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Effects of Antimycin A on Receptor-Activated Calcium Mobilization and Phosphoinositide Metabolism in Rat Parotid Gland

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SUMMARY

The effects of the mitochondrial poison, antimycin A, on responses of parotid acinar cells to cholinergic stimuli were examined. Antimycin A (10 μM) partially inhibited the agonist-induced increase in ^{86}Rb efflux. Specifcally, the initial transient phase of the response, believed to arise from intracellular calcium release, was partially inhibited, while the sustained phase of the response, believed to result from calcium entering from the extracellular space, was completely blocked. The stimulation of ^{45}Ca influx by a cholinergic agonist was also completely blocked. Antimycin A (10 μM) caused a rapid loss of [^{32}P] polyphosphoinositides. Stimulation of [^{32}P]phosphatidylinositol breakdown and [^{32}P] phosphatidate synthesis by methacholine was blocked by antimycin A. Breakdown of [^{32}P]phosphatidylinositol-4,5-bisphosphate in response to cholinergic stimulation was partially inhibited. These results indicated that the activation by cholinergic agonists of cellular calcium mobilization as well as effects on phosphoinositide metabolism are similarly inhibited by antimycin A. Furthermore, this presumably indicates a role for ATP in receptor-activated calcium mobilization and phosphoinositide turnover.

INTRODUCTION

The rat parotid gland can be activated *in vitro* through any of four known receptor pathways: *beta*-adrenergic, *alpha*-adrenergic, peptidergic (substance P), and muscarinic-cholinergic (1). All except the *beta*-adrenoceptor mobilize calcium by mechanisms involving intracellular calcium release and the opening of surface membrane calcium channels or "gates." The increase in cytosolic calcium activates (among other things) permeability pathways for Na^+ and K^+ which are presumably involved in the secretion of water and electrolytes in the intact gland (2).

These same calcium-mobilizing receptors also profoundly affect the turnover of phosphoinositides and phosphatidic acid. On the basis of circumstantial evidence, it has been postulated that changes in these phospholipids may play a role in coupling receptor activation to cellular calcium release and to the activation of surface membrane calcium gates (3, 4). This hypothesis deviates from simpler views of the relationship of receptors and ion channels in proposing one or more metabolically dependent steps linking the receptor to the process of calcium mobilization. However, Hawthorne (5) has argued that there is no apparent thermodynamic basis for expenditure of cellular energy in calcium gating, and therefore that phosphoinositide turnover is unlikely to serve such a function.

In this study, we have sought to determine whether the process of receptor-activated calcium mobilization depends on cellular metabolism by examining the effects of a mitochondrial oxidation-reduction inhibitor, antimycin A, on potassium and calcium movements in parotid cells. In parallel studies, we have examined the actions of this toxin on phospholipid metabolism. The results indicate that both receptor-activated calcium mobilization and phospholipid turnover are rapidly inhibited by antimycin A. These findings add to the body of circumstantial evidence supporting a link between receptor-mediated alterations in phospholipid metabolism and cellular calcium mobilization.

METHODS

All experiments were performed with parotid glands removed from anesthetized (sodium pentobarbital, 50 mg/kg i.p.) male Sprague-Dawley rats. The basic Ringer medium used had the following composition (millimolar): NaCl, 120; KCl, 5.0; $CaCl_2$, 1.0; $MgCl_2$, 1.2, sodium β-hydroxybutyrate, 5.0; Tris, 20.0; sufficient HCl to adjust pH to 7.40 at 37°; bovine serum albumin, 0.5% (w/v); gas phase, 100% O_2.

The methods employed for estimation of unidirectional efflux of ^{86}Rb as a marker for K^+ efflux have been described (6). Briefly stated, slices of rat parotid gland were equilibrated with ^{86}Rb and then transferred through

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a series of nonradioactive incubations. From the radioactivity released during these incubations, apparent first-order rate coefficients were calculated.

For net calcium flux measurements or phosphoinositide studies, dispersed acinar cells were prepared as described previously (7) and were incubated in a rotary shaker bath at 37°.

