MOLECULAR PHARMACOLOGY, 26:261–266

Phorbol Ester-Induced Protein Secretion in Rat Parotid Gland

Relationship to the Role of Inositol Lipid Breakdown and Protein Kinase C Activation in Stimulus-Secretion Coupling

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Received February 1, 1984; Accepted April 16, 1984

SUMMARY

When added to rat parotid gland slices incubated *in vitro*, 4$^{\alpha}$-phorbol-dibutyrate (PDBu) induced a dose-dependent increase in protein secretion, but did not affect membrane permeability to K^+ (as determined by ^{86}Rb efflux). The response to PDBu was unaffected by the removal of extracellular Ca^{2+} and was not markedly potentiated by incubation with the phosphodiesterase inhibitor, methylisobutylxanthine. PDBu did not activate phospholipase C breakdown of inositol lipids as shown by a failure to increase formation of soluble inositol phosphates. When applied in combination with the Ca^{2+} ionophore, ionomycin, a secretory rate was obtained that was greater than the predicted sum of rates obtained when the two drugs were given alone. These results, when taken with the reported results of others, are consistent with an action of PDBu in activating protein kinase C and suggest that this enzyme plays an important role in the pathway linking receptor activation to protein secretion, but not K^+ flux, in the parotid gland.

INTRODUCTION

The rat parotid gland can be stimulated to secrete amylase and other proteins through any of four known receptor mechanisms: *alpha*-adrenergic, *beta*-adrenergic, muscarinic-cholinergic, or substance P (1). The *beta*-adrenoceptor mechanism is believed to involve cyclic AMP as secondary messenger (1), although evidence has been presented for a role of Ca^{2+} in stimulus-secretion coupling distal to cyclic AMP formation (2). The muscarinic-, *alpha*-adrenergic-, or substance P-induced enzyme secretion appears to involve Ca^{2+} more directly (1). In addition, these Ca^{2+}-mobilizing agonists induce a Ca^{2+}-dependent K^+ efflux response which is believed to reflect ionic fluxes involved in water secretion (1). These latter three receptors also induce the degradation of inositol lipids in the parotid by a phospholipase C mechanism (3, 4). Nishizuka and his colleagues (5, 6) have suggested that the diacylglycerol formed from phosphoinositide breakdown can serve to activate protein kinase C which catalyzes phosphorylation of a protein important in triggering exocytosis. Such a proposal is reasonable for the parotid gland, as previous reports have provided circumstantial evidence implicating protein phosphorylation in stimulus-secretion coupling in this system (7, 8).

The phorbol esters induce secretion from a variety of cell types (9, 10). Recent studies have provided evidence that the phorbol esters produce their characteristic effects by binding to, and activating, protein kinase C (11, 12). The phorbol esters, therefore, may be useful pharmacological tools for determining the role of protein kinase C in cellular responses. Here we describe studies of the action of a phorbol ester, PDBu,[1] in rat parotid gland. The results suggest a role for protein kinase C in protein secretion, but not in the K^+ release response.

METHODS

All experiments were caried out with slices of parotid gland taken from pentobarbital-anesthetized male rats and incubated in Tris-buffered Ringer's medium with 2 mM $CaCl_2$ at 37° under 100% O_2, as described previously (13–15). The technique for measuring ^{86}Rb efflux (13), the method for pulse-chase labeling secretory proteins to measure protein secretion (15), and the techniques used for preservation of tissue for morphological studies (14) have also been described previously. Formation of [^{3}H]inositol phosphates by dispersed parotid cells was determined with recently published techniques (16, 17).

Phorbol esters were obtained from Sigma Chemical Company (St. Louis, Mo.), and were dissolved in Me_2SO to a concentration of 20 mM. On final dilution in Ringer's solution, the Me_2SO the DMSO never exceeded 0.1%, which was included in controls and at which concentra-

These studies were supported in part by National Institutes of Health Grant DE-05764. Electron microscopy for this study was done with a Zeiss EM 10CA transmission electron microscope purchased with funds from Biomedical Research Support Grants 2507RR05724, 1897RR05430, and 2507RR05697.

