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Incorporation of Inorganic [^{32}P]Phosphate into Rat Parotid Phosphatidylinositol

Induction through Activation of *Alpha* Adrenergic and Cholinergic Receptors and Relation to K^+ Release

YORAM ORON, MARGALITH LÖWE, AND ZVI SELINGER

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

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SUMMARY

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Epinephrine and carbamylcholine, which cause K^+ release in rat parotid slices, also increase the incorporation of $^{32}P_i$ into phosphatidylinositol. The effects of epinephrine on K^+ release and $^{32}P_i$ incorporation were inhibited by phentolamine but not by atropine, whereas the effects of carbamylcholine were inhibited by atropine but not by phentolamine. Epinephrine at 20 μM caused a half-maximal increase in the incorporation of $^{32}P_i$ into phosphatidylinositol; this is similar to the value previously determined for the *alpha* adrenergic response of K^+ release. Induction of massive enzyme secretion by isoproterenol of $N^6,O^{2'}$-dibutyryladenosine 3',5'-monophosphate (dibutyryl cyclic AMP) had no effect on the incorporation of $^{32}P_i$. No changes in the composition of the major phospholipids of the parotid gland were observed. The epinephrine-induced K^+ release was dependent upon calcium and reached a steady state within 5 min. On the other hand, the increased incorporation of $^{32}P_i$ into phosphatidylinositol showed a lag of about 10 min, was inhibited by Ca^{2+}, and was maximally increased in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid. It is concluded that the K^+ release is neither a prerequisite for nor the direct result of the increased incorporation of $^{32}P_i$ into phosphatidylinositol.

INTRODUCTION

Hokin and Hokin (1) first reported that hormones which induce enzyme secretion from pigeon pancreas also cause an increased incorporation of $^{32}P_i$ into phospholipids. This response was later found in a variety of tissues and could be elicited by many different hormones and neurotransmitters (2-12). In most tissues increased $^{32}P_i$ incorporation was found to be specific for acidic phospholipids. Although the enzymatic reactions stimulated by these hormones are not known, possible mechanisms have been suggested in several recent reports (13-15).

In the rat parotid gland three distinct receptors have been identified within the same acinar cell. The *beta* adrenergic re-

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ceptor mediates enzyme secretion via cyclic 3',5'-AMP (16), the *alpha* adrenergic receptor mediates K^+ release (17, 18), and the cholinergic receptor also mediates K^+ release (19). It has been shown that K^+ release in response to these agents can be simulated by the divalent cation ionophore A-23187, which introduces Ca^{2+} into the cell (20). Each of the hormone responses mentioned above has been characterized by specific morphological changes demonstrated by electron microscopy (18).

We have recently reported that increased incorporation of $^{32}P_i$ into phosphatidylinositol in rat parotid was associated with *alpha* adrenergic receptor activation (12). In the present work the kinetics and requirements for induction of K^+ release and for increased incorporation of $^{32}P_i$ into phosphotidylinositol are compared. The increased incorporation of $^{32}P_i$, although mediated by both *alpha* adrenergic and cholinergic receptors, does not require the release of K^+ from the cell.

MATERIALS AND METHODS

The sources of the neurotransmitters, inhibitors, and $N^6,O^{2'}$-dibutyryl cyclic AMP have been reported previously (17, 18, 20). The ionophore A-23187 was kindly contributed by Dr. M. Gorman of Eli Lilly & Company, and $^{32}P_i$ was purchased from the Nuclear Center, Negev, Israel.

Potassium release was assayed by atomic absorption spectrophotometry (20), inorganic phosphate was determined according to a modification of Bartlett's method (21), and ^{32}P radioactivity was measured in a liquid scintillation spectrometer utilizing Cerenkov radiation.