The method used for measurement of net ^{45}Ca flux has been described in detail elsewhere (8). Cells were preincubated with ^{45}Ca-containing medium for 55 min, by which time net ^{45}Ca flux is in a quasi-steady state (slow linear net influx). Drugs were added to some suspensions according to the particular experiment, and at various times aliquots were taken and the cells and their associated ^{45}Ca were separated from the medium by dilution and rapid centrifugation (8).

Binding of [^{3}H]QNB,[3] a muscarinic-cholinergic receptor ligand, to parotid acinar cells was measured according to a previously described method (9). In previous studies, [^{3}H]QNB binding was determined after incubation in the presence of the ligand for 30 min in order to achieve equilibrium binding. In the studies reported here, the incubation time was only 10 min so that effects of antimycin A on binding could be determined with a time frame relevant to other experimental protocols. However, relative changes in receptor number or agonist affinity would still be detected. Specific antagonist binding was measured as the difference in the quantity of [^{3}H]QNB (0.5 nM) bound in the presence and absence of 1 μM scopolamine. Relative agonist binding was measured by the ability of agonists to inhibit specific [^{3}H]QNB binding. The fractional inhibition of specific binding by methacholine was corrected for the concentration of [^{3}H]QNB used (0.5 nM) and the receptor affinity of [^{3}H]QNB (0.79 nM) as described previously (9).

Net changes in radiolabeled PIP or PIP_2 were determined as described previously (10). Cells were incubated in medium containing 10 μM $^{32}PO_4$ for 1 hr, after which time PIP and PIP_2 appeared to be labeled to near equilibrium. Changes in radioactivity seen after 60 min appear to reflect net changes in mass of these rapidly turning over phospholipids (10).

In order to measure net breakdown of radioactive PI a procedure was used similar to that described for the exocrine pancreas by Marshall *et al* (11). Cells were incubated in medium containing 10 μM $^{32}PO_4$ in the presence of 0.1 mM epinephrine for 30 min, and subsequently washed twice by gentle (about 50 × *g*) centrifugation with a nonradioactive medium containing 100 μM PO_4, no epinephrine, and 100 μM phentolamine. The cells were then incubated without drugs in nonradioactive medium containing 100 μM PO_4 for 60 min, after which time the application of a cholinergic-muscarinic agonist (methacholine) invariably caused net loss of radioactivity from PI relative to controls. All of the methods used for quantitative extraction and purification (by thin-layer chromatography) of PI, PA, PIP, and PIP_2 have been described (10, 12).

[3] The abbreviations used are: QNB, L-quinuclidinyl benzilate; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PI, phosphatidylinositol; PA, phosphatidic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid.

Radioactive materials were purchased from New England Nuclear Corporation (Boston, Mass.). Carbachol, methacholine, scopolamine, antimycin A, and phospholipids (standards) were obtained from Sigma Chemical Company (St. Louis, Mo.). Antimycin A was prepared as a 10 mM stock in ethanol. On dilution to 10 μM in Ringer's solution the final ethanol concentration was 0.1%, which had no discernible effects on any of the parameters measured in this study. Statistical comparisons were made by analysis of variance; $p = 0.05$ or less was considered statistically significant.

RESULTS

The effects of antimycin A (10 μM) on the K^+ efflux response to carbachol (100 μM) were determined (measured as ^{86}Rb efflux; ref. 6). The results are shown in Fig. 1. Under control conditions, carbachol causes a rapid, transient increase in ^{86}Rb efflux, followed by a sustained or slowly falling phase (ref. 6; Fig. 1A). Calcium is believed to be a second messenger for both phases. The first phase is attributed to cellular calcium release; the second phase, to activation of surface membrane calcium gates (2). Antimycin A had no discernible effects on the basal release of ^{86}Rb (Fig. 1). When carbachol was applied 5 min after antimycin A, the response was depressed but not totally blocked (Fig. 1A). It appeared as if the initial transient phase of the response persisted in part, whereas the later, calcium dependent, phase was prevented. Similar results were obtained with 3 mM KCN and by rapidly switching the gas phase from 100% O_2 to 100% N_2 (results not shown). When the time of pretreatment with antimycin A was increased from 5 min to 10 min, the transient response was further inhibited, but a significant response still remained (Fig. 1B). The response was not further decreased by the omission of extracellular calcium (Fig. 1C). This finding shows that the response resistant to antimycin A was the transient phase of the ^{86}Rb efflux response, which does not require extracellular calcium.

