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ENHANCEMENT OF CYCLIC AMP MODULATED SALIVARY AMYLASE SECRETION BY PROTEIN KINASE C ACTIVATORS

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Abstract—The effect of protein kinase C activators on isoproterenol-induced amylase secretion were investigated in isolated rat parotid cells. Pretreatment with phorbol dibutyrate potentiated isoproterenol-induced amylase secretion. This effect of phorbol dibutyrate was mimicked by dioctanoylglycerol or carbachol. Phorbol dibutyrate also potentiated secretion evoked by the adenylate cyclase activator forskolin and by dibutyryl cAMP. Neither phorbol dibutyrate nor carbachol enhanced isoproterenol-induced cAMP accumulation. The present study reveals a coordinate interaction between cAMP and protein kinase C at a step in the secretory mechanism distal to cAMP generation.

Cyclic AMP generated through beta-adrenoceptor action appears to be a critical modulator of amylase secretion by the rat parotid gland. By contrast, Ca^{2+}-mobilizing agonists that express their actions through muscarinic- and alpha-adrenergic receptors cause a predominance of water and electrolyte release [1]. Activation of this latter pathway takes place via stimulation of phospholipase C and results in the production of inositol 1,4,5-trisphosphate ($InsP_3$)† and diacylglycerol. $InsP_3$ mobilizes cellular Ca^{2+} which together with diacylglycerol activates phospholipid-dependent protein kinase (protein kinase C) to modulate physiological responses, including secretion [2, 3].

Experimentally, phorbol esters or synthetic diacylglycerol derivatives activate protein kinase C by decreasing cytosolic protein kinase C and increasing the level of enzyme associated with the particulate fraction [3]. A previous study conducted on rat parotid slices has shown that phorbol 12,13-dibutyrate (PDBu) modestly stimulates amylase release without activating adenylate cyclase or Ca^{2+} mobilization, thus suggesting a role for protein kinase C in salivary amylase secretion [4]. Moreover, the finding that activation of muscarinic receptors, but not beta adrenoceptors, in parotid lobules results in the redistribution of cytoplasmic protein kinase C to a particulate fraction supports an involvement of protein kinase C in salivary amylase secretion selectively elicited by Ca^{2+}-mobilizing agonists [5].

The present report is concerned with an analysis in rat parotid cells of the interactions between agents that raise tissue cAMP levels and those that activate protein kinase C. In this paper, we describe experiments demonstrating that protein kinase C and cAMP are synergistic cellular signals for salivary amylase secretion, interacting at a step distal to the generation of cAMP.

MATERIALS AND METHODS

Parotid cell preparation. Acinar cells were prepared from parotid glands of male Sprague–Dawley rats (150 g) by the method of Putney *et al.* [4]. The basic incubation medium had the following composition (mM): NaCl, 116; KCl, 5.4; $MgSO_4$, 0.8; $CaCl_2$, 1.8; NaH_2PO_4, 0.96; $NaHCO_3$, 25; beta-hydroxybutyrate, 5; plus 1% bovine serum albumin. All of the chemicals used to prepare the incubation medium were of analytic grade.

Enzyme and biochemical assays. For measurement of amylase release, cells were centrifuged through Nyosil oil in a microfuge at 12,000 *g*, and the supernatant fraction was analyzed for alpha amylase activity, according to the method of Bernfeld [6]. A cell aliquot (450 μl) treated with 0.2% Triton-X was also analyzed for total amylase content. Amylase activity was defined as milligrams of maltose formed, which averaged 163 ± 29 mg per mg cell protein. In each experiment, control samples were taken, from which basal secretion was determined and subtracted from drug-stimulated values (except for the data depicted in Fig. 1).