[1] The abbreviations used are: PDBu, 4$^{\alpha}$-phorbol-dibutyrate; Me_2SO, dimethyl sulfoxide; MIX, methylisobutylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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tion these and previous studies have shown Me_2SO to be pharmacologically inert. Methacholine and MIX were also obtained from Sigma Chemical Company. Ionomycin was kindly donated by S. J. Lucania, of the Squibb Institute for Medical Research (Princeton, N. J.). The ^{86}Rb and [^{3}H]leucine were purchased from New England Nuclear Corporation (Boston, Mass.).

Summarized data are reported as arithmetic means; dispersions shown indicate ± 1 SEM and in general are included only when they exceed 10% of the mean. Statistical analyses were performed by analysis of variance or by Student's *t*-test, paired or unpaired, as appropriate. Values for F or t were considered statistically significant when they exceeded those tabulated for $p = 0.05$.

RESULTS

PDBu stimulated ^{3}H-protein secretion from rat parotid gland over the concentration range 0.2–20 μM (Fig. 1A). Another phorbol ester, phorbol myrystate acetate, caused significant stimulation only at 20 μM (Fig. 1B), whereas phorbol had no effect at 20 μM (data not shown). ^{86}Rb efflux was not affected by 20 μM PDBu. The drug did not increase basal ^{86}Rb efflux nor did it significantly affect the response to methacholine (Fig. 2).

Possible roles of Ca^{2+} or cyclic nucleotides in the secretory response to PDBu were investigated in experiments summarized in Figs. 3 and 4. In the absence of extracellular Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA), 20 μM PDBu stimulated protein secretion at a rate similar to that obtained in the presence of 2 mM Ca^{2+} (Fig. 3). Secretion due to 100 μM methacholine was significantly, but only partially, inhibited by Ca^{2+} omission (Fig. 3).

The phosphodiesterase inhibitor, MIX (0.1 mM), slightly but significantly increased ^{3}H-protein secretion. When applied in combination with 20 μM PDBu, a slightly (approximately 50%) greater than additive effect was obtained (Fig. 4B). A similar result was obtained in two experiments with a limiting (1 μM) concentration of methacholine (Fig. 4C). In contrast, 0.1 mM MIX caused a >10-fold augmentation of secretion due to a limiting concentration (0.1 μM) of the *beta*-adrenoceptor agonist, isoproterenol (Fig. 4A).

Possible interactions between PDBu, the cholinergic agonist methacholine, and the Ca^{2+}-ionophore ionomycin were examined. When applied in combination, the effects of methacholine (1 μM) and PDBu (2 μM) or methacholine (1 μM) and ionomycin (2.67 μM) were less than additive (data not shown). However, the combination of PDBu and ionomycin resulted in a secretory response substantially greater than the predicted sum of responses of the two agents given alone (Fig. 5). When PDBu was applied with ionomycin and ^{86}Rb efflux was monitored, no such potentiation was seen (Fig. 6).

As argued under Discussion, these results can be taken to indicate that ionomycin does not provide a complete (or optimal) message for protein secretion, but does for ^{86}Rb efflux. Thus a quantitative comparison was made of the relative abilities of supramaximal concentrations (18, 19) of methacholine and ionomycin to stimulate protein secretion and ^{86}Rb flux. The results (Table 1) indicate that, relative to methacholine, ionomycin is a more efficient agonist for activating ^{86}Rb efflux than protein secretion. Ionomycin (13.3 μM) caused a slightly but significantly greater efflux of ^{86}Rb than did 0.1 mM methacholine, but the ionophore caused significantly less protein secretion (Table 1).

The possible ability of PDBu to activate phospholipase C degradation of inositol lipids was determined by examining formation of the water-soluble products of inositide breakdown, inositol phosphates. LiCl (10 mM) was used to prevent degradation of inositol-1-phosphate (20) and thereby to assure that a transient activation would not be missed. Following 30 min of stimulation, methacholine (100 μM) caused substantial increases in the radioactivity associated with all three of the inositol phosphate fractions; however, PDBu (20 μM) had no effect (Table 2).