The effects of antimycin A on net ^{45}Ca influx in dispersed parotid cells were determined. The results are

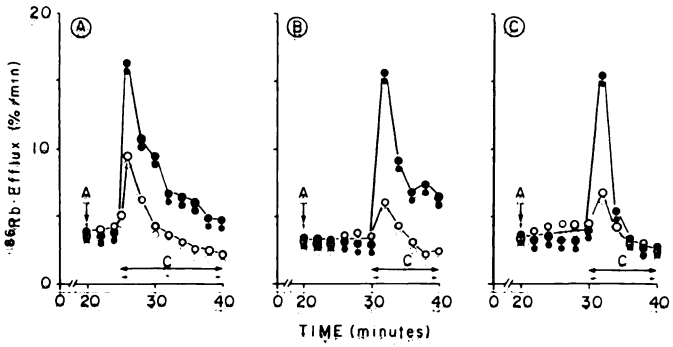

FIG. 1. *Effect of antimycin A on cholinergic stimulation of ^{86}Rb efflux from rat parotid gland slices*

Unidirectional efflux of ^{86}Rb was measured as described under Methods (for details, see ref. 6). Each curve represents the means from four experiments; standard errors of the mean averaged less than 10% of the means. Carbachol (100 μM) was added from 25 to 40 min (A) or from 30 to 40 min (B and C). In C, the medium contained 10^{-4} M EGTA and no added calcium. ●, Control; ○, 10 μM antimycin, 20–40 min.

TABLE 1

Stimulation of net ^{45}Ca influx by carbachol (100 μM) and ionomycin (2.67 μM) in parotid acinar cells

For experimental details see text. Values are means of net influx ± standard error of the mean ($n = 4$).

	Control	Carbachol	Ionomycin
	pmoles/mg protein/min		
+ Antimycin A	11.5 ± 7.2	−6.2 ± 7.1	722.5 ± 191.1[a]
− Antimycin A	33.0 ± 8.0	101.0 ± 10.6[a]	Not done

[a] Significantly greater than control.

summarized in Table 1. Cells were incubated in medium containing ^{45}Ca for 55 min, after which time readily exchangeable calcium pools are in a quasi-steady state (8). At 55 min, 10 μM antimycin A was added to some of the cells. At 60 min, 100 μM carbachol was added. Antimycin A did not increase the slow resting influx rate normally obtained under quasi-steady state conditions. Antimycin A blocked the stimulation of net ^{45}Ca influx induced by carbachol (Table 1). However, in the continued presence of antimycin A, the calcium ionophore ionomycin (75–85 min) induced a rapid ^{45}Ca influx in excess of 700 pmoles/mg of protein per minute (Table 1). Similar effects and interactions of antimycin A and the calcium ionophore A23187 on calcium fluxes have been described for the exocrine pancreas by Stolze and Schulz (13).

In experiments summarized in Table 2, cells were again incubated in ^{45}Ca for 55 min, at which time excess (10 mM) EGTA was added to the suspension. Under these conditions, only ^{45}Ca extrusion is seen, and agonist-induced release of ^{45}Ca is most clearly observed (8). When 100 μM carbachol was added to the suspension 5 min after the addition of 10 μM antimycin A, no acceleration of efflux was obtained; however, antimycin A alone caused a significant increase in ^{45}Ca efflux (Table 2).

The relative effects of antimycin A on muscarinic receptor binding were determined with [^{3}H]QNB. This ligand requires approximately 60 min to label muscarinic receptors on parotid cells to equilibrium (9). However, it was of interest in these studies to determine the effects of shorter times of exposure to antimycin A (5–10 min). Longer exposures to antimycin A caused morphological changes[4] that might result in receptor alterations not relevant to the effects seen in shorter incubations. Thus, in these experiments the quantity of [^{3}H]QNB bound in 10 min was measured assuming that substantial effects of antimycin A on receptor affinity or number would be revealed proportionately in this value. The data in Table 3 show that the addition of 10 μM antimycin A 5 min prior to the addition of [^{3}H]QNB inhibited binding by about 15%. This presumably represents a maximal estimate of loss in receptor number assuming no effect on QNB affinity; if QNB affinity is impaired, the loss of sites could be somewhat less. The fraction of receptors occupied by an agonist, in this case 1 μM methacholine, was also determined. In the cells treated with antimycin A, methacholine (1 μM) occupancy was inhibited by about 50%, which presumably reflects a decrease in receptor affinity for this agonist. When the agonist concentration

[4] B. A. Leslie and J. W. Putney, Jr., unpublished observations.

