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Alpha$_1$-Adrenergic Stimulation of Phosphatidylinositol-Phosphatidic Acid Turnover in Rat Parotid Cells

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SUMMARY

The regulation of phosphatidylinositol turnover by *alpha*-adrenergic agonists in rat parotid acinar cell aggregates was examined with respect to kinetics and agonist-antagonist interactions. Phosphatidylinositol turnover was followed by the changes in the specific activities of [^{32}P]phosphatidic acid and [^{32}P]phosphatidylinositol. The specific activity of phosphatidic acid increased rapidly (within 1 min) after addition of epinephrine (10^{-5} M), reached a maximal level within 12–16 min, and then decreased. Incorporation of ^{32}P into phosphatidylinositol exhibited a lag phase of about 5 min and then increased continuously for an additional 40 min. The absolute amounts of phosphatidic acid and phosphatidylinositol did not change. The concentrations of epinephrine needed to stimulate ^{32}P incorporation into phosphatidic acid and phosphatidylinositol, measured at 15 and 30 min, respectively, were similar; K_a values of $2.05 \pm 0.46 \times 10^{-6}$ M for phosphatidic acid and $2.98 \pm 0.30 \times 10^{-6}$ M for phosphatidylinositol were found. The effects of agonists on ^{32}P labeling of phosphatidylinositol, in order of potency, were epinephrine $\geq$ norepinephrine $>$ phenylephrine $\gg$ normetanephrine. When various adrenergic antagonists were evaluated for their ability to inhibit 10^{-5} M epinephrine-stimulated ^{32}P incorporation into both phosphatidic acid and phosphatidylinositol, the order of antagonist potency was prazosin $\geq$ phenoxybenzamine $>$ phentolamine $\geq$ yohimbine $>\gg$ propranolol. These findings indicate that phosphatidylinositol-phosphatidic acid turnover in the rat parotid gland is mediated by the *alpha*$_1$-adrenergic receptor system.

INTRODUCTION

Hokin and Hokin (1) first reported that specific neurotransmitters stimulated the incorporation of $^{32}P_i$ into PI[3] in pancreas sections. Since this initial observation, enhanced PI turnover was demonstrated in many tissues following exposure to a variety of agents (2). The mechanism by which these effectors increased PI turnover is poorly understood, but it was shown to be distinct from the adenylate cyclase-cyclic AMP system (2). Instead, it was suggested that the increased turnover of PI was associated with an alteration in cellular calcium metabolism or in the promotion of Ca^{2+} entry into, or mobilization of Ca^{2+} within, responsive cells, leading to a rise in cytosolic Ca^{2+} (3, 4). In the rat parotid gland, stimulation of exocrine secretion by *alpha*-adrenergic agents was found to be concomitant with increased PI turnover (5, 6). However, neither the time course of the phospholipid alterations nor its relationship to secretory events was reported. In addition, *alpha*-adrenergic receptors were recently subdivided into two subtypes (*alpha*$_1$ and *alpha*$_2$), and distinct mechanisms were proposed for the mediation of their respective actions (7–10). *Alpha*$_1$-adrenergic receptors were implicated in the turnover of PI in adipocytes and hepatocytes (11). The subtype of *alpha*-adrenergic receptor involved in parotid cell phospholipid turnover was not reported. This question took on added importance with the recent finding that the *alpha*$_1$-adrenergic receptor system regulates K^+ secretion in rat parotid cells (12). Thus, a more detailed examination of the turnover of PI in this cell was prompted. In this study the kinetics of the response is described and evidence is presented suggesting that *alpha*$_1$-adrenergic receptors also regulate PI turnover in rat parotid cells.

MATERIALS AND METHODS

Animals. Three month-old male rats, a Wistar-derived strain supplied from the colony of the Gerontology Research Center, National Institute on Aging, were used for these studies. Animals were maintained on National Institutes of Health/Purina laboratory chow and water ad libitum until sacrifice.

Chemicals. Purified collagenase (Type CLSPA) was

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[3] The abbreviations used are: PI, phosphatidylinositol; (±)-normetanephrine, 3-*O*-methylnorepinephrine hydrochloride; HBSS, Hanks' balanced salt solution; PA, phosphatidic acid.