For measurement of tissue cAMP, cells were incubated for various intervals in the presence or absence of drugs, and the reaction was terminated by the addition of 600 μl of ice-cold EtOH. The cells were pelleted by rapid centrifugation in microfuge tubes, and the supernatant fraction was withdrawn. The pellet was washed with 1 ml EtOH:H_2O (2:1), and the supernatant fractions were combined and dried under nitrogen. The residue was resuspended in 50 mM Tris buffer containing 4 mM EDTA (pH 7.5), and cAMP was measured by a modification of a protein binding method [7], for which a commercial

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† Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; PDBu, phorbol 12,13-dibutyrate; and DMSO, dimethyl sulfoxide.

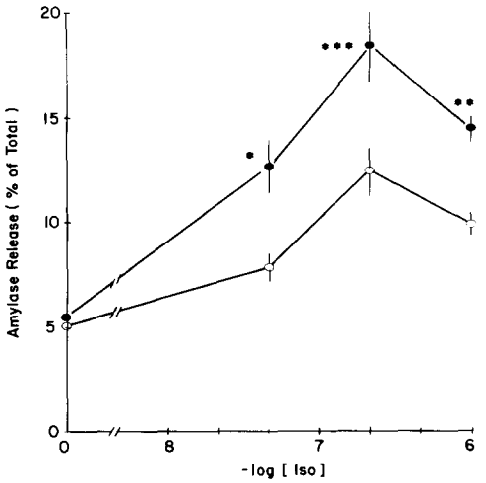

Fig. 1. Log concentration–response curve for isoproterenol on isolated parotid acinar cells and the effect of PDBu. Amylase release was determined in response to a 15-min exposure to various concentrations of isoproterenol in the presence (●) or absence (○) of 1 μM PDBu. The phorbol ester was added 10 min prior to isoproterenol. Each point represents the mean (±SE) for four to seven separate experiments. Key: (*) $P < 0.02$; (**) $P < 0.01$; and (***) $P < 0.001$ (Student's paired t-test).

kit was employed (Amersham Corp., Arlington Heights, IL). The concentration of cAMP in the experimental tubes was read against a standard curve for each assay and was expressed as picomoles per milligram of protein. The protein content of a representative sample from each experiment was determined [8], using bovine serum albumin as the standard.

Chemicals and drugs. Isoproterenol, carbachol, forskolin, dioctanoylglycerol, and the sodium salt of dibutyryl cAMP were obtained from the Sigma Chemical Co. (St. Louis, MO), while collagenase (CLSPA 0.28 units/mg) was purchased from Boehringer-Mannheim (Indianapolis, IN). Phorbol esters were obtained from LC Services (Woburn, MA). Dobutamine HCl was supplied by Lilly Research Laboratories (Indianapolis, IN). Dimethyl sulfoxide (DMSO, grade 1 Sigma), the vehicle for phorbol esters and dioctanoylglycerol, had no effect on amylase release. The maximal final concentration of DMSO used was 0.5%. All other reagents were the highest grade commercially available.

Statistical analyses. Values are expressed as means ± SE and the significance of the difference between means was determined using Student's paired t-test or two-way analysis of variance. $P < 0.05$ was considered significant; N is the number of experiments.

RESULTS

The secretory response to a range of isoproterenol concentrations is depicted in Fig. 1. Threshold and maximal responses were obtained with 50 and 200 nM isoproterenol respectively. Analysis of the time course indicated that the secretory response was still not complete at 15 min. In the absence of isoproterenol, mean basal secretion after 15 min averaged 4.9% of the total cell content (Fig. 1).