TABLE 2

Effect of antimycin A (10 μM) on stimulation of ^{45}Ca efflux by carbachol (100 μM)

For experimental details see text. Values are means of net efflux of ^{45}Ca ± standard error of the mean ($n = 4$). Radioactivity was converted to apparent mass by using the specific radioactivity of the ^{45}Ca loading medium.

	Control	Carbachol
	pmoles/mg protein/min	
+ Antimycin A	57.7 ± 4.5[a]	46.2 ± 12.5
− Antimycin A	26.2 ± 5.7	105.5 ± 37.8[b]

[a] Significantly greater than in the absence of Antimycin A.
[b] Significantly greater than control.

was increased to 100 μM, methacholine caused complete inhibition of specific [^{3}H]QNB binding with or without antimycin A (three experiments; data not shown).

The effects of antimycin A and a cholinergic stimulus (methacholine) on [^{32}P]PI breakdown and [^{32}P]PA synthesis are shown in Figs. 2 and 3. The protocol described under Methods was used by which a stimulus is employed during the prelabeling period, and $^{32}PO_4$ is "chased" with a 1-hr incubation in nonradioactive phosphate. This procedure apparently does not completely eliminate radioactivity from ATP because the net synthesis of [^{32}P]PA due to methacholine is still apparent (Fig. 3). However, it does increase the specific radioactivity of PI to a sufficient extent such that the net breakdown of [^{32}P]PI due to receptor activation is not totally obscured by resynthesis (Fig. 2).

The data in Fig. 2 confirm earlier reports (14, 15) that cholinergic agonists induce a net decrease in tissue PI (15–20%). The presence of 10 μM antimycin A (added 5 min before the addition of methacholine) prevented any statistically significant loss of [^{32}P]PI. There was no apparent effect of antimycin A on the retention of $^{32}PO_4$ by PI in the absence of methacholine (Fig. 2). A similar result was obtained by Hokin (16) for the effects of 2,4-dinitrophenol on PI breakdown in exocrine pancreas.

The data in Fig. 3 show that antimycin A accelerated the loss of $^{32}PO_4$ from PA under control conditions. There was no significant effect of methacholine on [^{32}P]PA in the presence of antimycin A. In the absence of antimycin A, methacholine provoked a rapid increase (about 50%) in PA radioactivity (Fig. 3), qualitatively confirming earlier reports (12, 17).

The effects of antimycin A on metabolism of PIP and PIP_2 were investigated under near steady-state labeling conditions rather than with a pulse-chase procedure as used for PI and PA. Thus, cells were incubated in 10 μM

TABLE 3

Effect of antimycin A (10 μM) on muscarinic-cholinergic receptor binding in rat parotid acinar cells

Values are means ± standard error of the mean from nine experiments.

	Control	+ 10 μM Antimycin A
[^{3}H]QNB specifically bound (fmoles/mg P)	89.1 ± 4.2	76.2 ± 3.6[a]
% Occupancy by methacholine (1 μM)	55.9 ± 3.0	21.3 ± 2.7[a]

[a] Significantly less than control.