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obtained from Worthington Biochemical Corporation (Freehold, N. J.). Bovine testicular hyaluronidase (Type 1-S), bovine serum albumin (Fraction V), (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, (−)-phenylephrine hydrochloride, (±)-normetanephrine, and yohimbine hydrochloride were purchased from Sigma Chemical Company (St. Louis, Mo.). Phenoxybenzamine hydrochloride and (±)-propranolol hydrochloride were obtained from Smith Kline & French Laboratories (Philadelphia, Pa.) and Ayerst Laboratories (New York, N. Y.), respectively. Prazosin hydrochloride and phentolamine mesylate were gifts from Pfizer Laboratories Division (New York, N. Y.) and Ciba Pharmaceutical Company (Summit, N. J.), respectively. Precoated silica gel thin-layer plates (0.25 mm thick, Silica Gel 60) were purchased from E. Merck (Darmstadt, West Germany). [^{32}P]Orthophosphoric acid was obtained from New England Nuclear Corporation (Boston, Mass.). All other chemicals used were the highest grade commercially available.

Experimental procedure. Parotid cell aggregates were prepared, as previously reported, by using collagenase and hyaluronidase digestion (12). Following incubation with enzymes, cell aggregates were washed twice with 4% bovine serum albumin in HBSS (136.7 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, and 4.2 mM $NaHCO_3$, pH 7.4 at 20°C), twice with HBSS containing 0.022% bovine serum albumin, and twice more with incubation medium: HBSS with 30 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid substituted for phosphate. All solutions were previously oxygenated with 95% O_2-5% CO_2 for at least 30 min.

Parotid cell aggregates from one rat were preincubated for 1 hr at 37° in 3 ml of incubation medium containing 10 μCi of $^{32}P_i$ in plastic centrifuge tubes. Following preincubation, cell aggregates were washed twice with the incubation medium, without P_i, divided into several portions, and resuspended with 1 ml of incubation medium containing the effectors described in the individual experiments.

In antagonist experiments, cell aggregates were incubated with antagonists for 15 min prior to the addition of epinephrine. During all stages of incubation, cell aggregates were gassed at 15-min intervals (95% O_2-5% CO_2) and continuously shaken (Dubnoff metabolic shaker, 100 rpm).

Analytical methods. Experiments were terminated by removal of incubation media after brief centrifugation (15 sec at 40 × *g*) and the addition of 1 ml of chloroform-methanol (2:1, v/v), followed by homogenization with a Brinkman Polytron (Speed 5, 10 sec). The lipids from cell aggregates were extracted according to the method of Folch *et al.* (13). All further manipulations were carried out at room temperature. Subsequent lipid extracts were evaporated under a stream of nitrogen and lipids redissolved in chloroform-methanol (2:1, v/v). PI was separated with magnesium acetate-impregnated silica gel thin-layer plates (14) prepared by spraying 5% magnesium acetate onto silica gel plates, followed by heating at 120° for 1 hr. The developing solvent used was chloroform-methanol-concentrated ammonia-water (65:35:3:2, v/v) (14). By using magnesium acetate-impregnated plates, PI was clearly separated from sphingomyelin, PA, phosphatidylserine, and other phospholipids. PA was separated on silica gel plates with a solvent system containing chloroform-pyridine-formic acid (50:30:5, v/v) (15). After development, spots were visualized with iodine vapor and scraped off for determination of phosphorus by the method of Rouser *et al.* (16). The ^{32}P content of these same samples was measured by mixing, after spectrophotometric quantitation of phosphorous, 1.0 ml of the assay mixture with 10 ml of Aquassure (New England Nuclear Corporation) and counting in a liquid scintillation spectrometer. The intense blue color caused negligible loss of radioactivity. Data were calculated as counts per minute of ^{32}P per nanomole of phosphorus in each phospholipid.

The DNA content of cellular homogenates was determined by the diphenylamine method, as modified by Richards (17), using calf thymus DNA (Sigma Chemical Company) as a standard.

