J. Membrane Biol. 144, 49–58 (1995)

The Journal of
Membrane
Biology

Lysophosphatidic Acid Induces Inositol Phosphate and Calcium Signals in Exocrine Cells from the Avian Nasal Salt Gland

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Received: 11 February 1994/Revised: 24 October 1994

Abstract. We tested lysophosphatidic acid (LPA), known to induce inositol phosphate generation and calcium signals as well as rearrangements of the cytoskeleton and mitogenic responses in fibroblasts, for its ability to activate phospholipase C in an exocrine cell system, the salt-secreting cells from the avian nasal salt gland. LPA (>10 nmol/l) caused the generation of inositol phosphates from membrane-bound phosphatidylinositides. The resulting calcium signals resembled those generated upon activation of muscarinic receptors, the physiological stimulus triggering salt secretion in these cells. However, close examination of the LPA-mediated calcium signals revealed that the initial calcium spike induced by high concentrations of LPA (>10 μmol/l) may contain a component that is not dependent upon generation of inositol (1,4,5)-trisphosphate (Ins(1,4,5)P_3) and may result from calcium influx from the extracellular medium induced by LPA in a direct manner. Low concentrations of LPA (<10 μmol/l), however, induce inositol phosphate generation, Ins(1,4,5)P_3-mediated release of calcium from intracellular pools and calcium entry. These effects seem to be mediated by a specific plasma membrane receptor and a G protein transducing the signal to phospholipase C in a pertussis-toxin-insensitive manner. Signaling pathways of the muscarinic receptor and the putative LPA-receptor seem to merge at the G-protein level as indicated by the fact that carbachol and LPA trigger hydrolysis of the same pool of phosphatidylinositol (4,5)-bisphosphate (PIP_2) and mobilize calcium from the same intracellular stores.

Key words: Signal transduction — Lysophospholipids — Plasma membrane receptor — Phospholipase C-activation — Calcium influx — Calcium entry

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Introduction

Lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphate; LPA) is a naturally occurring phospholipid that has hormone- or growth factor-like activities in a variety of different cell types (Benton et al., 1982; Jalink, Van Corven & Moolenaar, 1990; Van Corven et al., 1992; Ridley & Hall, 1992; Cook et al., 1993; Kumagai et al., 1993). The mechanisms of LPA action at the plasma membrane are not fully characterized. In several cell types, LPA causes generation of water-soluble inositol phosphates from membrane-bound phosphatidylinositides and, as a consequence, intracellular calcium signals. In fibroblasts, these responses seem to be mediated by an, as yet, unidentified plasma membrane receptor (Van der Bend et al., 1992) coupled to a pertussis-toxin-insensitive G protein (Van Corven et al., 1989). Moreover, LPA activates $p21^{ras}$ (Van Corven et al., 1993), MAP-kinases (Kumagai et al., 1993), and induces the formation of actin stress fibers (Ridley & Hall, 1992) and cell proliferation (Van Corven et al., 1992) in fibroblasts involving either a pertussis toxin-sensitive G protein or a protein tyrosine kinase-dependent pathway. Due to its molecular structure, LPA is soluble in aqueous environments and seems to form micelles only at concentrations above 100 μmol/l (Jalink et al., 1990; Das & Hajra, 1992). Moreover, LPA binds to carrier molecules like serum albumins (Tigyi & Miledi, 1992). Its apolar acyl-chain, however, makes it soluble in lipid phases, explaining the partitioning of externally added LPA between extracellular medium and the lipid bilayer of the cell membrane (Van der Bend et al., 1992). While there is experimental evidence that some of the biological responses mentioned above are mediated by specific cell surface receptors for LPA, it seems possible that others are due to nonreceptor mechanisms of LPA at the plasma membrane (Moolenaar, Jalink & Van Corven, 1992).

Most of the studies of LPA-dependent biological

activities were performed using smooth muscle, blood cells, fibroblasts or neuronal cells (Moolenaar et al., 1992). In the present study, a primary epithelial cell type, the exocrine cells from the avian nasal salt gland, has been used to study the characteristics of LPA effects on intracellular signaling mechanisms. Salt secretion in the salt gland is under control of cholinergic nerves (Borut & Schmidt-Nielsen, 1963). Agonist binding to muscarinic acetylcholine receptors (mAChR) on the surface of the secretory cells activates phospholipase C (Fisher et al., 1983; Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991) involving a G_q-type GTP-binding protein (Hildebrandt & Shuttleworth, 1993). This results in the accumulation of water-soluble inositol phosphates in the cytosol (Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1992) and a characteristic sequence of intracellular calcium signals (Shuttleworth & Thompson, 1989). Upon addition of the agonist, the intracellular free calcium concentration ($[Ca^{2+}]_i$) displays a transient peak originating from the depletion of intracellular calcium stores by Ins(1,4,5)P_3 (Shuttleworth & Thompson, 1989), whereas the sustained portion of the calcium signal following the initial peak is dependent upon calcium influx from the extracellular space into the cytosol (Shuttleworth, 1990). It is this prolonged elevation in $[Ca^{2+}]_i$ that sustains salt secretion in these exocrine cells.