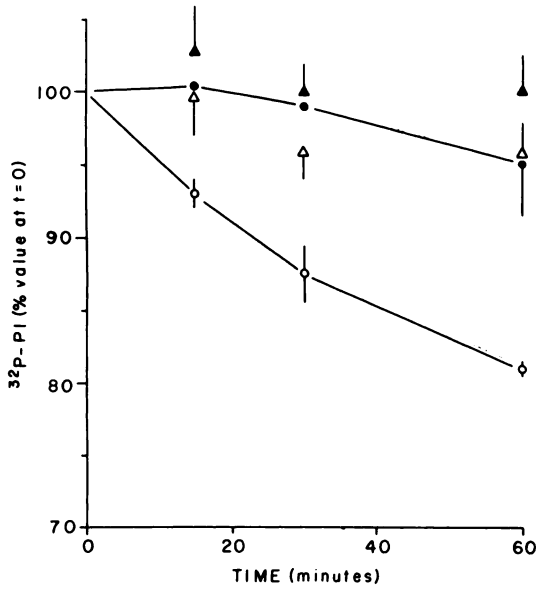

FIG. 2. *Effects of cholinergic stimulation and antimycin A on PI breakdown*

Cellular PI was made radioactive with a $^{32}PO_4$ pulse-chase procedure described under Methods. ●, ▲, control; △, ○, 100 μM methacholine at $t = 0$; ●, ○, unpoisoned cells; ▲, △, 10 μM antimycin 5 min prior to $t = 0$. Results are means from four experiments ± standard error of the mean.

$^{32}PO_4$ for 60 min, at which time radioactivity in PIP and PIP_2 reaches a plateau (10). At this time net change in radioactivity in these lipids presumably reflects net changes in tissue content and, as long as $^{32}PO_4$ is present, ATP specific radioactivity will be constant (10, 12).

The addition of 10 μM antimycin A to parotid acinar cells caused a rapid and immediate net loss of PIP and PIP_2 (Fig. 4). The kinetics of this effect appeared complex. There were small fractions of labeled PIP (about

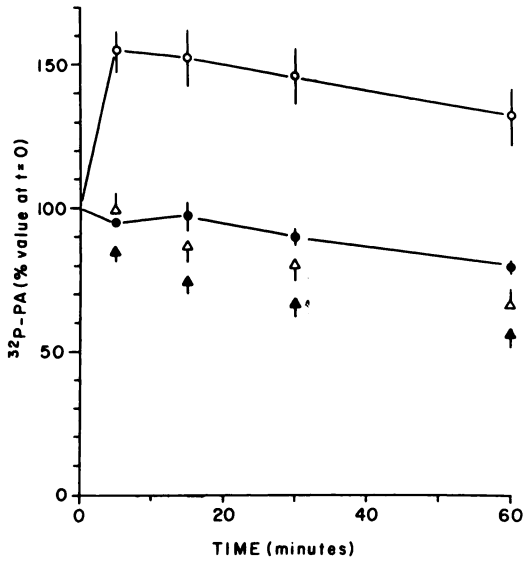

FIG. 3. *Effects of cholinergic stimulation and antimycin A on PA synthesis*

Cellular PA was made radioactive with a $^{32}PO_4$ pulse-chase procedure described under Methods. ●, ▲, control; ○, △, 100 μM methacholine at $t = 0$; ●, ○, unpoisoned cells; ▲, △, 10 μM antimycin A 5 min prior to $t = 0$. Results are means from four experiments ± standard error of the mean.

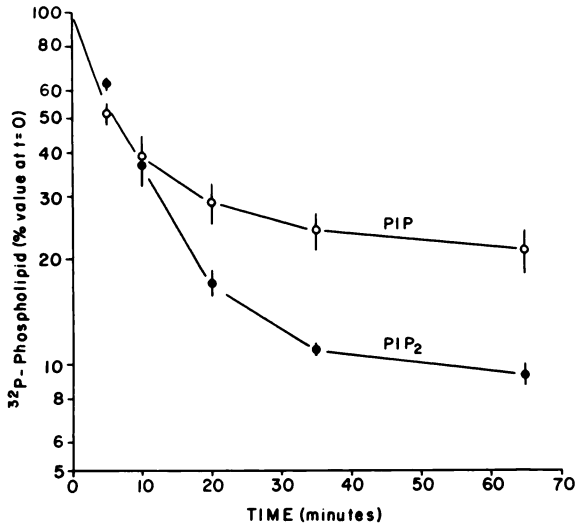

FIG. 4. *Effect of antimycin A on $^{32}PO_4$-labeled PIP and PIP_2*

Cellular PIP and PIP_2 were labeled to steady state (60 min) with $^{32}PO_4$ as described under Methods. Antimycin A (10 μM) was added at $t = 0$. Results are means ± standard error of the mean from four experiments.