All experiments were conducted on male albino rats (150–250 g) fed ad libitum. In some experiments reserpine-treated rats were used (18). Parotid slices were prepared as described elsewhere (22). Krebs-Ringer bicarbonate medium was used at all stages of the experiments. Unless otherwise specified, Ca^{2+} and P_i were omitted from this medium [calcium was omitted in order to reduce the release of endogenous neurotransmitters (23)]. The parotid slices were rapidly agitated in a New Brunswick rotary shaker bath at 37° and vigorously gassed with 95% O_2–5% CO_2. The slices to be used in different experimental systems were first incubated together for 30 min in the medium at a ratio of 1 gland/ml. This medium contained approximately 50 μCi of $^{32}P_i$ per milliliter. The $^{32}P_i$ was subsequently removed by three consecutive washings, 10 ml of buffer medium per gland being used in each wash. Thereafter, depending on the number of experimental incubation conditions to be evaluated, the slices were divided into the desired number of portions, each equal to approximately four glands. An additional 60-min incubation was usually carried out in 50-ml Erlenmeyer flasks containing various additions. Preliminary experiments had shown that this procedure resulted in equivalent labeling of ATP under all incubation conditions It also prevented possible effects of altered cellular permeability to $^{32}P_i$. The incubation was terminated by removal of the medium, which was then replaced with 10 ml of methanol at approximately −50°. The tissue in each flask was ground with a Polytron (Kinematica GMBH) mincer, and lipids were extracted in a single step according to Burger *et al.* (24). The extract was filtered through a Whatman GF/c filter. The filtrate was taken to dryness in a rotary evaporator, dissolved in 5 ml of chloroform–methanol–0.1 M aqueous KCl, 86:14:1 by volume (lower phase), and washed three times with chloroform–methanol–0.1 M aqueous KCl, 3:48:47 by volume (upper phase) (25). The extract was deacylated according to Wells and Dittmer (26), and the dried hydrolysate was dissolved in 5 ml of 5 mM sodium tetraborate solution. An aliquot of the hydrolysate was chromatographed on an anion-exchange resin, and the glycerylphosphorylinositol fraction was eluted as described by Chang and Sweeley (27).

It was found that anion exchange chromatography of deacylated phospholipids was superior to two-dimensional thin-layer chromatography, since it gave much better separation between glycerylphosphorylinositol and glycerylphosphorylserine. The over-all recovery of phos-

phatidylinositol phosphorus through the deacylation and chromatography steps was 85% or more, which is close to the recovery reported by Wells and Dittmer (26).

The following modifications were introduced in the chromatography step. Dowex AG21K, 50–100 mesh, was used, and the column was reduced to 0.8 ml bed volume. Elution was conducted in two steps, first with 25 ml of sodium tetraborate, 5 mM, and ammonium formate, 15 mM, followed by 20 ml of sodium tetraborate, 5 mM, and ammonium formate, 40 mM. The last 15-ml eluate, containing the majority of the glycerylphosphorylinositol peak, was dried overnight at 100°, digested for 45–60 min in 2 ml of 70% $HClO_4$, and assayed for ^{32}P specific radioactivity. The data are expressed as the average specific radioactivity in duplicate slice systems. When experiments were conducted on different batches of slices there were differences in the extent of $^{32}P_i$ incorporation into phosphatidylinositol in the basal state. Therefore each experiment includes a control without stimulation. Epinephrine and carbamylcholine caused 3–5-fold stimulation with respect to the basal incorporation. Because of this variability, the results were not pooled. Instead, results of typical individual experiments, each repeated at least three times, are shown in the figures. In 35 experiments run in duplicate under various incubation conditions, the average difference from each mean was 6% and the standard deviation was ±5%. It is possible that the differences noticed in $^{32}P_i$ incorporation in the basal state are related to variations in release of endogenous neurotransmitters.

RESULTS

Stimulation and inhibition of $^{32}P_i$ incorporation into phosphatidylinositol. Epinephrine and carbamylcholine caused an approximately 4-fold increase in the incorporation of $^{32}P_i$ into phosphatidylinositol in rat parotid slices. Phentolamine, an *alpha* adrenergic blocking agent, abolished the effect of epinephrine, but not that of carbamylcholine. Atropine, an inhibitor of the muscarinic-cholinergic receptor, abolished the action of carbamylcholine, but not that of epinephrine. Both isoproterenol, a specific activator of the *Beta* adrenergic receptor, and dibutyryl cyclic AMP, which cause massive enzyme secretion (16), did not affect the incorporation of $^{32}P_i$ into phosphatidylinositol (Fig. 1). This indicates that the increased incorporation of $^{32}P_i$ could have been due to independent activation of either the *alpha* adrenergic or the cholinergic receptor, but precludes association with enzyme secretion.