The purpose of this study was to characterize the mechanism of inositol phosphate production and calcium signaling in salt gland cells following extracellular application of LPA. The results indicate that inositol phosphate production and calcium signaling induced by low concentrations of LPA (up to 10 μmol/l) are mediated by a plasma membrane receptor mechanism, whereas higher LPA concentrations may facilitate calcium entry into the cytosol by mechanisms that do not depend on receptor-mediated hydrolysis of polyphosphoinositides.

Materials and Methods

Cells

Cells from the nasal salt gland of ducklings (*Anas platyrhynchos*), 5–15 days old, were isolated as previously described (Shuttleworth & Thompson, 1989) and stored at 38°C in air-equilibrated HEPES-buffered saline containing (in mmol/l) 141.5 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 15.0 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 6.0 glucose until used. The protein concentration in these cell suspensions was usually 1.5 mg/ml.

Binding Assay for Ins(1,4,5)P_3

The cellular content of Ins(1,4,5)P_3 was determined in samples of isolated cells using a specific receptor binding assay as described (Hildebrandt & Shuttleworth, 1991). Unless stated otherwise in the figure legends, aliquots of cell suspension (200 μl) were incubated with the respective drugs for 15 sec at 38°C. It has been shown previously (Hildebrandt & Shuttleworth, 1992) that the turnover in the phosphoinositide-cycle reaches a maximum within this time period in carbachol-stimulated salt gland cells. Reactions were terminated by addition of 200 μl ice-cold 1 mol/l trichloroacetic acid (TCA) solution. Samples were vortexed and kept on ice for 15 min. The TCA precipitate was pelleted by centrifugation at 5,000 × g for 10 min. The supernatant was transferred to another polypropylene-tube, extracted three times with water-saturated diethylether and finally neutralized using 200 μl 65 mmol/l $NaHCO_3$ solution. Aliquots (200 μl) of this preparation were used in the binding assay. The TCA pellet was dissolved in 500 μl 3 mol/l NaOH at room temperature and protein concentration was determined according to the method of Bradford (1976) using BSA (fraction V) as a standard. Some batches of cells were suspended in HEPES-buffered saline and incubated for 4 h at 38°C in the presence or absence of 500 ng/ml pertussis toxin (PTX) before being stimulated and extracted for determination of Ins(1,4,5)P_3 content.

HPLC Assay of Inositol Phosphates

Cells were loaded with tritium-labeled inositol (200 μCi/ml) for 2 hr at 38°C, washed and resuspended in air-equilibrated HEPES-buffered saline containing 10 mmol/l LiCl. Aliquots of cell suspension (200 μl) were treated with LPA or carbachol for various periods of time. Reactions were terminated by addition of 200 μl ice-cold 1 mol/l TCA-solution containing 2.5 g/l sodium phytate to improve the recovery of higher phosphorylated inositol phosphate species (Hughes, Takemura & Putney, 1988). Samples were prepared as described above for the receptor binding assay. The entire volume of the extracted and neutralized aqueous supernatant was filtered through a nylon filter (0.45 μm) before being injected onto a Partisphere SAX 5 μm HPLC column (Whatman) protected by a guard column filled with the same material. Inositol phosphates were eluted using the following gradient with distilled water as eluent A and 2.5 mol/l sodium dihydrogen phosphate-solution, pH 3.8 as eluent B: 0–6 min 0% B, 6–25 min 0–4% B, 25–26 min 4–9% B, 26–53 min 9–16% B, 53–54 min 16–20% B, 54–90 min 20–41% B. Flow rate was 1 ml/min and 0.5 ml fractions were collected directly into miniscintillation vials. After adding 5 ml scintillant (Rotiscint), radioactivity was quantified in a liquid scintillation counter (LKB). Counts (cpm) were corrected for background and converted to decays per minute (dpm) using a previously prepared quench curve with known amounts of [^{3}H] radioactivity in the HPLC fractions.

Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined in suspensions of isolated salt gland cells as previously described (Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991). Cells were suspended in air-equilibrated HEPES-buffered saline and loaded in individual batches with 5 μmol/l of indo-1/AM (acetoxymethylester form of the calcium-sensitive dye indo-1; Grynkiewicz, Poenie & Tsien, 1985) for 20 min at 38°C in the dark. Cells were washed, resuspended in fresh saline and left for 20 min at 38°C in the dark. Cells were washed 2× in fresh saline and resuspended in 3 ml saline in the cuvette of a spectrofluorimeter (Perkin-Elmer LS-5). The cell suspension was stirred and kept at 38°C throughout the measurement. Excitation and emission wavelengths were set at 332 nm and 400 nm respectively. Fluorescence data from the spectrofluorimeter were recorded on a pen recorder and simultaneously digitized and stored in a personal computer. Calibration of fluorescence signals was performed as described previously (Shuttleworth & Thompson, 1989).