20%) and PIP_2 (about 10%) that appeared resistant to antimycin A. When cells were pretreated with antimycin A for 5 min, methacholine caused an acceleration of PIP_2 breakdown. The effect was most apparent 1 min after methacholine and was still statistically significant 5.0 min after methacholine (Table 4). Net breakdown of PIP was not accelerated by methacholine (Table 4).

DISCUSSION

In this report we describe a series of effects on rat parotid cells of brief (5–10 min) exposures to 10 μM antimycin A. This concentration of antimycin A completely blocks parotid O_2 consumption in less than 10 sec.[5] The results of this study are discussed in the belief that they reflect the effects of a decrease in intracellular ATP. Although this seems a reasonable working hypothesis, it is at present unproven because (a) ATP levels have not been measured and (b) the possibility exists that antimycin A could have other direct pharmacological effects unrelated to blockade of respiration. However, relevant to the first point Bdolah and Schramm (18) and Feinstein and Schramm (19) have demonstrated that parotid tissue appears to be completely dependent on oxidative phosphorylation for energy production, and that enzyme secretion ceases immediately on addition of other mitochondrial poisons. As discussed below, the rapid fall in cellular PIP and PIP_2 content induced by antimycin A would suggest that ATP levels fall quickly after respiration is blocked.

In analyzing the effects of antimycin A on ^{86}Rb efflux (Fig. 1), an understanding of the biphasic nature of receptor-activated responses in the parotid is necessary. Previous studies have shown that activation of muscarinic-cholinergic receptors in the rat parotid gland causes a biphasic increase in membrane permeability to K^+. A receptor-activated mobilization of calcium is believed to mediate this effect (20, 21). The effect manifests itself in

[5] J. W. Putney, Jr., unpublished observation.

TABLE 4

Effect of methacholine (100 μM) on breakdown of radioactive PIP and PIP_2 in parotid acinar cells, with or without antimycin A (10 μM) added 5 min previously

Values are means of percentage decrease ± standard error of the mean (n = 4) from samples taken just prior (<2 sec) to methacholine addition.

Time after methacholine	Antimycin	PIP		PIP_2	
		Control	Methacholine	Control	Methacholine
1.0 min	+	9.0 ± 2.7	10.8 ± 1.9	7.0 ± 3.6	23.7 ± 3.1[a]
	−	−4.0 ± 2.0	4.0 ± 3.0	−6.3 ± 4.9	29.6 ± 2.3[a]
5.0 min	+	26.1 ± 7.0	28.0 ± 4.5	44.7 ± 4.9	58.4 ± 1.2[a]
	−	−7.0 ± 5.0	0.0 ± 3.0	10.4 ± 4.8	46.6 ± 2.7[a]

[a] Significantly greater than control.

the experiments shown in Fig. 1 as an accelerated efflux of ^{86}Rb. The rapid increase in ^{86}Rb efflux occuring during the first 2 min after drug application does not require the presence of extracellular calcium. It is presumed to result from the intracellular release of calcium (22), which has been suggested to come from the plasma membrane (8). Following this initial transient phase, a sustained or slowly falling phase follows which requires the presence of millimolar extracellular concentrations of calcium. This phase of the response is believed to be mediated by calcium entering the cell through receptor-activated calcium channels or gates (2, 6).

Thus one interpretation of the results in Fig. 1 would be that antimycin A inhibits partially that phase of the response resulting from calcium release and inhibits completely the phase due to calcium gating. That the small residual ^{86}Rb efflux response does in fact represent the calcium release phase of the response is demonstrated by the failure of omission of calcium to reduce the response (compare Fig. 1C with 1B).

