma et al. [27] noted that adrenaline specifically inhibited the biosynthesis of only four major proteins, and that most other cellular proteins appeared to be resistant to inhibition by this agonist. Secretory proteins constitute up to 90 % of the total protein synthesized by the rat parotid gland [1]. Concerted inhibition of amino acid incorporation into these proteins alone could therefore account for our inability to detect differential regulation of the biosynthesis of amylase and other parotid proteins. Further studies defining the effects of cholinergic receptor activation on amino acid incorporation into several different parotid proteins, including non-secretory proteins, will provide a clearer understanding of the nature and specificity of the mechanisms that mediate this response.

The data presented in this report are consistent with the proposal that agonist binding to the muscarinic receptor regulates amylase biosynthesis by two distinct post-transcriptional mechanisms. In the short-term, the decline in the rates of amylase and total protein synthesis probably reflects rapid modulation of the translational process. Thus, inhibition of amino acid incorporation occurred almost immediately upon stimulation of the cholinergic receptor, with near maximal inhibition of about 60 % being achieved in less than 5 min. The response was also readily reversed if a muscarinic receptor antagonist such as atropine was added within 10 min of carbamylcholine. This rapid onset and reversal of inhibition argue against the possibility that gross changes in cellular mRNA levels are involved. Moreover, there was no evidence that carbamylcholine causes significant repression of gene transcription in the parotid cell since [³H]uridine incorporation into cellular RNA was unaffected by this agonist. Activation of the β -adrenergic receptor, which has been shown to elevate amylase mRNA levels and stimulate the synthesis of amylase and other secretory proteins [11,28], however, resulted in a 2–2.5-fold increase in RNA synthesis. Almost total repression of gene transcription with actinomycin D also caused only a slight decline in the rate of amylase biosynthesis even after 1 h. It is therefore very likely that modulation of the translational process rather than transcriptional regulation is the primary mechanism mediating the rapid and substantial inhibition of amylase biosynthesis in carbamylcholine-treated parotid cells.

Numerous studies have established that both protein kinase C and Ca^{2+} ions are key elements regulating protein synthesis in eukaryotic cells. Protein kinase C phosphorylates several translational factors including the mRNA specific factors such as eIF-4B and eIF-4F and activation of protein kinase C with phorbol esters stimulates protein synthesis [16,29]. In contrast, a vast body of evidence shows that Ca^{2+} mobilization generally results in inhibition of translation in a variety of tissues [16,17] including the rat parotid [14]. Our data demonstrating that A23187 but not phorbol 12-myristate 13-acetate causes inhibition of amino acid incorporation into amylase as well as other parotid proteins are consistent with these earlier findings and suggest that the carbamylcholine-induced inhibition of amylase and total protein synthesis are consequences of receptor-mediated Ca^{2+} mobilization. In many cell types, phosphorylation of elongation factor eEF-2 by a calcium-calmodulin-dependent protein kinase III results in inhibition of protein synthesis [29,30]. Increased phosphorylation of eEF-2 has also been observed in rat parotid cells stimulated with Ca^{2+} -mobilizing agonists such as carbamylcholine, substance P, ATP or A23187 [15]. Hincke and Nairn have therefore suggested that Ca^{2+} -dependent phosphorylation of this factor may be important in mediating Ca^{2+} -dependent inhibition of protein synthesis in parotid cells. In an alternative

model Brostrom and Brostrom have proposed that mobilization of endoplasmic reticular Ca^{2+} rather than elevation of cytosolic Ca^{2+} is the key event triggering translational inhibition in eukaryotic cells [16,31]. These workers have provided convincing evidence that protein synthesis is inhibited not only by agonists that activate the phosphoinositide pathway and cause Ca^{2+} mobilization but also by agents that deplete the endoplasmic reticular Ca^{2+} pool such as EGTA or thapsigargin [16,31,32]. The inhibition has also been correlated with decreased rates of translational initiation and inactivation of eEF-2B by phosphorylated eIF-2 α [16–18,31]. Although the relative importance of eEF-2 and eIF-2 phosphorylation in regulating the biosynthesis of amylase and other proteins in the parotid gland remains to be defined, it is clear that both regulatory mechanisms are likely to influence the rate of global protein synthesis rather than amylase biosynthesis alone.