To decrease the variability in the results of the calcium measure-

ments for the dose-response curve due to different degrees of filling of the intracellular calcium stores, these experiments were conducted following a strict time protocol. After resuspension of cells in the cuvette, salt gland cells were maximally stimulated using 500 µmol/l carbachol, an agonist for muscarinic acetylcholine receptors, generating inositol phosphates by activation of phospholipase C followed by cytosolic calcium signals (Shuttleworth & Thompson, 1989; Hildebrandt & Shuttleworth, 1991). After 3 min, receptor occupation by carbachol was terminated by addition of 100 µmol/l atropine, a high-affinity muscarinic antagonist, and refilling of intracellular calcium stores was allowed for a period of 3 min after which LPA was added. Final LPA concentrations were between 1 nmol/l and 0.5 mmol/l.

Trypan Blue Uptake

Isolated salt gland cells were resuspended in HEPES-buffered saline containing (in mmol/l) 100 KCl, 20 NaCl, 2 $MgCl_2$, 25 HEPES (free acid), 0.96 NaH_2PO_4, 10 EGTA, 6.96 $CaCl_2$, 0.75 g/l ATP, 2 g/l BSA, 0.2 g/l trypsin inhibitor, pH 7.2. The free calcium concentration in this solution was approximately 100 nmol/l. Aliquots of cell suspension were treated with LPA (up to 300 µmol/l) or digitonin (10 µg/ml) or left untreated. After 1 min, a solution of trypan blue was added to a final concentration of 0.05% (w/v). The mixture was incubated at 38°C for 15 min. Cells were spun down and resuspended in fresh saline without dye, transferred to a Neubauer counting chamber and viewed under a microscope.

Materials

Pertussis toxin was obtained from Biomol (Hamburg, FRG). Hexadecylphosphocholine, phenylmethylsulfonyl fluoride (PMSF) and HEPES (Na salt and free acid) were purchased from Calbiochem (Bad Soden, FRG) and suramin was from Bayer (Leverkusen, FRG). Thapsigargin and Ins(1,4,5)P_3 were supplied by LC Services (Woburn, MA), tritium-labeled inositol by ARC (St. Louis, MO). Indo-1/AM was obtained from Molecular Probes (Eugene, OR). L-α-Lysophosphatidic acid (oleoyl-) was obtained from Sigma (Deisenhofen, FRG). The preparation was checked for impurities immediately after arrival and after several weeks of storage (–20°C) as a solution in deionized water by thin layer chromatography (Whatman Silica K5D) using a solvent system containing chloroform, methanol, distilled water and acetic acid (50/40/4/3, v/v/v/v) (Jalink et al., 1990). LPA travelled as a single spot (Rf value of 0.89) without any additional signals as checked by staining with iodine or molybdate reagent, respectively, indicating that the LPA preparation used in the experiments was not degraded. All other chemicals were also from Sigma (Deisenhofen, FRG).

Results

Inositol Phosphate Generation

In isolated salt gland cells, the basal level of Ins(1,4,5)P_3 was 18.7 ± 1.7 pmol/mg protein (mean ± SE, n = 6) as determined with the receptor binding assay (Fig. 1). A similar Ins(1,4,5)P_3 content in unstimulated cells has been reported previously (Hildebrandt & Shuttleworth, 1991). Upon stimulation with 10 µmol/l lysophosphatidic acid, Ins(1,4,5)P_3 accumulation reached maximal levels at 15 sec (35.3 ± 3.6 pmol/mg protein, n = 6).

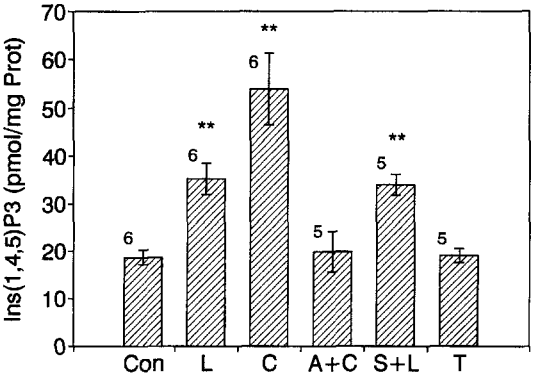

Fig. 1. Accumulation of Ins(1,4,5)P_3 in intact salt gland cells. Isolated cells were incubated for 15 sec at 38°C with 10 µmol/l lysophosphatidic acid (*L*), 500 µmol/l carbachol (*C*), 100 µmol/l atropine plus 500 µmol/l carbachol (*A* + *C*), 100 µmol/l suramin plus 10 µmol/l LPA (*S* + *L*) or treated with 500 nmol/l thapsigargin for 1 min (*T*) or with vehicle as controls (*Con*). Reactions were terminated by adding ice-cold TCA solution and water-soluble inositol phosphates were extracted as described in Materials and Methods. The content of Ins(1,4,5)P_3 was analyzed using a specific receptor-binding assay. **Significant changes from the control (Student's *t*-test) ($P < 0.01$) (means ± SE, *n*).