The effects of PDBu on morphology of the parotid gland were examined. Electron micrographs of parotid slices incubated under control conditions and for 10 min in the presence of 20 μM PDBu are shown in Fig. 7. There was a marked increase in the presence of exocy-

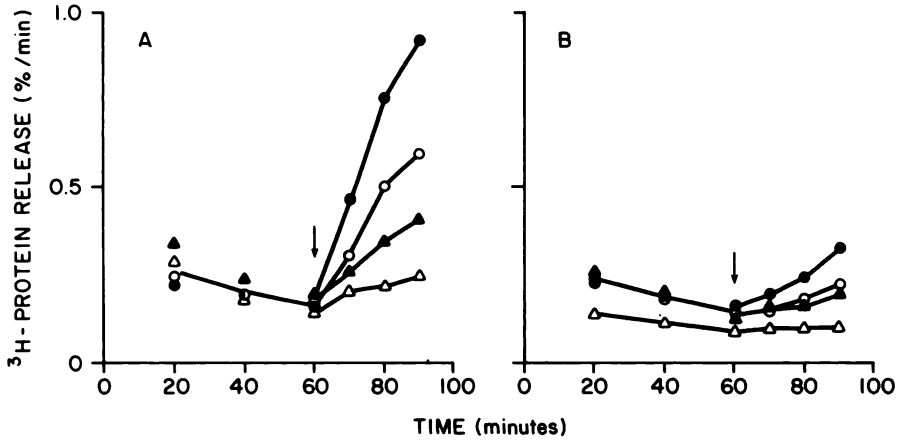

FIG. 1. *Induction of ^{3}H-protein secretion by phorbol esters*

Parotid slices whose secretory proteins were prelabeled with [^{3}H]leucine were transferred through a series of 20- or 10-min incubations, and rates of ^{3}H-protein release were measured as described previously (15). At 60 min, PDBu (A) or phorbol myristate acetate (B) was added at the following concentrations (micromolar): ●, 20; ○, 2; ▲, 0.2; △, 0.02. Each point represents the mean of three experiments; standard errors averaged 10–20% of the means.

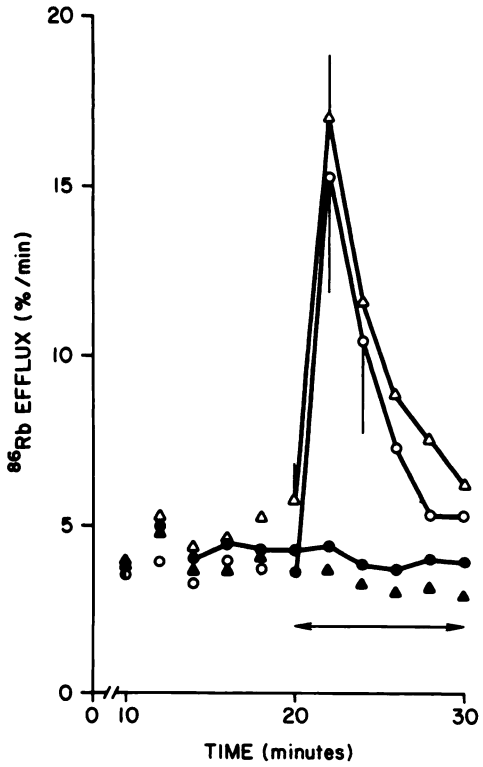

FIG. 2. *Effects of methacholine and PDBu on ^{86}Rb efflux*

Efflux of ^{86}Rb was measured as described previously (13). Agonists were present from 20 to 30 min. ●, Control (no agonist); ○, 100 μM methacholine; ▲, 20 μM PDBu; △, 100 μM methacholine + 20 μM PDBu. Each point represents the mean of three experiments; standard errors, where not shown, were less than 10% of the means.