RESULTS

Time course of PA and PI labeling. Figure 1 shows the time course of ^{32}P incorporation into PA and PI of parotid cells incubated with epinephrine. Data are presented as the percentage increase in radioactivity relative to the 0-min time point. The specific radioactivities, at 0 min, were 623 ± 36 cpm/nmole of PA and 207 ± 32 cpm/nmole of PI (n = 7). The specific activity of PA was always higher than that of PI over the 45-min time course. The labeling of PA increased rapidly, and a significant increase in specific activity was found as early as 1 min after the addition of hormone (Fig. 2). The level of specific activity reached a peak within 12–16 min and then decreased. However, the labeling of PI showed a lag

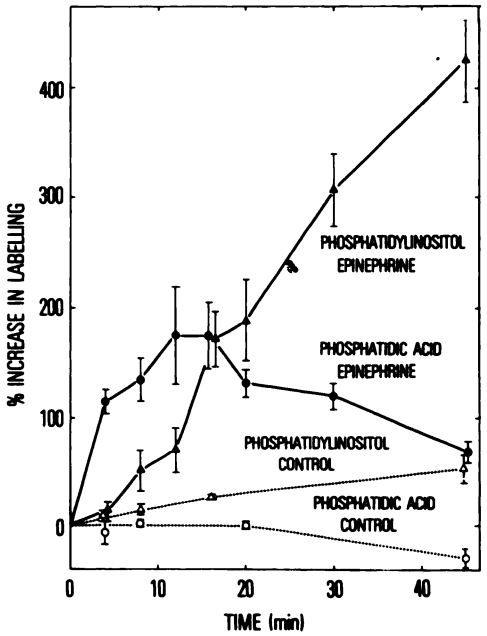

FIG. 1. *Time course of ^{32}P incorporation into PA and PI*
The concentration of epinephrine used was 10^{-5} (●,▲); ○, △, control. Each point represents the mean ± standard error calculated from data of three or four experiments. ●, ○, PA; ▲, △, PI.

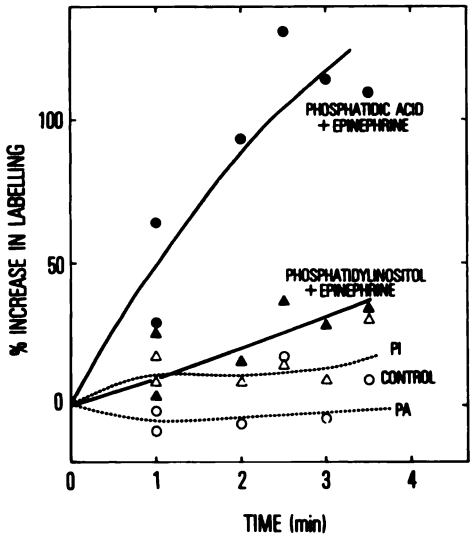

FIG. 2. *Early time course of ^{32}P incorporation into PA and PI*
Symbols are the same as in Fig. 1.

of about 5 min and then increased continuously over the remaining 40 min of incubation.

The absolute amounts of PA and PI were 46.0 ± 9.8 pmoles/μg of DNA (n = 7) and 491 ± 29 pmoles/μg of DNA (n = 6), respectively. There was no significant change in these amounts following the 45-min exposure of cells to epinephrine.

Specific activities of phosphatidylcholine plus phosphatidylethanolamine and sphingomyelin were also determined and were 80 cpm/nmole during the entire incubation period. No change in the specific radioactivity of these phospholipids was detected after stimulation with epinephrine.

Effect of varying concentrations of epinephrine on phospholipid labeling. The effects of different concentrations of epinephrine on the specific activity of PA and PI, measured at 15 and 30 min, respectively, are shown in Fig. 3. The results are presented as the percentage of maximal increase in specific activity (i.e., 10^{-4} M of epinephrine = 100%). The concentrations of epinephrine resulting in half-maximal response were 2.05 ± 0.46 × 10^{-6} M for PA and 2.98 ± 0.30 × 10^{-6} M for PI. The absolute amounts of PI and PA did not change after incubation with the different concentrations of epinephrine (data not shown).

Effects of various adrenergic agonists and antagonists on PI turnover. The stimulation in the incorporation of label into PI was dependent on the nature of the *alpha*-adrenergic agonist (Fig. 4). The order of potency, indicated by the concentration of agonist required for half-maximal as well as maximal stimulation, was epinephrine ≥ norepinephrine > phenylephrine ≫ normetanephrine. Normetanephrine had no detectable effect on PI turnover. Table 1 compares the actions of the different *alpha*-adrenergic agonists in stimulating PI turnover with their actions in enhancing K^+ efflux from rat parotid cells and in displacing the binding of [^{3}H] prazosin to rat parotid membranes. Definite correlations were evident.