A number of explanations are possible for this effect. In terms of our present understanding of the cellular events involved, antimycin A could (a) prevent the occupancy of the receptor by agonist, (b) prevent receptor-mediated calcium mobilization, (c) prevent activation of K^+ channels by calcium, or (d) completely disrupt cell and membrane integrity resulting in the destruction of all ionic gradients. Alternative d seems unlikely, since antimycin A did not appreciably affect basal permeability to K^+ (Fig. 1) or calcium (Table 1). Alternative a seems unlikely as well. Antimycin caused a loss of 15% of muscarinic receptors, at the most (Table 3). However, the parotid has a considerable excess of muscarinic receptors such that only about 10% need be occupied to activate maximal calcium mobilization under normal conditions (9). Also, the apparent decrease in receptor affinity (about 2-fold; Table 3) is not sufficient to interfere with a receptor occupancy by agonists when used in supramaximal concentrations (100 μM). However, these subtle changes in receptor binding properties may suggest an alteration in the ability of the receptor to couple to the appropriate effector, whatever that may be. Alternative c cannot be ruled out completely but cannot explain the entire effect, since a component of the transient phase remains. This suggests that some responsiveness of the K^+ channels to calcium remains intact.

For the inhibition of the calcium gating phase of the response, the ^{45}Ca flux data strongly support Alternative b: that antimycin A disrupts the mechanism of activation of calcium gating by occupied receptors. Thus, antimycin A completely prevented the stimulation of net ^{45}Ca uptake by carbachol. The fact that a subsequent application of an ionophore causes rapid ^{45}Ca uptake suggests that a considerable inward gradient for ^{45}Ca still exists in the presence of antimycin A. Also, when extracellular calcium is chelated with excess EGTA, a net outward calcium gradient should exist. Accordingly, any increase in membrane permeability to calcium due to carbachol should result in an accelerated rate of efflux; again, however, no effect was observed (Table 2).

In the ^{45}Ca efflux experiments, one might expect some acceleration of ^{45}Ca efflux (as seen in previous studies; ref. 8) due to intracellular calcium release. The ^{86}Rb experiments suggest that some release should still occur. However, net ^{45}Ca loss under such conditions requires not only ^{45}Ca release internally, but probably also requires active extrusion of the ^{45}Ca by an ATP-dependent pump. This active extrusion mechanism may not be fully operable in antimycin A-treated cells. This is consistent with the ionophore effect in Table 1; in unpoisoned cells, ionomycin causes considerably less gain of ^{45}Ca (23), suggesting that antimycin A has inhibited active calcium extrusion.

Summarizing to this point, then, these data would suggest that a 5-min incubation of parotid acinar cells in the presence of 10 μM antimycin results in a partial failure of receptor occupation to cause cellular calcium release, and a complete failure of receptor-mediated membrane calcium gating.

Many, if not all, of the effects of antimycin A on labeled phospholipids measured in these experiments may result from a lack of cellular ATP. The most obvious of such effects are (a) the rapid fall in tissue content of PIP and PIP_2 (Fig. 4) and (b) the failure of cholinergic stimulation to cause stimulation of net [^{32}]PA synthesis (Fig. 3). The effects of antimycin A on agonist-stimulated PIP_2 breakdown are more difficult to analyze. As soon as antimycin A is added, PIP_2 radioactivity begins to fall at a rate of about 7–8%/min, which presumably reflects a cessation of PIP_2 synthesis. Note that the initial rate of agonist-stimulated net PIP_2 breakdown in unpoisoned cells is much faster, in the range of 30–50%/min (ref. 10; Table 4). This suggests that the agonist effect is at least in part due to stimulation of PIP_2 degradation and is not due solely to an inhibition of synthesis.

When methacholine was added to cells 5 min after antimycin A, decay of PIP_2 was significantly accelerated, while PIP was unaffected (Table 4). This result is quali-

tatively similar to that reported previously for unpoisoned cells (10). However, the magnitude of the effect was diminished considerably. In unpoisoned cells, methacholine causes a net breakdown of 45–50% of labeled PIP_2 (ref. 10, Table 4). Here, the increase in breakdown (minus the control rate) was only 17% of the radioactivity remaining after 5 min in antimycin A. Since the data in Fig. 4 show that only 63% of labeled PIP_2 remains after 5 min in antimycin A, the net breakdown caused by methacholine would be only about 10% if referred to the initial tissue radioactivity. Thus, about all that can be said of this result is that after a 5-min treatment with antimycin A, cells can still respond to methacholine with a brief acceleration of PIP_2 breakdown, but the magnitude of this effect appears diminished when compared with the effect is unpoisoned cells. One possible explanation for this is that the pool of PIP_2 which is sensitive to receptor activation is only a part of the total radioactive PIP_2. If, in the poisoned cells, this pool of PIP_2 is lost more rapidly than other pools, then responsiveness to stimulation would decline faster than total PIP_2 radioactivity.