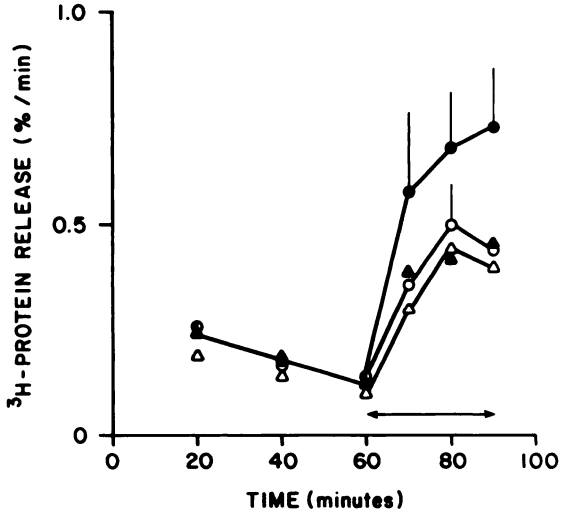

FIG. 3. *Effects of Ca^{2+} omission on ^{3}H-protein secretion due to methacholine or PDBu*

The protocol was similar to that for Fig. 1. ●, ▲, 2 mM Ca^{2+} present; ○, △, 40–90 min, no added Ca^{2+} + 10^{-4} M EGTA. Agonists were present from 60 to 90 min. ○, ●, 100 μM methacholine; △, ▲, 20 μM PDBu.

totic figures associated with the luminal membrane in the PDBu-treated tissues. Other aspects of cell ultrastructure were apparently unaffected by the drug. Characteristic morphological changes induced by cholinergic stimulation of the parotid gland such as changes in mitochondrial configuration, Golgi vacuolation, and condensation of nuclear chromatin (14) were not observed in PDBu-treated cells.

DISCUSSION

The phorbol ester used in this study, PDBu, activates protein secretion by a mechanism that appears different from the previously described Ca^{2+}-mobilizing or adenylate cyclase-activating agents. The concentrations of PDBu required for optimal responses appear somewhat greater than those required in other systems, and this may be due to diffusion barriers in the slice system. In support of this, dispersed parotid acinar cells have been found to be about 1 order of magnitude more sensitive to the drug than are slices.[2] The final secretory response is apparently due to exocytosis rather than a nonspecific toxic effect of the drug, since morphological studies showed no noticeable effects on cell ultrastructure with the exception of exocytotic figures (Fig. 7) and since PDBu did not affect basal ^{86}Rb flux (Figs. 2 and 6). The most efficient mechanism for induction of parotid enzyme secretion is through *beta*-adrenoceptor activation of adenylate cyclase (1). However, although the phosphodiesterase inhibitor, MIX, markedly potentiates isoproterenol-induced secretion, PDBu-induced secretion was little affected, indicating that PDBu probably does not act by activation of adenylate cyclase.

In the rat parotid gland, protein secretion can also be stimulated through activation of muscarinic (cholinergic), *alpha*-adrenergic, or substance P receptors (1). These receptors cause a phospholipase C degradation of polyphosphoinositides and, it is believed, an elevation in cytosolic Ca^{2+} concentration (1, 4). Recent reports suggest that the solubilized headgroup of the cleaved inositide may be the signal for internal Ca^{2+} mobilization (21, 22). However, PDBu did not appear to activate phosphodiesteratic breakdown of inositol lipids or Ca^{2+} mobilization in the parotid gland. There was no augmented formation of [^{3}H]inositol phosphates by PDBu (Table 2), which is probably the most sensitive indicator of phospholipase C activity (20, 21). Furthermore, PDBu did not activate ^{86}Rb efflux or affect the ^{86}Rb efflux response to methacholine (Fig. 2). As the ^{86}Rb efflux is believed to reflect the activity of Ca^{2+}-sensitive K^{+} channels in the parotid gland, this finding argues that PDBu probably does not bring about a generalized increase in cytosolic Ca^{2+}. In support of this conclusion, secretion due to PDBu was not impaired by the omission of Ca^{2+} from the incubation medium (Fig. 3).

Thus, the data suggest that PDBu stimulates protein secretion without activating adenylate cyclase, phosphoinositide breakdown, or Ca^{2+} mobilization. These findings are consistent with reports for other secretory systems in which the effects of phorbol esters have been found to be relatively Ca^{2+}-independent (23, 24).

A mechanism previously suggested for phorbol ester-induced secretion in platelets (11) and neutrophils (24) involves activation of the Ca^{2+}-activated, phospholipid-dependent, protein kinase C (6). Nishizuka (6) has suggested that this enzyme plays a central role in secretory mechanisms. The sensitivity of the enzyme to Ca^{2+} is

[2] J. W. Putney, Jr., and J. S. McKinney, unpublished observation.