Phospholipid composition of rat parotid gland. Activation of the adrenergic receptors in the parotid gland causes marked morphological changes in the cellular membranes (18). It was therefore of interest to determine whether these morphological changes are also associated with changes in the relative amounts of particular phospholipids. Epinephrine, alone or in the presence of the adrenergic inhibitors phentolamine and propranolol, did not cause any major changes in the phospholipid composition. The synthetic catecholamine isoproterenol also had no effect (Table 1).

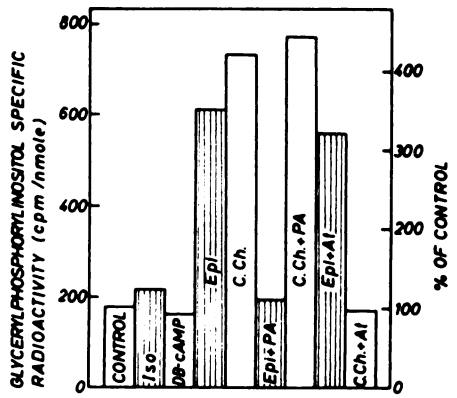

FIG. 1. *Effects of activators and inhibitors of adrenergic and cholinergic receptors on incorporation of $^{32}P_i$ into phosphatidylinositol*

Parotid slices were labeled with $^{32}P_i$ for 30 min (see the text). Thereafter reagents were added to the medium to give the following concentrations: isoproterenol (Iso), epinephrine (Epi), and carbamylcholine (C.Ch) 0.1 mM; phentolamine (PA) and atropine (At) 0.02 mM; dibutyryl cyclic AMP (Db-cAMP), 1 mM. After further incubation for 60 min, lipids were extracted and the specific radioactivity of phosphatidylinositol was determined.

TABLE 1

Phospholipid composition of rat parotid slices incubated in the presence of catecholamines and their inhibitors

Parotid slices were incubated and lipids were extracted as described in the text, except that two consecutive lipid extractions were performed. The combined phospholipid extract was separated into phospholipid classes by two-dimensional thin-layer chromatography on silica gel H (Merck) plates, using chloroform-methanol-H_2O (60:35:5 by volume) in one direction and chloroform-methanol-NH_4OH (30:10:2 by volume) in the second direction). The concentrations of epinephrine and isoproterenol were 0.1 mM; phentolamine and propranolol, 0.02 mM; the incubation time was 60 min. The results are given as the mean percentage of total recovered phospholipid phosphorus ± standard deviations.

Additions	Recovered phospholipid phosphorus					
	Lysophosphatidyl choline	Sphingomyelin	Phosphatidylcholine	Phosphatidylinositol + phosphatidylserine	Phosphatidylethanolamine	Phosphatidic acid + cardiolipin
	%	%	%	%	%	%
None (9)[a]	3.77 ± 1.51	7.86 ± 1.54	45.03 ± 5.56	14.17 ± 4.05	22.09 ± 2.63	3.29 ± 1.37
Epinephrine (7)	3.66 ± 1.88	7.60 ± 1.48	47.93 ± 5.14	12.94 ± 2.21	23.59 ± 4.86	3.03 ± 0.71
Epinephrine + phentolamine (3)	7.5	8.7	45.0	15.3	20.9	1.5
Epinephrine + propranolol (3)	3.7	7.0	46.1	14.6	25.2	2.5
Phentolamine (2)	5.4	8.6	43.4	13.9	24.6	2.7
Propranolol (2)	4.5	7.5	45.7	14.6	24.6	3.6
Isoproterenol (2)	3.6	7.9	45.8	15.9	21.2	3.5

[a] Numbers in parentheses indicate number of experiments.