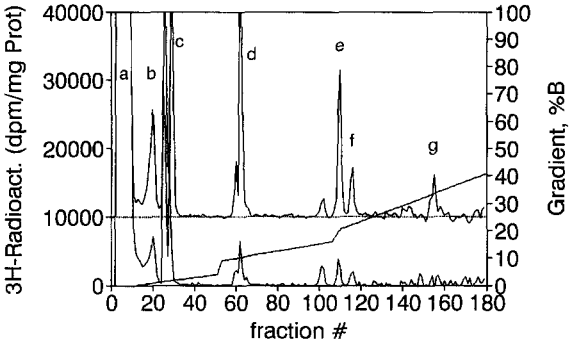

Fig. 2. Representative HPLC chromatograms of radiolabeled inositol phosphates extracted from unstimulated control cells (lower trace) or cells stimulated with 10 µmol/l LPA for 1 min (upper trace). The baseline of the upper trace has been set to 10,000 dpm/mg protein for clarity. Peak identity was verified by running authentic tritiated standard substances (NEN-Du Pont) using the same gradient: a—Inositol, b—Glycerophosphoinositol, c—Inositol monophosphates, d—Inositol bisphosphates, e—Ins(1,3,4)P_3, f—Ins(1,4,5)P_3, g—Ins(1,3,4,5)P_4. LPA-stimulation of salt gland cells caused the accumulation of label in Ins(1,4,5)P_3 as well as in Ins(1,3,4,5)P_4 and their breakdown products.

This value was significantly lower ($P < 0.05$) than the Ins(1,4,5)P_3 content in cells stimulated with carbachol for the same period of time (53.9 ± 8.0 pmol/mg protein, n = 6) (Fig. 1). Brief preincubation of cells with 100 µmol/l atropine, a high-affinity antagonist at muscarinic receptors, suppressed the carbachol effect on Ins(1,4,5)P_3 accumulation. However, preincubation of salt gland cells with suramin, a drug that reportedly inhibits binding of growth factors to their receptors (Coffey et al., 1987) and suppresses LPA-mediated inositol phosphate gener-

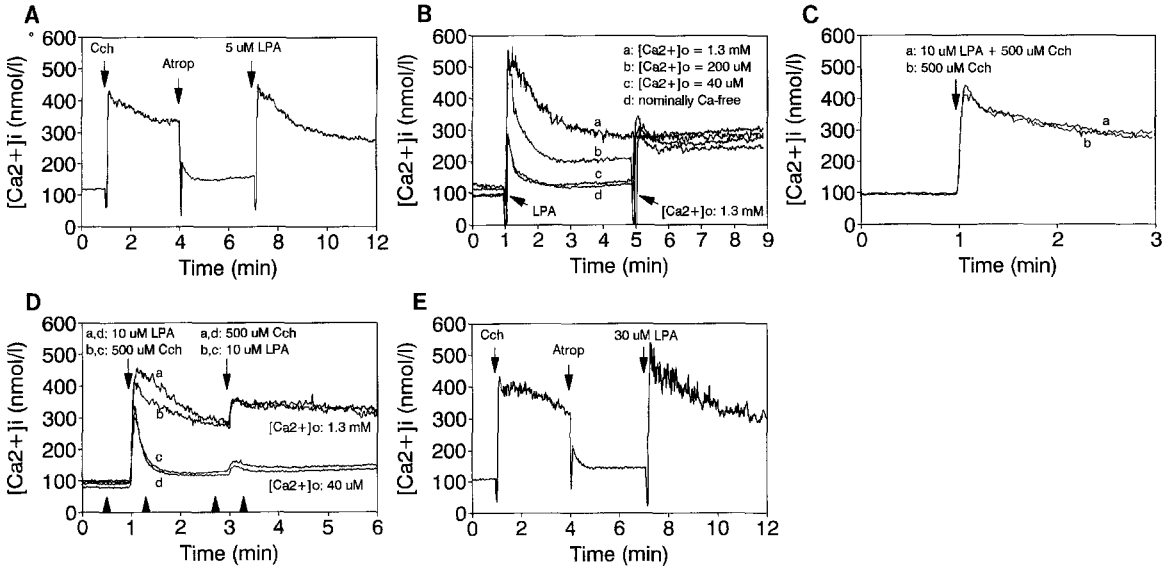

Fig. 3. Intracellular calcium signals in LPA-stimulated salt gland cells. (A) $[Ca^{2+}]_i$ signals in indo-1-loaded salt gland cells stimulated with carbachol, an agonist for muscarinic acetylcholine receptors, or lysophosphatidic acid. Isolated cells were loaded with indo-1 as described in Materials and Methods and resuspended in HEPES-buffered saline in the cuvette of a Perkin-Elmer LS-5 spectrofluorimeter. At 1 min, cells were stimulated by addition of 500 µmol/l carbachol. At 4 min, 100 µmol/l atropine was added. At 7 min, cells were stimulated with 5 µmol/l LPA. Both drugs induced a rapid increase in $[Ca^{2+}]_i$ followed by a sustained plateau phase. The curve represents the mean of four experiments on separate batches of salt gland cells. (B) $[Ca^{2+}]_i$ signals in indo-1-loaded salt gland cells suspended in HEPES-buffered saline containing different concentrations of free calcium ($[Ca^{2+}]_0$) and stimulated with 10 µmol/l LPA. In the last three experiments, a concentrated calcium chloride solution was added to the cuvette at 5 min to reestablish the normal extracellular calcium concentration of 1.3 mmol/l. Representative traces from experiments with aliquots of the same batch of cells are shown. Note the marked reduction in the size of the initial calcium peak due to very low extracellular calcium concentrations. (C) $[Ca^{2+}]_i$ signals in indo-1-loaded salt gland cells stimulated with a combination of LPA and carbachol (Trace *a*) or carbachol alone (Trace *b*). The traces shown represent means of 5 (*a*) or 3 (*b*) experiments on different preparations of cells. Note the lack of additive effects when cells are stimulated with both agonists at the same time. (D) $[Ca^{2+}]_i$ signals in salt gland cells resuspended in saline with normal (1.3 mmol/l) or reduced (40 µmol/l) calcium concentration, successively stimulated with LPA and carbachol (or in reversed order, respectively). Traces represent means of three experiments each on different preparations of cells. The arrowheads mark the time points of corresponding (Trace *b*) determinations of cellular $Ins(1,4,5)P_3$ (*cf.* Table 1). (E) Same experiment as described in Fig. 3*A*, but a higher LPA-concentration (30 µmol/l) was used to stimulate cells. The curve represents the mean of three experiments on separate batches of cells.