A previous study of rat parotid cells has shown that PDBu, even at relatively high concentrations, induces only a low level of salivary amylase release [4]. Our findings confirmed that exposure of parotid cells to 1 μM PDBu for 25 min elicited a very modest but significant increase in amylase secretion (Table 1). However, when parotid cells were pretreated with PDBu for 10 min prior to isoproterenol, amylase release elicited by the beta-adrenoceptor agonist was enhanced markedly (Fig. 1). Enhancement of secretion was observed even with maximum stimulatory concentrations of isoproterenol. Table 1 documents that the amount of amylase released by a combination of PDBu and isoproterenol was substantially greater than the sum of the release by the two agents given alone. Figure 2 shows that the enhancement of isoproterenol-evoked amylase was a function of the PDBu concentration. The concentration of PDBu that gave the maximum enhancement was approximately 200 nM, whereas the threshold concentration was 3 nM. These values coincide with the concentration range over which PDBu stimulates protein kinase C in a broken cell brain preparation [3]. PDBu alone did not raise mean basal secretion by more than 1% (data not shown), except at the highest concentration employed (1 μM) (Table 1). Thus, PDBu facilitated isoproterenol-evoked secretion even when basal release was not elevated by the phorbol ester.

The ability of PDBu to enhance isoproterenol-induced secretion was compared with that of 4-alpha-phorbol 12,13-dibutyrate, a phorbol ester that is inactive with respect to protein kinase C [9]. 4α-PDBu alone was unable to elevate secretion significantly above basal levels and failed to produce a significant change in isoproterenol-evoked amylase release (Table 1).

Since phorbol esters have actions on the cell in addition to the activation of protein kinase C [3, 10], the effect on amylase secretion of the synthetic diacylglycerol analogue dioctanoylglycerol was also examined. Dioctanoylglycerol (100 μM) alone elicited a modest release of amylase (Table 1); however, amylase secretion in the presence of dioctanoylglycerol and isoproterenol was greater than the additive effect of each stimulus alone (Table 1). The similar effects of PDBu and dioctanoylglycerol on isoproterenol-stimulated secretion suggest that they share a common mechanism of action.

Figure 3 shows that a threshold concentration of carbachol (1 μM) with regard to amylase secretion also caused a modest but significant augmentation in the secretory response to isoproterenol. This stimulatory action of carbachol on isoproterenol-induced amylase secretion was abolished in the presence of 1 μM propranolol, indicating that the effect of isoproterenol was mediated by beta adrenoceptors.

The rat parotid beta adrenoceptor has been characterized as belonging predominantly to the $beta_1$ subtype [11]. The ability of 1 μM carbachol to enhance the secretory response to beta-adrenoceptor stimulation was also demonstrable when the putative selective $beta_1$-agonist dobutamine was employed

Table 1. Effects of PDBu, 4α-PDBu, and dioctanoylglycerol (DOG) on isoproterenol-stimulated amylase release

Additions	Amylase release (% of total)		P*
	Control	Isoproterenol	
None	—	5.5 ± 1.5	<0.01
PDBu (1 μM)	2.5 ± 0.8	14.1 ± 2.9	<0.01
4α-PDBu (1 μM)	0.6 ± 0.3	7.4 ± 2.1	>0.05
DOG (100 μM)	0.7 ± 0.3	10.0 ± 1.9	<0.01

Results show amylase release from four to six different preparations. Parotid cells were exposed for 10 min to either phorbol ester or DOG, and then 200 nM isoproterenol or vehicle was added for an additional 15 min. Values are expressed as means ± SE. In each experiment basal release, which averaged 6.8 ± 0.5%, was subtracted from drug-stimulated release.

* P is the probability, calculated by Student's paired *t*-test, that isoproterenol-stimulated amylase release in the presence of phorbol ester or DOG was significantly different from that obtained in the presence of isoproterenol alone.