Saturation curve for epinephrine and time kinetics of $^{32}P_i$ incorporation into phosphatidylinositol. All these experiments were conducted on rats treated with reserpine, which depletes endogenous catecholamines (23). Furthermore, in order to diminish the oxidation of catecholamines which are present at low concentrations, the incubation time in the presence of epinephrine was reduced to 30 min. The incubation medium contained 0.2 mM EGTA[1] and 1 μM atropine, which blocked the effect of endogenous acetylcholine. The increased incorporation of $^{32}P_i$ into phosphatidylinositol, as a function of epinephrine concentration, described a rectangular hyperbola (Fig. 2). Half the maximal effect occurred at an epinephrine concentration of 20 μM. A similar value was previously determined for epinephrine-induced K^+ release (16). The epinephrine-induced increase of $^{32}P_i$ incorporation into phosphatidylinositol followed a triphasic time

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

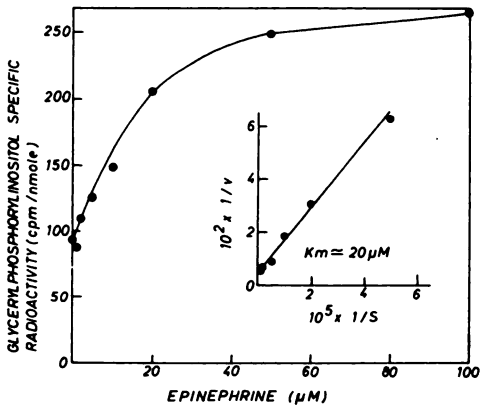

FIG. 2. *Effects of various epinephrine concentrations on incorporation of $^{32}P_i$ into phosphatidylinositol*

Parotid glands from reserpine-treated rats were used. All systems contained atropine, 1 μM, and EGTA, 0.2 mM. Incorporation was terminated 30 min after the addition of epinephrine. The value for v due to epinephrine stimulation in the Lineweaver-Burk plot was defined as the increment in specific radioactivity of phosphatidylinositol in the presence of epinephrine over that of control slices incubated without epinephrine.

course: a 7–10-min lag, followed by a rapid increase for 10 min, and then a less steep linear increase for at least another 40 min (Fig. 3). The shape of the curve was not changed by a second addition of epinephrine after 30 min, nor by the presence of 2.5 mM Ca^{2+} and atropine in the medium (not shown). In contrast, K^+ release in the presence of 2.5 mM Ca^{2+} reached a steady state in about 5 min (Fig. 3). When phentolamine (20 μM) was added 5 and 15 min after the addition of epinephrine, there was no further increase in the incorporation of $^{32}P_i$ into phosphatidylinositol over that of the control. However, phentolamine did not cause a reduction in the specific radioactivity of the phosphatidylinositol.

Effect of Ca^{2+} on incorporation of $^{32}P_i$ into phosphatidylinositol. Epinephrine caused a maximal increase in the incorporation of $^{32}P_i$ into phosphatidylinositol in the presence of 5 mM EGTA (Fig. 4). This is in contrast to the epinephrine-induced K^+ release, which is dependent on the presence of millimolar concentrations of

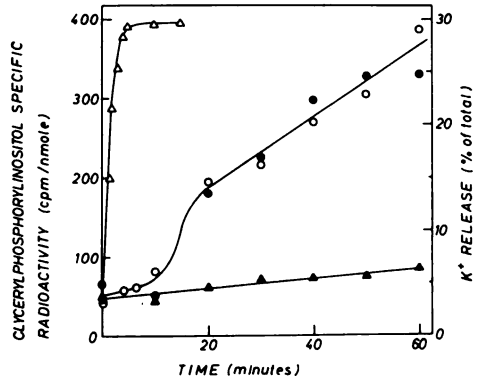

FIG. 3. *Time curve of $^{32}P_i$ incorporation into phosphatidylinositol in the presence and absence of epinephrine*

Slices equivalent to four parotid glands were withdrawn from the incubation medium at the indicated times and processed for determination of phosphatidylinositol specific radioactivity. ▲, control; ●, epinephrine at a final concentration of 0.1 mM added at zero time, with the same amount of epinephrine added 30 min later; O, epinephrine at a final concentration of 0.1 mM added at zero time; Δ, K^+ *release.* $^{32}P_i$ incorporation was measured in slices incubated in Krebs-Ringer-bicarbonate medium from which Ca^{2+} was omitted. K^+ release was measured in the presence of 2.5 mM Ca^{2+}.