ation in fibroblasts (Van Corven et al., 1992), failed to affect the LPA-induced accumulation of $Ins(1,4,5)P_3$ in salt gland cells (Fig. 1). This indicates that the mechanism of phospholipase C (PLC) activation in salt gland cells is somewhat different from the one in fibroblasts. To exclude the possibility that generation of $Ins(1,4,5)P_3$ was solely due to PLC stimulation by elevated intracellular calcium concentrations, a mechanism present in several other cell types (Morris et al., 1990; Fain, 1990), salt gland cells were incubated with 0.5 µmol/l thapsigargin for 1 min. Although this treatment caused a sustained increase in the free calcium concentration in the cytosol (Fig. 6), no $Ins(1,4,5)P_3$ generation was observed (Fig. 1). This finding is consistent with previously reported results in parotid acinar cells (Takemura et al., 1989).

LPA activated not only the generation of $Ins(1,4,5)P_3$ but also its metabolism. Salt gland cells were loaded with radiolabeled inositol and stimulated for 1 min with 10 µmol/l LPA. As shown in representative HPLC chromatograms of inositol phosphate extracts from unstimulated (Fig. 2, lower trace) and LPA-stimulated cells (Fig. 2, upper trace), LPA induced the accumulation of radiolabeled $Ins(1,4,5)P_3$ (peak f) and also the generation of $[^3H]$-$Ins(1,3,4,5)P_4$ (peak g), a phosphorylation product of $[^3H]$-$Ins(1,4,5)P_3$, as well as increases in radioactivity in their breakdown products. These results indicate that LPA activates the entire inositol phosphate cascade in salt gland cells in a similar manner as carbachol (Hildebrandt & Shuttleworth, 1992).

LPA-mediated Calcium Signals

In isolated indo-1-loaded salt gland cells, addition of LPA elicited calcium signals that resembled those upon addition of carbachol in size as well as in kinetics (Fig. 3*A*). Blockage of muscarinic receptors by addition of 100 µmol/l atropine did not affect the LPA-induced calcium signals indicating that LPA acted through a different mechanism than cholinergic agonists. The initial $[Ca^{2+}]_i$-peak upon addition of LPA was present even in

the nominal absence of extracellular calcium (Fig. 3*B*) indicating that it was due to calcium release from intracellular stores. However, the size of the initial calcium peak was significantly reduced when the extracellular calcium concentration was low (<40 μmol/l). A possible explanation for this observation is that influx of extracellular calcium contributed to the initial calcium signal. A similar observation has been made previously in carbachol-activated salt gland cells (Shuttleworth & Thompson, 1989). The sustained calcium signal, however, was entirely dependent upon influx of calcium from the extracellular space since removal of extracellular calcium virtually abolished this portion of the calcium signal whereas readdition of calcium to a final extracellular concentration of 1.3 mmol/l restored it (Fig. 3*B*).

Simultaneous addition of carbachol (500 μmol/l) and LPA (10 μmol/l) to a suspension of indo-1-loaded salt gland cells had no additive effects on the resulting calcium signals (Fig. 3*C*). Furthermore, cells resuspended in low-Ca^{2+} (40 μmol/l) saline showed exactly the same sequence of calcium signals when stimulated with 10 μmol/l LPA and, 2 min later, with 500 μmol/l carbachol compared with cells stimulated with both agonists in the reversed order (Fig. 3*D*). These results indicate that LPA and carbachol mobilized calcium ions from the same calcium pools inside the cells. The same experiments, performed on cells resuspended in saline with a normal calcium concentration, showed that the second agonist, regardless of the order of application, caused an additional increase in $[Ca^{2+}]_i$ (Fig. 3*D*). Sequential addition of the same agonist, however, did not produce such an effect (*not shown*). Measurements of cellular Ins(1,4,5)P_3 contents were performed to determine whether these changes in $[Ca^{2+}]_i$ were correlated with similar changes in the inositol phosphate generation rate. Following addition of carbachol to the cell suspension, there was a rapid increase in cellular Ins(1,4,5)P_3 (Table 1). However, 2 min later, Ins(1,4,5)P_3-content had declined to approximately 65% of the initial level, although the agonist was still present in the medium. Addition of LPA at this point restored the full Ins(1,4,5)P_3-signal (Table 1).