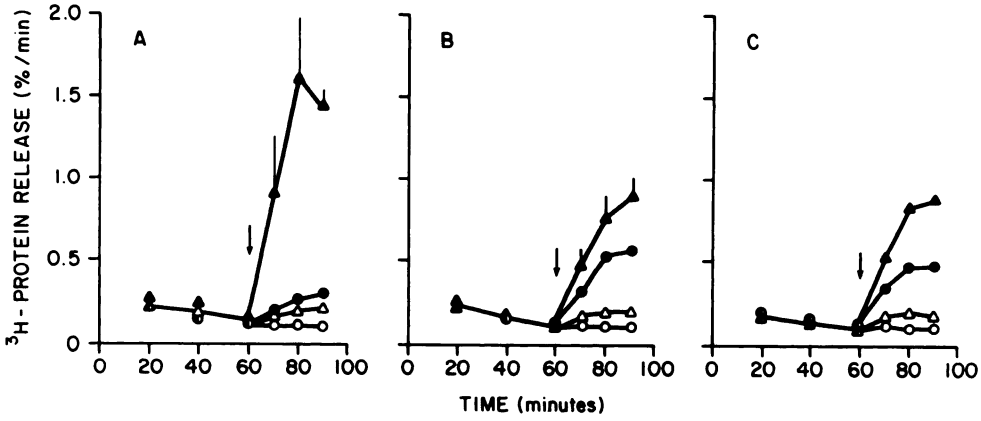

FIG. 4. *Effect of MIX on ^{3}H-protein secretion due to isoproterenol, PDBu, or methacholine*
The protocol was similar to that for Figs. 1 and 3. Agonists were added, with or without MIX, from 60 to 90 min and were as follows: A, isoproterenol (0.1 μM); B, PDBu (20 μM); C, methacholine (1 μM). ○, Control (no additions); △, MIX alone; ●, agonist alone; ▲, MIX + agonist. Each point represents the mean of three to six experiments, except for methacholine and methacholine + MIX, which are the means of two experiments; standard errors, where not shown, averaged 0.05%/min.

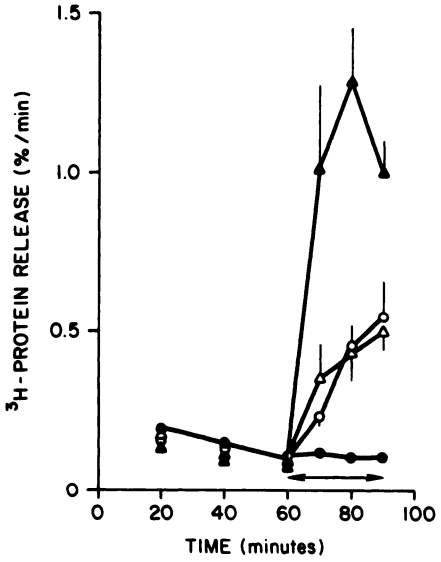

FIG. 5. *Effects of PDBu and ionomycin on ^{3}H-protein secretion*
The protocol was similar to that for Figs. 1, 3, and 4. Agonists were present from 60 to 90 min. ●, Control (no addition); ○, 2 μM PDBu; △, 2.67 μM ionomycin; ▲, 2 μM PDBu + 2.67 μM ionomycin. Each point represents the mean ± standard error of three experiments; standard errors for control were ≤0.02%/min.