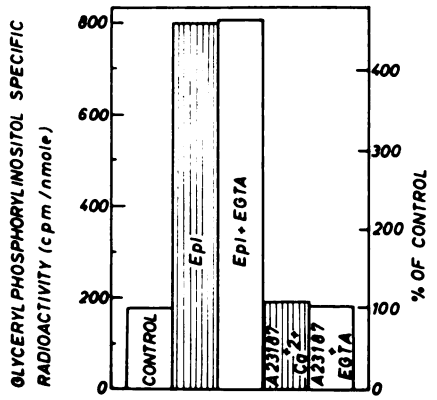

FIG. 4. *Effects of divalent cation ionophore A-23187 and Ca^{2+} on incorporation of $^{32}P_i$ into phosphatidylinositol*

Parotid slices labeled with $^{32}P_i$ as described under MATERIALS AND METHODS were incubated for 20 min in the presence or absence of 6 μg/ml of A-23187 and then transferred to systems containing the following additions: epinephrine (Epi), 0.1 mM; epinephrine, 0.1 mM, plus EGTA, 5 mM; A-23187, 6 μg/ml, plus Ca^{2+}, 2.5 mM; A-23187, 6 μg/ml, plus EGTA, 1 mM. After further incubation for 60 min lipids were extracted and the specific radioactivity of phosphatidylinositol was determined.

Ca^{2+} in the medium. Moreover, increasing the Ca^{2+} concentration markedly inhibited incorporation, inhibition reaching 90% at 20 mM Ca^{2+} (Fig. 5). Epinephrine caused essentially the same K^+ release at 2.5 mM and 20 mM Ca^{2+}.

In the presence of Ca^{2+} the divalent cation ionophore A-23187 caused efficient K^+ release independently of the *alpha* adrenergic and cholinergic receptors (20). Induction of K^+ release by the ionophore A-23187 and Ca^{2+} did not significantly change the specific radioactivity of phosphatidylinositol (Fig. 4). This indicates that K^+ release does not cause increased incorporation of $^{32}P_i$ into phosphatidylinositol.

DISCUSSION

The increased incorporation of $^{32}P_i$ into acidic phospholipids was first noted in tissues that have secretory functions (2–4). This observation led many investigators to connect the so-called phospholipid effect with secretion (13). Despite this, under certain experimental conditions there is no

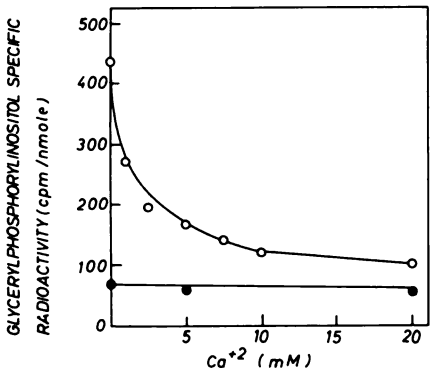

FIG. 5. *Effect of epinephrine on incorporation of $^{32}P_i$ into phosphatidylinositol at various Ca^{2+} concentrations*

To prevent precipitation of $CaCO_3$ the bicarbonate in the incubation medium was replaced by an equivalent amount of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, and the gas mixture was changed to 100% O_2. Incubation time was 60 min, and the epinephrine concentration was 0.1 mM, with a second addition of epinephrine at 30 min. ●, control; O, epinephrine.

correlation between these two phenomena (28–32), and a cause-and-effect relationship has thus been questioned (30, 32, 33).