Peak values of the initial calcium signal upon addition of more than 10 μmol/l LPA seemed higher than those obtained upon addition of maximal concentrations of carbachol (Fig. 3*E*). Since the inositol phosphate production reached a maximum at an LPA concentration of approximately 1 μmol/l (Table 2), it was obvious that this overshoot in the initial $[Ca^{2+}]_i$ peak was not a result of calcium release from intracellular stores or of calcium influx dependent upon PIP_2 hydrolysis. Measurements of $[Ca^{2+}]_i$-peak heights in response to stimulation of salt gland cells with different LPA concentrations between 1 and 0.5 mmol/l (Fig. 4) revealed the presence of two mechanisms affecting the calcium peak upon LPA stimulation of cells in different concentration ranges. The first mechanism showed an EC_{50} of approximately 20 nmol/l for calcium release from intracellular stores. At LPA concentrations higher than 10 μmol/l, peak values of $[Ca^{2+}]_i$ rose much higher (Fig. 4) reaching maximal values well above 500 nmol/l at LPA concentrations between 100 and 500 μmol/l.

Table 1. Accumulation of Ins(1,4,5)P_3 in salt gland cells before and after sequential stimulation with carbachol and LPA

Carbachol/ LPA stimulation	pmol Ins(1,4,5)P_3/mg protein
(1) Aliquot (Control)	12.9 ± 0.7
(2) Aliquot (15 s Carbachol)	40.6 ± 3.9
(3) Aliquot (105 s Carbachol)	27.4 ± 2.0
(4) Aliquot (135 s Carbachol, 15 s LPA)	41.3 ± 3.0

Corresponding calcium signals are shown in Fig. 3*D*, trace *b*). Isolated salt gland cells were resuspended in HEPES-buffered saline ($[Ca^{2+}]_i$ = 1.3 mmol/l). Aliquots of this cell suspension were kept at 38° C. Cells of the (1) aliquot were left unstimulated, cells of the (2) and (3) aliquots were stimulated with 500 μmol/l carbachol for 15 or 105 s, respectively. Cells of the (4) aliquot were stimulated with 500 μmol/l carbachol for 2 min and, at this time point, with 10 μmol/l LPA for additional 15 sec. Reactions were terminated by addition of 1 mol/l trichloroacetic acid solution, and samples were processed as described in Materials and Methods. Results are given in pmol Ins(1,4,5)P_3/mg protein (means ± SE, n = 5).

Table 2. Accumulation of Ins(1,4,5)P_3 in salt gland cells stimulated with different concentrations of LPA for 15 sec

LPA concentration	pmol Ins(1,4,5)P_3/mg protein
Control	16.3 ± 2.4
1 μmol/l LPA	35.7 ± 3.6
10 μmol/l LPA	37.1 ± 2.9
50 μmol/l LPA	41.1 ± 3.1

Each value for stimulated cells was significantly different from the control (Student's *t*-test, $P < 0.01$), whereas the Ins(1,4,5)P_3 contents in cells stimulated with 1, 10 or 50 μmol/l LPA were not significantly different from each other (ANOVA, $s < 0.9$). (Mean ± SE, n = 9).

Checks for Cell Integrity

To test whether LPA is able to permeabilize salt gland cells, calcium measurements were performed using LPA concentrations up to 1 mmol/l. In all of these experiments, indo-1-fluorescence declined after the initial calcium peak to a stable plateau with $[Ca^{2+}]_i$ values below 500 nmol/l (e.g., Fig. 3*E*). However, upon addition of 50 μmol/l digitonin, cells were rapidly permeabilized as judged from the increase in fluorescence to maximal values by exposure of the dye to the extracellular calcium concentration of 1.3 mmol/l. This indicates that LPA did

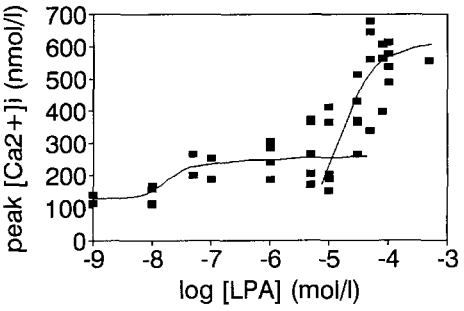

Fig. 4. Size of the initial $[Ca^{2+}]_i$ peak upon stimulation of salt gland cells with different concentrations of LPA in the range between 1 nmol/l and 0.5 mmol/l. Each data point represents the result of an individual determination. The dose-response curves (solid lines) were fitted by eye. The discontinuity in the dose-response function indicates the presence of two independent mechanisms by which LPA affects $[Ca^{2+}]_i$ in salt gland cells.

not allow unlimited access of extracellular calcium ions to the intracellularly trapped dye or liberate the dye from the cytosol despite indo's low molecular weight of 800 g/mol.

Another series of experiments was performed using trypan blue as a marker for cell integrity. Less than 3% of unstimulated salt gland cells showed trypan blue uptake after a 15 min incubation period in 0.05% trypan blue in HEPES-buffered saline at 38°C. Upon stimulation with 300 μmol/l LPA, there was no change in the number of cells containing trypan blue whereas virtually all cells became intracellularly stained upon incubation with 10 μg/ml digitonin (five experiments with different batches of cells).