Breakdown of PI due to cholinergic stimulation is not statistically detectable in antimycin A-treated cells. If PI breakdown is an event directly linked to receptor activation, then there would seem to be no readily apparent explanation for this effect. However, Kirk *et al.* (24) have raised the possibility that PI breakdown may be an event secondary to PIP_2 breakdown. Thus, in unpoisoned cells, PI would be consumed by sequential phosphorylation to form PIP_2, which would be continually broken down directly. This idea is consistent with the results obtained here, since any metabolic linkage between PI and PIP_2 would require a continuous supply of ATP. However, it should be mentioned that the measurement of PI loss is inherently less precise than the measurement of PIP_2 loss, and thus small effects of brief duration might well pass undetected.

In summarizing to this point, the data suggest that a 5-min exposure of parotid cells to antimycin A causes a rapid net loss of polyphosphoinositides, partially inhibits the acceleration of PIP_2 breakdown due to cholinergic stimulation, and completely blocks cholinergic stimulation of PA synthesis and (possibly) PI breakdown. Since this metabolic poison also caused a substantial impairment of receptor activated ion movements, these results are compatible with the idea that receptor-mediated effects on phospholipids may be necessary for the coupling of receptor occupation to calcium mobilization and subsequent calcium-mediated responses.

Speculating further, in recent reports we and others have raised the possibilities that (a) the receptor-mediated release of calcium from the parotid plasma membrane may result from the breakdown of PIP_2 (10), and (b) that receptor-mediated calcium gating may be mediated by PA functioning as a calcium ionophore (25, 26). The results reported here are consistent with the latter idea, since the 5-min incubation in 10 μM antimycin A appeared to cause a complete block of PA synthesis (Fig. 3) and calcium gating (Fig. 1; Tables 1 and 2). The involvement of PIP_2 in calcium release is more difficult to analyze. The net breakdown of PIP_2 due to methacholine was partially inhibited by antimycin A, as was cellular calcium release (Fig. 1; Table 2). The accelerated breakdown of PIP_2 due to antimycin A was also associated with an elevation in calcium efflux rate (Table 2), but did not alone trigger any ^{86}Rb response (Fig. 1). This may be due to the fact that antimycin A-induced PIP_2 breakdown is considerably slower than receptor-induced breakdown.

The results of this study bear relevance to a recently published criticism of the phosphoinositide-calcium gating hypothesis (5). It was suggested that stimulated phospholipid turnover should not be necessary to open calcium gates, since thermodynamically calcium entry could proceed without intervention of complex metabolic reactions and thus such a scheme would not be energetically economical. The results reported here, while not directly linking the calcium mobilization process to phosphoinositide metabolism, clearly indicate a metabolic component in the process of calcium gating. That is, arguments of cellular economics notwithstanding, it would appear that cellular ATP is absolutely required for coupling receptor occupation to the activation of membrane calcium gates.

Pertinent to this same argument are the results of a previous study relating stimulation of parotid cell O_2 consumption to receptors and calcium mobilization (27). In this prior study, agonists acting on calcium-mobilizing receptors stimulated O_2 consumption by 40–50%. The evidence suggested that this increase in O_2 consumption is a calcium-mediated response (27). However, the phosphoinositide response in the parotid is clearly not a calcium-mediated response (10, 12, 28). This would suggest that the energy cost of the phosphoinositide effect is quite small compared with the energy expended in cellular events arising subsequent to calcium mobilization. A similar argument, raised on theoretical grounds, has been given by Michell (29).

The major conclusions of this report may be summarized as follows. Antimycin A, a penetrating inhibitor of mitochondrial respiration, when applied at 10 μM concentration to parotid acinar cells, blocks activation by cholinergic stimuli of calcium gating and PA synthesis. In addition, receptor-mediated calcium release and PIP_2 breakdown appear to be partially, but not completely, inhibited. Presumably, these effects result from an inability of the cells to synthesize ATP. The results of this study add to the body of circumstantial evidence relating receptor-mediated phosphoinositide turnover and cellular calcium mobilization in the rat parotid gland.

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