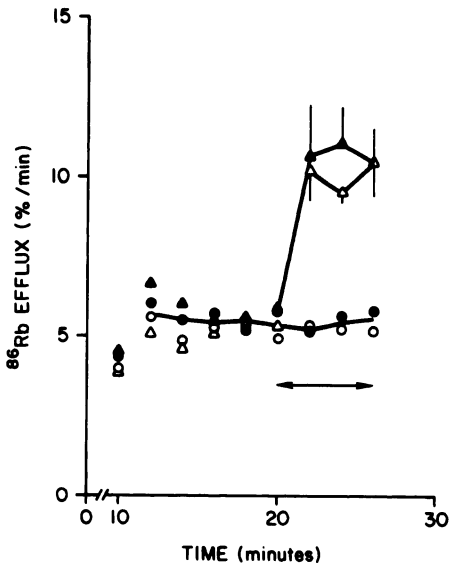

FIG. 6. *Effects of PDBu and ionomycin on ^{86}Rb efflux*
The protocol was similar to that for Fig. 2. Agonists were present from 20 to 26 min. ●, Control (no addition); ○, 2 μM PDBu; △, 2.67 μM ionomycin; ▲, 2 μM PDBu + 2.67 μM ionomycin. Each point represents the mean ± standard error for three experiments; For ● and ○, standard errors were <0.5%/min.

greatly enhanced by diacylglycerol, which is formed from inositol lipid breakdown following receptor activation (6, 25). Phorbol esters have been shown to activate protein kinase C, presumably in the same manner as diacylglycerol (11, 12). Accordingly, it has been suggested that the actions of phorbol esters in intact cells may be due entirely to activation of this enzyme (11, 12).

The results obtained in this study are totally consistent with this hypothesis. Enzyme secretion induced by methacholine is only partially inhibited in low Ca^{2+} media and continues at a significant rate under these conditions for 30 min (Fig. 3). There is a previously documented release of intracellular Ca^{2+} in the parotid gland with muscarinic receptor activation (1, 13); however, in the absence of extracellular Ca^{2+}, this pool is emptied in less than 5 min (13). This may suggest therefore that the muscarinic receptor mechanism generates a signal other than Ca^{2+} which participates in activating exocytosis. Diacylglycerol would seem to be the likely candidate for this signal, and protein kinase C would be its target. Mimicking of this Ca^{2+}-independent secretion by PDBu supports this idea. Furthermore, the failure of PDBu to affect ^{86}Rb efflux can be taken to indicate that Ca^{2+} activation of membrane K^+ channels does not involve protein kinase C.

An argument against a role for diacylglycerol and protein kinase C in secretion is that secretion can be stimulated by Ca^{2+} ionophores, which in the parotid gland, at least, do not activate phospholipase C (4). This apparent inconsistency can be resolved by suggesting that, under normal conditions, agonists provide both Ca^{2+} and diacylglycerol, which in some way act in concert

TABLE 1

Stimulation of ^{86}Rb efflux and ^{3}H-protein secretion by supramaximal concentrations of methacholine (100 μM) and ionomycin (13.3 μM)

Data were calculated as -fold increases over control to facilitate comparsions of ^{86}Rb flux to ^{3}H-protein secretion. Basal ^{3}H-protein secretion was 0.120 ± 0.008 %/min and basal ^{86}Rb flux was 5.37 ± 0.05 %/min. For ^{86}Rb flux, data during the first 2 min after stimulation were not included, to eliminate the large increase due to internal Ca^{2+} release which only occurs with receptor-active agonists (1). ^{86}Rb Flux was determined from 2 to 6 min after stimulation, and ^{3}H-protein, from 0 to 10 min. Values are means ± standard error of the mean; number of replications is shown in parentheses.

	Methacholine	Ionomycin
^{86}Rb Flux	2.07 ± 0.15 (4)	2.88 ± 0.16 (3)[a]
^{3}H-Protein secretion	5.49 ± 0.42 (6)	3.39 ± 0.19 (5)[b]

[a] Significantly greater than methacholine.
[b] Significantly less than methacholine.

TABLE 2

Effect of methacholine (100 μM) and PDBu (20 μM) on formation of [^{3}H]inositol phosphates in parotid acinar cells prelabeled with [^{3}H] inositol for 60 min, as described previously (16, 17)

Drugs were present for 30 min. LiCl (10 mM) was present to block inositol-1-phosphate phosphatase (20). Values are means ± standard error of the mean of three experiments.