In the parotid, in which three distinct receptors initiate specific physiological responses, a separation between the phospholipid effect and enzyme secretion has been achieved at the receptor level. Activation of a *beta* adrenergic receptor mediates enzyme secretion but has no effect on the incorporation of $^{32}P_i$ into phospholipids. The *alpha* adrenergic and cholinergic receptors, however, cause K^+ release from the cell, in addition to a specific increase in the incorporation of $^{32}P_i$ into phosphatidylinositol. Although the phospholipid effect in the rat parotid is related to activation of the *alpha* adrenergic and cholinergic receptors, it is not dependent on K^+ release from the cell. The divalent cation ionophore A-23187, which introduces Ca^{2+} into the cell, thus causing K^+ release (20), has little, if any, effect on the incorporation of $^{32}P_i$ into phosphatidylinositol. Conversely, the phospholipid effect is maximal in the absence of Ca^{2+} in the medium, when there is no K^+ release from the cell.

A mechanism compatible with these results would imply that the phospholipid effect is an early event, enabling Ca^{2+} to enter the cell, this in turn causing K^+ release. Although this mechanism could account for the phospholipid effect in the absence of Ca^{2+}, it is not compatible with the kinetics of the effect. In the parotid slice system the incorporation of $^{32}P_i$ into phosphatidylinositol showed a 7–10-min lag, while in the same system K^+ release started immediately after hormone addition and reached a steady state within 5 min. Since phosphatidic acid is the precursor of phosphatidylinositol, it is possible that the incorporation of $^{32}P_i$ into phosphatidic acid preceded the incorporation into phosphatidylinositol. This possibility was difficult to evaluate because of the very low incorporation of $^{32}P_i$ into phosphatidic acid in the parotid system. However, Trifaro (32) has shown that in the adrenal medulla acetylcholine-induced catecholamine release reaches a maximal rate before the onset of an increase in the incorporation of $^{32}P_i$ into both phosphatidic acid and phosphatidylinositol. On the other hand, Friedel and Schanberg (34) have shown that in the whole animal, 5 min following the injection of $^{32}P_i$ and acetylcholine into the cisterna magna of the rat brain *in situ*, there is an increased incorporation of $^{32}P_i$ into phosphatidic acid and phosphatidylinositol. These findings, however, were not correlated with any physiological phenomena.

It has been postulated that the phospholipid effect represents a secondary adaptive response to major physiological changes in the cell (30). Since the maximal increase in incorporation of $^{32}P_i$ into phosphatidylinositol in the parotid could be demonstrated under conditions when no K^+ was released, we propose a different sequence of events. It is suggested that upon interaction of the neurotransmitter with the receptor, the first molecular event occurs at the receptor level, producing as yet unidentified changes in the cell membrane. Following this interaction two parallel and independent responses could result: (a) the permeability of the cell membrane to Ca^{2+} is increased, so that whenever Ca^{2+} is present in the medium it

enters into the cell and causes K^+ release; (b) the same interaction with the receptors initiates a chain of reactions resulting in increased incorporation of $^{32}P_i$ into acidic phospholipids. The latter response does not depend on the presence of Ca^{2+} and is in fact maximally stimulated in its absence. Consequently the increased phospholipid metabolism need not be confined to the cell membrane, nor does it have to follow the kinetics of the physiological response.

This hypothesis is compatible with the following, apparently unrelated findings. The phospholipid effect was demonstrated in a wide spectrum of tissues and elicited by a variety of stimuli which produce different physiological responses. In all these systems a Ca^{2+} requirement is implicated in the physiological response (35), and in some Ca^{2+} has been shown to act as the intracellular messenger which transduces receptor activation into cellular response (20, 36–38). The effect is not limited to phosphatidylinositol. In other systems phospholipids such as phosphatidic acid and phosphatidylglycerol have been shown to undergo intensive turnover upon stimulation (2, 8–11, 32). This observation tends to strengthen the assumption that the phospholipid effect does not represent the mechanism of a receptor-activated Ca^{2+} entrance into the cell, but is rather an independent event, modulated by the specific biochemical characteristics of each particular tissue.

Finally, studies on the cellular localization of the phospholipid effect in human lymphocytes and guinea pig pancreas showed no clear-cut association of the labeled phosphatidylinositol with the cell membrane (11, 39). The greatest intensity of labeling was associated with intracellular membranes. Preliminary studies in our laboratory have confirmed similar results in the parotid system.

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