These results indicate that LPA, even when applied in extremely high concentrations, did not permeabilize salt gland cells in a general manner.

Experiments with Structural Analogues of LPA

Calcium measurements in indo-1-loaded salt gland cells treated with compounds with structural similarity to LPA were performed to evaluate the specificity of the observed effects. Addition of 10 μg/ml phosphatidic acid (PA), as obtained from Sigma Chemicals, elicited a small but significant calcium signal (Fig. 5*A*). The same PA preparation was subjected to TLC analysis as described in Materials and Methods. The major fraction was detected at an Rf value of 0.83. In addition, a smear of substances was detected extending from Rf 0.83 to 0.95. Unstained material from parallel PA lanes was scraped (at Rf 0.83 and at the Rf value of authentic LPA, 0.89), collected separately into microreaction tubes and eluted with a methanol/chloroform mixture (1:1, v/v). Silica was removed by centrifugation and lipids were resuspended in deionized water following evaporation of the original solvent. Mass determination using a phosphate assay system revealed that 72% of the loaded PA was recovered at Rf 0.83 while only 6% was found in the fraction at Rf 0.89. It was assumed that the fraction at Rf 0.83 represented pure phosphatidic acid, whereas the fraction at Rf 0.89 was mainly lysophosphatidic acid. Addition of 10 μg/ml purified PA to a suspension of indo-1-loaded salt gland cells did not result in any detectable calcium signal, while the Rf 0.89 fraction of PA generated a measurable elevation in $[Ca^{2+}]_i$ (Fig. 5*A*). These results indicate that pure phosphatidic acid did not mobilize calcium in salt gland cells and that the calcium response observed following addition of original PA was due to the presence of impurities, presumably LPA, in the preparation.

Addition of 50 μmol/l of the phospholipid analogue hexadecylphosphocholine, a substance that showed no toxic effects on Madin-Darby canine kidney cells at concentrations as high as 200 μmol/l (Haase et al., 1991), readily permeabilized salt gland cells (Fig. 5*B*). Addition of 50 μmol/l lysophosphatidylcholine to salt gland cells caused the slow development of a sustained calcium signal similar to that following LPA stimulation, but the rapid initial component of the calcium signal observed upon addition of LPA or carbachol to salt gland cells was absent (Fig. 5*C*). These results indicate that the observed calcium signals, especially the initial portion of the LPA-mediated calcium signal, were brought about by an LPA-specific mechanism.

LPA-mediated Calcium Signaling not Depending on PIP_2 Hydrolysis

It is widely accepted that calcium influx from the external medium into the cytosol of nonexcitable cells is dependent upon the depletion of intracellular calcium stores (Putney, 1993). Since LPA mediated release of calcium from intracellular stores by an $Ins(1,4,5)P_3$-dependent mechanism, thereby causing calcium influx as well, it was difficult to decide whether LPA opens an additional pathway for calcium entry into cells. To investigate whether such a pathway exists or not, salt gland cells were treated with thapsigargin, a substance that inhibits calcium pumps located on intracellular calcium stores (Takemura et al., 1989), thereby indirectly causing a net loss of calcium from the stores and subsequent activation of calcium entry. Salt gland cells treated with 500 nmol/l thapsigargin developed a sustained plateau of $[Ca^{2+}]_i$ (Fig. 6) without initiating inositol phosphate production (Fig. 1). Shortly before the $[Ca^{2+}]_i$ reached the usual plateau concentration, supposedly when intracellular calcium stores were completely emptied, LPA was added to the extracellular medium. Application of 10 μmol/l LPA under these conditions did not further enhance the effect of thapsigargin on $[Ca^{2+}]_i$, whereas addition of 50 μmol/l LPA caused a pronounced additional