	Control	Methacholine	PDBu
	(cpm/mg DNA) × 10^{-3}		
Inositol phosphate	5.8 ± 1.3	66.0 ± 7.9[a]	4.7 ± 1.1
Inositol bisphosphate	1.3 ± 0.5	33.8 ± 5.0[a]	1.3 ± 0.6
Inositol trisphosphate	1.5 ± 0.8	21.4 ± 3.7[a]	1.2 ± 0.7

[a] Significantly greater than control.

to bring about optimal rates of exocytosis. Two findings reported here support this view for the parotid gland. First, relative to methacholine, the Ca^{2+} ionophore, ionomycin, was found to be a somewhat more efficient inducer of ^{86}Rb efflux than was protein secretion (Table 1). This is as expected if optimal protein secretion (but not ^{86}Rb efflux) requires some diacylglycerol, which ionomycin cannot provide. Second, the combination of ionomycin and PDBu was found to be synergistic with respect to protein secretion, but not for ^{86}Rb efflux. This can be taken to indicate that this combination has in effect "reconstituted" the action of an agonist such as methacholine; the ionophore provides the Ca^{2+}, and the PDBu bypasses the normal diacylglycerol requirement by directly activating protein kinase C. Other investigators have recently reported a synergistic action of phorbol diesters and Ca^{2+} in activating secretion (26, 27). This synergism is consistent with the dual activation of the Ca^{2+} and protein kinase C pathways originally proposed by Nishizuka and colleagues (5, 6).

In summary, the data reported here show that the phorbol ester PDBu induces protein secretion in the rat parotid gland by a Ca^{2+}-independent mechanism. The results are consistent with the idea that phorbol esters directly activate the enzyme, protein kinase C, which plays a role in exocrine secretion. Furthermore, the data are interpretated to indicate that protein kinase C is important for protein secretion but not for Ca^{2+} activation of membrane K^{+} channels. It is thus suggested that in the parotid gland Ca^{2+} mobilizing secretagogues activate two signaling pathways that act in concert to induce protein secretion, these being diacylglycerol, which activates protein kinase C, and Ca^{2+}, whose molecular target remains a mystery. It is hoped that future investigation will clarify further the molecular mechanisms by which these and other intracellular mediators interact in regulating secretion in exocrine glands and other systems.

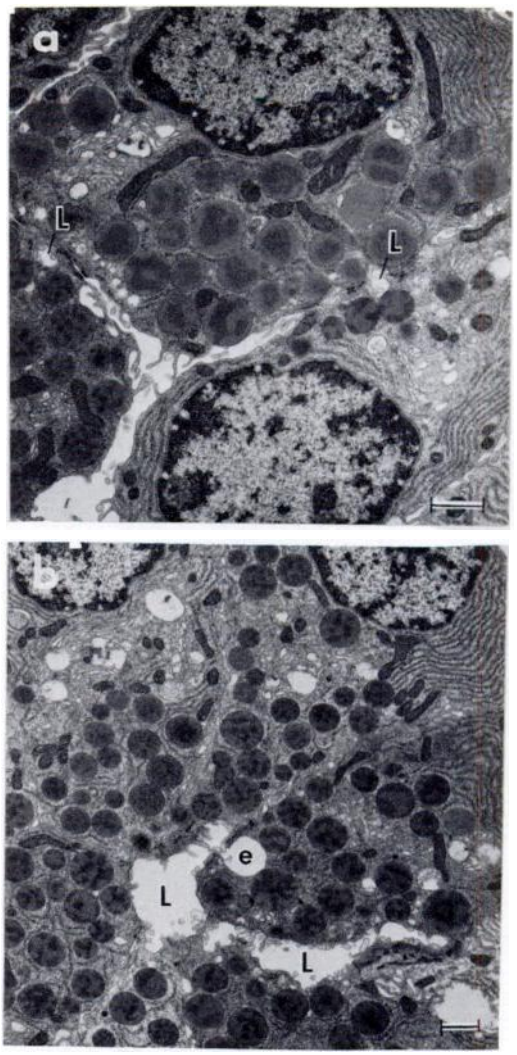

FIG. 7. *Electron micrographs of incubated parotid tissue slices*
The bar = 1 μm. a. Sample incubated under control conditions. Note especially the small lumina (*L*) (×6600). b. Sample incubated for 10 min in medium containing 2.0 μM PDBu. The only notable change in morphology is the evidence for exocytosis (*e*) of secretory granules into the lumen (*L*), enlarging the luminal space (×4600).

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