The inhibitory effects of adrenergic antagonists on PI turnover stimulated by 10^{-5} M epinephrine were in the order of prazosin ≥ phenoxybenzamine > phentolamine > yohimbine ≫ propranolol (Fig. 5A). Comparable results for antagonist effects on PA turnover were also observed (Fig. 5B). Analogous to the data with *alpha*-adrenergic agonists (Fig. 4 and ref. 12), the relationship

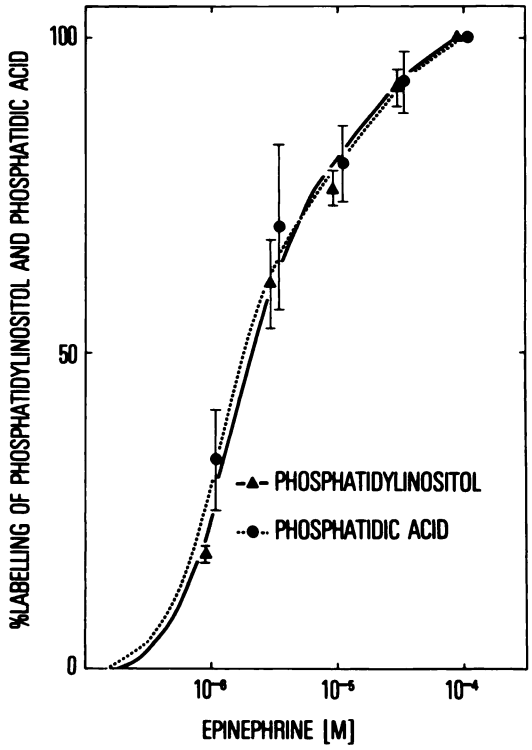

FIG. 3. *Effect of epinephrine concentration on PI turnover*
The incubation times with epinephrine were 15 min for PA (●) and 30 min for PI (▲). The results are the means ± standard error of six (PA) or seven (PI) experiments.

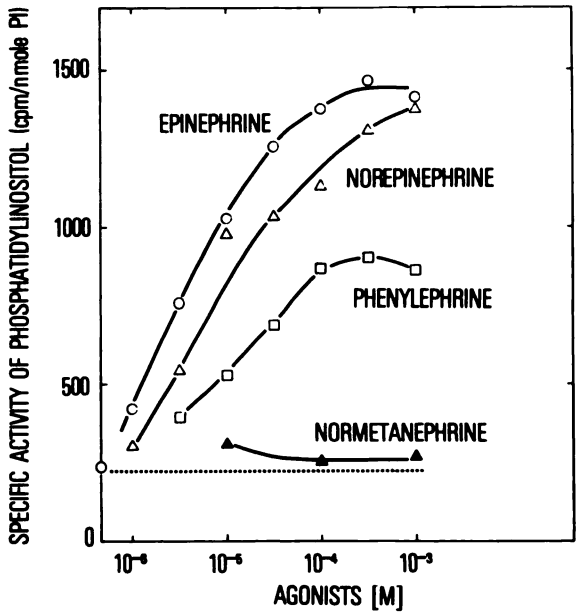

FIG. 4. *Stimulation of PI turnover by adrenergic agonists*
The incorporation of ^{32}P into PI was measured 30 min after the addition of individual agonists. Results are the means of two different experiments. ○, Epinephrine; △ norepinephrine; □, phenylephrine; ▲, normetanephrine; ○-----, buffer control.