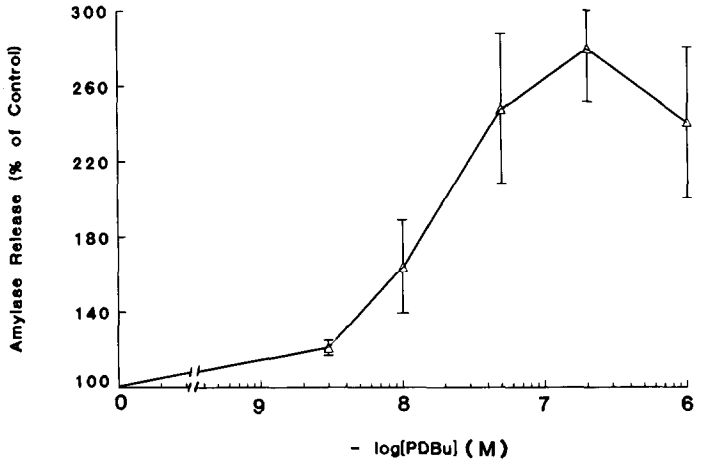

Fig. 2. Concentration-dependent enhancement of isoproterenol-evoked amylase secretion by PDBu. Acinar cells were pretreated for 10 min with the indicated concentrations of PDBu and then exposed to 100 nM isoproterenol for 15 min. Each point represents the mean ± SE of three to five separate experiments and is expressed as a percentage of the secretory response obtained with isoproterenol alone which averaged 5.0 ± 1.5% of the total amylase in the cell suspension. In each experiment, values for secretion elicited by the corresponding PDBu concentration (or vehicle) alone have been subtracted from values obtained for isoproterenol-stimulated release.

as a secretagogue. Dobutamine (10 μM) was less efficacious than isoproterenol as a secretory stimulus, elevating amylase secretion to 3.5% above basal levels after 15 min (N = 6). However, in the presence of 1 μM carbachol, the effect of dobutamine was enhanced by 262 ± 13% (N = 3). The lower efficacy of dobutamine in parotid cells may be attributed to an action as a partial agonist with relatively low intrinsic activity, which has been observed in other adrenergic preparations [12].

To provide further insight into the mechanism of action of PDBu, the effects of PDBu on amylase release induced by forskolin and dibutyryl cAMP were also examined. Figure 4 shows that the modest stimulatory action of 20 μM forskolin on amylase secretion was greatly enhanced by PDBu. This concentration of forskolin markedly augments cAMP levels in the rat parotid gland [13]. PDBu augmented forskolin-stimulated amylase by almost 4.5-fold. Dibutyryl cAMP increased amylase release to 2.1% above basal levels, and PDBu elevated dibutyryl cAMP-induced amylase release to 7.8%, resulting in an almost 4-fold elevation in secretion (Fig. 4). This potentiation of dibutyryl cAMP stimulated amylase by PDBu contrasts with the findings of Takuma and Ichida [14] who observed an additive effect of phorbol 12-myristate 13-acetate on salivary amylase release elicited by dibutyryl cAMP.

To determine whether alterations in cAMP levels would mediate the effects of PDBu and carbachol on amylase release, cells were incubated with isoproterenol alone or in the presence of PDBu for various intervals, and cAMP levels were measured. Isoproterenol produced a time-dependent increase in cAMP accumulation over a 15-min interval (Fig. 5). PDBu failed to elevate cAMP levels above basal

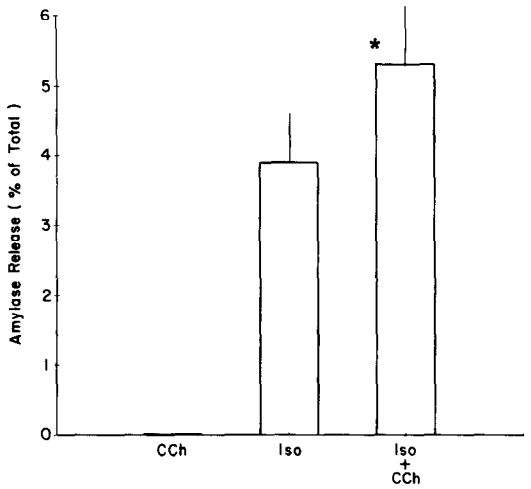

Fig. 3. Enhancement of isoproterenol-induced amylase release by carbachol. Cells were incubated in the presence or absence of 1 μM carbachol (CCh) and 200 nM isoproterenol (Iso) for 15 min. Each vertical bar represents a mean ± SE for three separate experiments. Basal values for amylase secretion have been subtracted. Key: (*) $P < 0.05$ (Student's paired t-test).