After prolonged incubation with carbamylcholine for 2 or 3 h, parotid cells displayed a slight recovery of translational activity. This recovery was inhibited in the presence of actinomycin D (P. Thiagarajah and S. C. Lim, unpublished work), suggesting that *de novo* synthesis of proteins such as GRP78 [31,32], which enable cells to recover translational activity after prolonged Ca^{2+} deprivation, may have occurred. Surprisingly, blockade of the muscarinic receptor 2 h after addition of carbamylcholine failed to restore amylase biosynthetic rates to control levels. The quantum of inhibition of amylase biosynthesis that persisted in atropine-treated cells was matched by a similar decline in amylase mRNA content. These results taken together suggest that inhibition of amylase biosynthesis in cells stimulated with carbamylcholine for extended periods cannot be explained by translational inhibition alone and that loss of amylase mRNA is a predominant factor. Interestingly, total protein synthesis also failed to show substantial recovery upon atropine treatment after 2 h. We therefore speculate that a decline in the concentration of some other major mRNA species may also have occurred.

In contrast to its rapid regulation of translation, the decrease in amylase mRNA content induced by carbamylcholine was detectable only after 1 h and was most pronounced after 2 h. This decline in the mRNA level cannot be explained by attenuation of amylase gene transcription since inhibition of transcription with actinomycin D resulted in only an 8 % decrease in the mRNA content after 2 h. The loss of amylase mRNA under conditions where transcription is arrested is determined essentially by its half-life. Since the amylase mRNA of actinomycin D-treated cells exceeded that of carbamylcholine-stimulated cells, it is likely that prolonged cholinergic stimulation results in destabilization of amylase mRNA.

It has become increasingly evident in recent years that regulation of messenger RNA stability is a major control mechanism governing mRNA levels. Although a number of different factors have been implicated, the molecular processes involved are not yet fully understood. Several mRNA species, especially those with relatively short half-lives, are destabilized by the presence of instability conferring AU-rich sequences in the 3' untranslated region or by shortening of the poly(A) tail [33,34]. Others, including that coding for parotid galactosyl transferase [35], require ongoing protein synthesis for maintaining stability, presumably because certain labile proteins are essential for maintaining mRNA stability. Many mRNA species which are sensitive to degradation by ribosome-associated nucleases are, however, stabilized under conditions where translation is attenuated. None of these previously described mechanisms could, however, be identified as playing a major role in regulating the stability of amylase mRNA. Amylase mRNA in parotid cells subjected to cholinergic stimulation showed no detectable

difference in molecular size compared with that of controls, indicating that there is little or no loss of the poly(A) tail. Accelerated decay of the amylase mRNA also appeared to be catalysed by mechanisms that are independent of the translational process since almost total inhibition of protein synthesis with cycloheximide had no effect on the amylase mRNA. It is therefore unlikely that destabilization of amylase mRNA observed in cholinergically stimulated parotid cells is a consequence of agonist-induced attenuation of protein synthesis.

It is evident from our studies that Ca^{2+} but not protein kinase C exerts a significant influence on the processes that regulate the stability of amylase mRNA. Thus a substantial loss of amylase mRNA occurred in cells incubated with A23187 whereas modulation of protein kinase C activity with phorbol myristate acetate or staurosporine failed to induce any change in amylase mRNA content. Staurosporine also failed to reverse the loss of amylase mRNA caused by carbamylcholine. These observations are consistent with the view that destabilization of amylase mRNA in cholinergically stimulated parotid cells is essentially a Ca^{2+} -regulated process. Ca^{2+} ionophores such as A23187 or ionomycin have been shown to enhance the stability of certain mRNA species [36,37]. Accelerated decay of eukaryotic mRNA by a Ca^{2+} -regulated process has not, however, been previously reported and may represent a novel mechanism for receptor-mediated regulation of mRNA stability.

In summary, the results presented in this study demonstrate that acute stimulation of the muscarinic receptor of parotid acinar cells results in inhibition of amylase biosynthesis by two different post-transcriptional mechanisms, both of which are apparently Ca^{2+} -regulated. In addition to rapid inhibition of amylase and total protein synthesis by modulation of the rate of translation, receptor activation also results in a decline in amylase mRNA caused by destabilization of the mRNA. The physiological significance of this rapid and sustained attenuation of amylase synthesis is not clear, but they may serve as mechanisms enabling the acutely stimulated cell to divert its energy resources towards more essential functions such as salivary secretion.

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