TABLE 1

Relationship of the different adrenergic agonists to displace [^{3}H]prazosin binding in rat parotid membrane, to effect K^+ release and to stimulate PI and PA labeling in rat parotid cell aggregates

Agonist	[^{3}H] Prazosin binding (K_D)[a]	K^+ release (EC_{50})[a]	PI labeling (EC_{50})[b]	PA labeling (EC_{50})[c]
	M	*M*	*M*	*M*
Epinephrine	2.5×10^{-8}	8.0×10^{-7}	2.98×10^{-6}	2.05×10^{-6}
Norepinephrine	8.3×10^{-8}	1.3×10^{-6}	7.9×10^{-6}	
Phenylephrine	1.0×10^{-6}	1.1×10^{-5}	1.0×10^{-5}	
Normetanephrine	1.7×10^{-5}	$>10^{-3}$	$\gg 10^{-3}$	—

[a] The data showing the adrenergic agonist displacement of [^{3}H]prazosin binding from membrane binding sites (K_D) and the ability (EC_{50}) to elicit K^+ release are from Ito *et al.* (12).

[b] The EC_{50} values were determined from data in Fig. 4.

[c] The EC_{50} value was determined from data in Fig. 3.

between *alpha*-adrenergic antagonist concentration and inhibition of epinephrine-stimulated PI turnover resembled the relationship between antagonist concentration and the inhibition of K^+ efflux and the displacement of [^{3}H]prazosin binding (Table 2).

DISCUSSION

Epinephrine stimulation of rat parotid gland acinar cells elicits an increase in incorporation of ^{32}P into both PI and PA with an EC_{50} of $\sim 2 \times 10^{-6}$ M. This concentration is about one-tenth lower than previously reported for rat parotid gland (6, 19) but is very close to the EC_{50} (0.8×10^{-6} M) reported for K^+ release from similarly prepared parotid cells (12). However, after epinephrine-stimulation, there was a lag time of at least 5 min prior to increases in the ^{32}P content of PI, whereas a significant change in PA turnover was observed as early as 1 min after addition of agonist. Although K^+ release is even more rapid, with a maximal increase 1 min after addition of agonist, the kinetics of PA alterations allow for some involvement of phospholipid turnover in facilitating *alpha*-adrenergic stimulation of ion transport and water movement.

The function of parotid glands, the secretion of saliva, is principally controlled by *alpha*- and *beta*-adrenergic and cholinergic agonists. Exocrine protein secretion is primarily regulated by *beta*-adrenergic receptors through the adenylate cyclase-cyclic AMP system (20). Conversely, release of water and electrolytes is mediated principally via *alpha*-adrenergic and cholinergic receptors (21, 22). *Alpha*-adrenergic receptors have recently been subdivided into $alpha_1$- and $alpha_2$-receptors on the basis of their different responsiveness to adrenergic agonists and antagonists (7–10). We have recently demonstrated the $alpha_1$-adrenergic regulation of K^+ release from these cells (12). The results presented here on PI turnover support the concept that PI turnover is also regulated via $alpha_1$-adrenergic receptors. For example, phenylephrine is reported to be a more active $alpha_1$-adrenergic agonist than normetanephrine (primarily an $alpha_2$-adrenergic agonist) (7, 23, 24). As shown in Fig. 4 and Table 1, phenylephrine was much more efficient in stimulating PI turnover than normetanephrine. In addition, norepinephrine was about 1.3 times more efficient

TABLE 2

Relationship of the different adrenergic antagonists to displace [^{3}H]prazosin binding in rat parotid membrane, to block K^+ release, and to inhibit PI and PA labeling in rat parotid cell aggregates

Antagonist	[^{3}H] Prazosin binding (K_D)[a]	K^+ release (K_i)[a]	PI labeling (K_i)	PA labeling (K_i)
	M	*M*	*M*	*M*
Prazosin	2.0×10^{-9}	7.4×10^{-10}	1.4×10^{-10}	2.0×10^{-11}
Phenoxybenzamine[b]	6.7×10^{-9}	1.1×10^{-9}	2.3×10^{-10}	—
Phentolamine	—	—	1.1×10^{-9}	—
Yohimbine	1.3×10^{-6}	3.0×10^{-7}	2.4×10^{-7}	3.2×10^{-7}
(±)−Propranolol	$>38 \times 10^{-6}$	$>7.4 \times 10^{-6}$	$\gg 10^{-3}$	—

[a] The data showing the adrenergic antagonist displacement of [^{3}H]prazosin binding from membrane binding sites and the (K_i) for inhibition of epinephrine-stimulated K^+ release (concentration of epinephrine = 10^{-5} M) are taken from Ito *et al.* (12). The K_i values of PI and PA labeling were determined from Fig. 5 according to the method of Cheng and Prusoff (18).