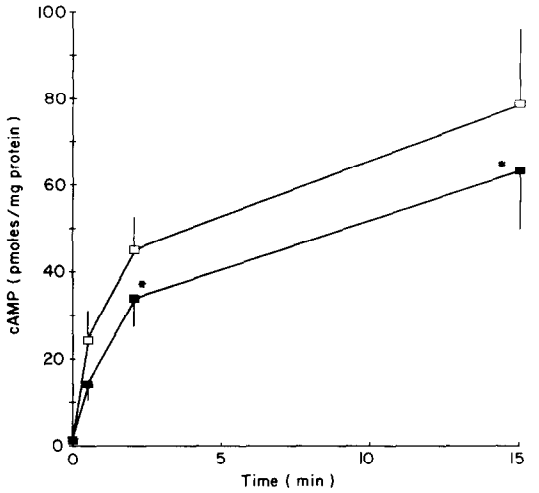

Fig. 5. Effect of PDBu on isoproterenol-stimulated cAMP levels. Parotid cells were preincubated in the presence (■) or absence (□) of 1 μM PDBu for 10 min and then exposed to 200 nM isoproterenol for various intervals. Each point represents a mean ± SE for three to four separate experiments. The amount of cAMP produced by untreated and PDBu-treated cells after 10 min averaged 1.7 (± 0.8) and 2.0 (± 0.6) pmol/mg protein respectively. Key: (*) $P < 0.01$ (Student's paired t-test).

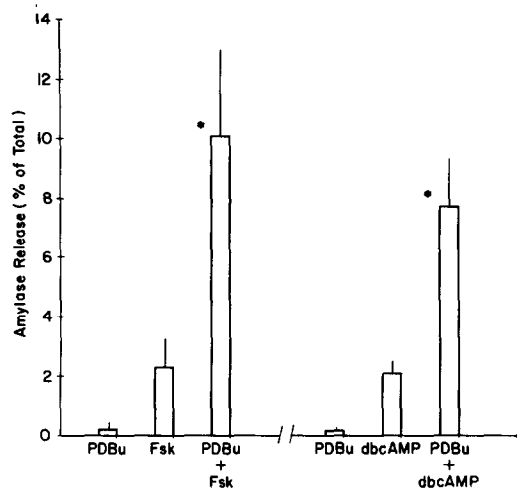

Fig. 4. Effect of PDBu on amylase release evoked by forskolin and dibutyryl cAMP. Cells were preincubated for 10 min in the presence or absence of 1 μM PDBu, and then 20 μM forskolin (FSK) or 2 mM dibutyryl cAMP (dbcAMP) was added for an additional 15 min. Basal values were subtracted from the data. Key: (*) $P < 0.05$ (Student's paired t-test) (mean ± SE, N = 4).

values, and the addition of PDBu in combination with isoproterenol reduced cAMP levels over the time course of the experiment (Fig. 5). This reduction was not significant when assessed by two-way analysis of variance, but the control and experimental values at both 2 and 15 min were significantly different when analyzed by Student's paired t-test.

The concentration of carbachol (1 μM) that enhanced the secretory response to isoproterenol (Fig. 3) failed to elicit a significant change in isoproterenol-induced cAMP accumulation. The amount of cAMP generated during a 2-min exposure to 200 nM isoproterenol in the presence of carbachol was 93 ± 7% of that observed in the absence of carbachol (N = 3). We were thus unable to confirm a carbachol-induced inhibition of isoproterenol-stimulated cAMP formation, previously reported in parotid slices [15].