[b] Since phenoxybenzamine is an irreversible *alpha*-adrenergic antagonist, the K_D and K_i values presented here are only apparent and are used to compare this antagonist with the other adrenergic antagonists.

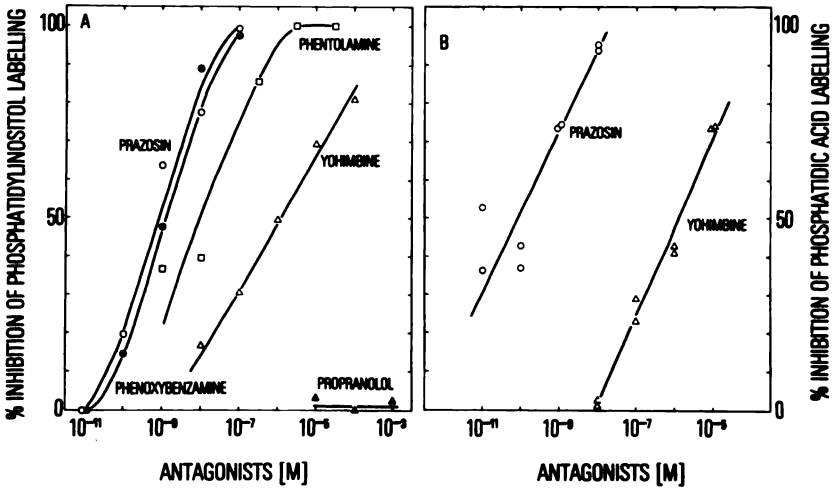

FIG. 5. *Inhibitory effects of adrenergic antagonists on turnover of PI (A) and PA (B).*

The concentration of epinephrine used was 10^{-5} M. Antagonists were added 15 min before the addition of epinephrine. The ^{32}P incorporation into PI and PA was measured at 30 min and 15 min, respectively. The results are from two experiments for PI and one for PA. ●, Prazosin; ○, phenoxybenzamine; □, phentolamine; △, yohimbine; ▲, propranolol.

than phenylephrine in eliciting PI turnover. The relative potencies of norepinephrine and phenylephrine are more compatible with those noted in *alpha*$_1$-adrenergic receptors (7-fold) than for those in *alpha*$_2$-adrenergic receptors (142-fold) (7). Agonist potency followed the order epinephrine ≥ norepinephrine > phenylephrine ≫ normetanephrine. The concept of *alpha*$_1$-receptor mediation of PI turnover is further supported by the relative potencies of antagonists in blocking epinephrine stimulation of PI labeling. The order of potency observed (prazosin ≥ phenoxybenzomine > phentolamine > yohimbine ≫ propranolol) and the much greater effectiveness of prazosin as compared with yohimbine towards both PI and PA labeling are indicative of involvement of *alpha*$_1$-adrenergic receptors (24). These relationships are also consistent with studies of the displacement of prazosin binding and K^+ release in rat parotid cell aggregates (Table 1 and ref. 12) and are analogous to results previously found by Garcia-Sainz *et al.* (25, 26) in white and brown adipocytes.

The enhanced incorporation of $^{32}P_i$ into PI and PA in secretory glands has been observed in many laboratories using a variety of regulatory agents. Hokin and Hokin (27) proposed that these acidic phospholipids would be related to the regulation of ion movements evoked by these compounds. However, the exact role for turnover in this process remains obscure. PA, produced by the phosphorylation of diacylglyceride resulting from the breakdown of PI, has been of particular interest. After the addition of epinephrine to rat parotid cell aggregates, the incorporation of ^{32}P into PA is significantly increased before detectable changes in PI are observed (Fig. 1). A similar rapid increase in the radiolabeling of PA has been shown in isolated stomach smooth muscle cells after carbamylcholine exposure (28) and in rabbit neutrophils after treatment with a chemotactic stimulant (29).

The mechanism coupling agonist-receptor interaction with PI turnover, alteration in Ca^{2+} metabolism, and K^+ release remains obscure. Additional studies are obviously needed. However, the present results indicate that the preparation of enzymatically dispersed cells from the parotid gland may be valuable to the further investigation of the mechanism of *alpha*-adrenergic-regulated physiological responses.

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