DISCUSSION

In the present study, the phorbol ester PDBu caused a potentiation of isoproterenol-stimulated amylase secretion from isolated parotid cells. The effect of PDBu on isoproterenol-induced amylase release is attributed to its ability to activate protein kinase C, since the degree of enhancement was related to the PDBu concentration and inactive phorbol ester 4α-PDBu did not potentiate isoproterenol-induced amylase secretion. Furthermore, the stimulatory effect of PDBu was mimicked by the diacylglycerol analogue dioctanoylglycerol, which is capable of readily entering cells, activating protein kinase C, and enhancing secretion [16–19]. Taken together, these results reveal that optimal secretory activity of isoproterenol in rat parotid acinar cells is expressed when protein kinase C is activated. These findings, which portray protein kinase C and cAMP as synergistic cellular signals, are at odds with a recent report which would lead us to believe that activators of protein kinase C and cAMP-dependent protein kinase C stimulate salivary amylase release independently [14].

Further insight into the mechanism involved in this synergism was provided by the finding that the stimulatory action of PDBu was also demonstrable

when forskolin was employed to raise cellular cAMP levels. Forskolin bypasses the beta adrenoceptor and directly activates the catalytic subunit of adenylate cyclase [20]. These data indicate that the site of interaction between these two cellular messenger systems is distal to the catalytic site of adenylate cyclase. This conclusion is fortified by our findings that PDBu also enhanced the secretory response to dibutyryl cAMP and the stimulatory action of PDBu on isoproterenol-stimulated secretion was not associated with an enhancement of cAMP levels. These results indicate that activators of protein kinase C alter amylase secretion in parotid cells through actions on cellular mechanisms distal to changes in cAMP accumulation. However, the inhibitory trend manifested by PDBu on isoproterenol-evoked cAMP accumulation may be attributed to an action on the beta receptor, since in several cell types phorbol esters cause receptor desensitization by promoting the phosphorylation of the beta adrenoceptor [21–23].

To ascribe physiological relevance to our findings, we addressed the question as to whether muscarinic receptor-mediated activation of phospholipase C enhances the effects of isoproterenol. Although diacylglycerol was not measured in this study, muscarinic agonists promote phosphoinositide hydrolysis in parotid acinar cells [24]. Our finding that low doses of carbachol potentiated isoproterenol- and dobutamine-stimulated amylase release supports the notion that an endogenous product of phosphoinositide hydrolysis, presumably diacylglycerol, acts to stimulate protein kinase C. The ability of carbachol to potentiate isoproterenol-evoked amylase release, but not the accumulation of cAMP, confirms previous reports [13, 15, 25, 26] and supports the concept that cAMP-dependent protein kinase is not the sole factor in the beta-adrenergic mechanism of salivary amylase release [25].

Salivary amylase release induced by beta-adrenoceptor stimulation is associated with an increase in Ca^{2+} availability [13, 27, 28]. Thus, cAMP and protein kinase C activation may raise cytosolic Ca^{2+} in parotid acinar cells either by promoting Ca^{2+} flux or modulating ATP-dependent Ca^{2+} transport [29]. In liver cells, the synergistic interaction between cAMP-dependent and Ca^{2+}-mobilizing hormones appears to occur at the level of Ca^{2+} gating [30]. On the other hand, the lack of effect of PDBu on Ca^{2+} mobilization in parotid gland [4] supports the supposition that secretion may be enhanced during protein kinase activation at a fixed concentration of cytosolic Ca^{2+} [31, 32].

In conclusion, this study describes in parotid cells a synergistic interaction between the two intracellular signalling systems that utilize cAMP, Ca^{2+}, and diacylglycerol as second messengers. Since norepinephrine, the physiological neurotransmitter for salivary amylase secretion, not only triggers the transduction reaction involving cAMP, but also activates alpha-receptors to promote phosphoinositide breakdown, the physiological implications of the present findings are apparent. The biochemical and cellular mechanisms underlying this synergistic interaction will be the focus of future investigation.

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