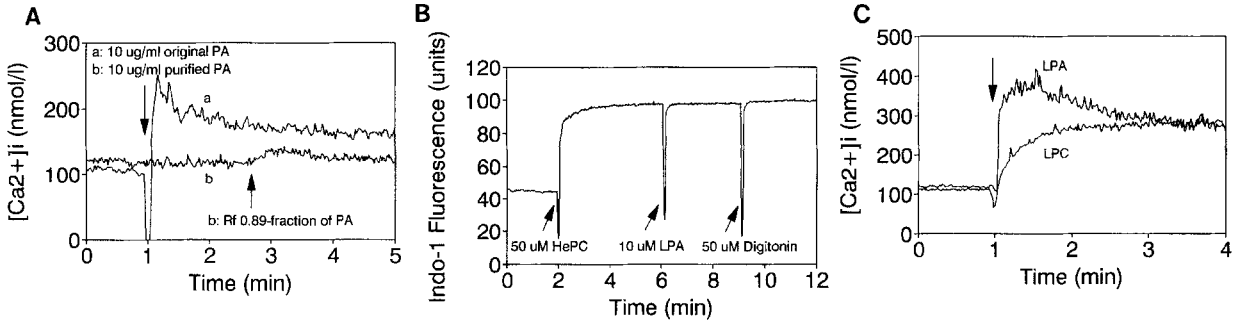

Fig. 5. Specificity of the $[Ca^{2+}]_i$ signals in LPA-stimulated salt gland cells. (A) $[Ca^{2+}]_i$ signals in indo-1-loaded salt gland cells following addition of phosphatidic acid (PA) or TLC-purified preparations of phosphatidic acid. Unpurified (Trace *a*) or TLC-purified (Trace *b*) phosphatidic acid was added at time point 1 min. At 3 min (Trace *b*), all of the recovered Rf 0.89 material scraped from one TLC lane (presumably representing the LPA fraction in the original phosphatidic acid preparation) was added. Traces are representative examples of three (Trace *a*) or two (Trace *b*) experiments with different preparations of cells. (B) Representative recording (total of three experiments) of indo-1-fluorescence in salt gland cells suspended in saline containing 1.3 mmol/l free calcium and treated with 50 µmol/l of the lysophospholipid-analogue hexadecylphosphocholine (HePC). Subsequent addition of 10 µmol/l LPA and 50 µmol/l digitonin did not change the maximum level of fluorescence induced by HePC indicating that this compound permeabilized salt gland cells instantly. (C) Comparison of intracellular calcium signals in indo-1-loaded salt gland cells induced by 5 µmol/l LPA or 5 µmol/l lysophosphatidylcholine (LPC) added at 1 min (arrow). Representative traces (total of four experiments) from experiments with aliquots of the same batch of cells are shown.

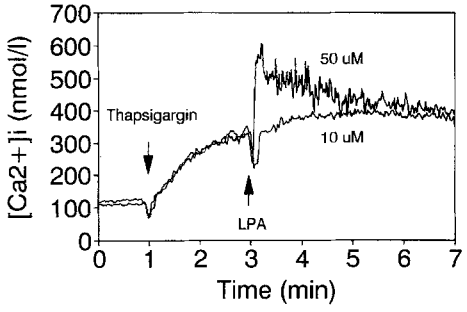

Fig. 6. Recordings of $[Ca^{2+}]_i$ in salt gland cells treated with 500 nmol/l thapsigargin for 2 min followed by stimulation with 10 or 50 µmol/l LPA. Development of the full $[Ca^{2+}]_i$ response to treatment of cells with thapsigargin was not disturbed by addition of 10 µmol/l LPA, but addition of 50 µmol/l LPA caused a profound additional increase in $[Ca^{2+}]_i$. Curves represent means of four (10 µmol/l LPA) or two (50 µmol/l) experiments on separate batches of cells.

increase in $[Ca^{2+}]_i$ to peak values above 500 nmol/l (Fig. 6). This increase was transient and after several minutes, a plateau value of $[Ca^{2+}]_i$ was established that was identical with those seen upon addition of 10 µmol/l LPA (Fig. 6) or thapsigargin alone (*not shown*).

PTX Sensitivity of LPA-mediated Phospholipase C Activation

It has been shown previously (Hildebrandt & Shuttleworth, 1993) that muscarinic receptor activation in salt gland cells activates phospholipase C involving a GTP-binding protein of the G_q family. Members of this group of G proteins are not prone to ADP ribosylation catalyzed by pertussis toxin (PTX) (Strathmann & Simon, 1990). To test whether inositol phosphate generation upon addition of LPA to isolated salt gland cells was sensitive to PTX treatment or not, cells were preincubated with 500 ng/ml PTX for 4 hr at 38°C, a procedure that has been shown to cause ADP ribosylation of more than 80% of all ribosylatable G proteins in salt gland cells (Hildebrandt & Shuttleworth, 1993). $Ins(1,4,5)P_3$ accumulation upon LPA stimulation was not affected by PTX pretreatment of cells (Table 3) indicating that the elements of the transmembrane signaling apparatus utilized by LPA are not sensitive to ADP ribosylation by PTX.

Discussion

LPA-mediated Inositol Phosphate and Calcium Signals

Lysophosphatidic acid (LPA) induced the generation of inositol phosphates as well as intracellular calcium signals in avian nasal salt gland cells (Figs. 1–3). These responses seem to be specific for LPA, since pure phosphatidic acid was not able to generate calcium signals in these cells (Fig. 5*A*) while other phospholipid analogues caused changes in $[Ca^{2+}]_i$ that were entirely unrelated to those seen after LPA application (Fig. 5*B* and *C*). These findings resemble previous results in other cell types suggesting the existence of specific cell surface receptors for LPA (Jalink et al., 1990; Van der Bend et al., 1992; Durieux et al., 1993). Although the $Ins(1,4,5)P_3$ accumulation in LPA-stimulated cells was somewhat attenuated in comparison with carbachol-stimulated cells (Fig. 1), the resulting calcium signals in salt gland cells ap-

Table 3. $Ins(1,4,5)P_3$-content in isolated salt gland cells

Condition	–PTX	+PTX
Unstimulated	24.6 ± 7.2	20.4 ± 2.0
10 μmol/l LPA	36.8 ± 6.9	32.9 ± 6.2
500 μmol/l Cch	47.9 ± 8.0	41.6 ± 2.9

Salt glands cells were incubated for 4 hr in the presence (+PTX) or absence (–PTX) of 500 ng/ml pertussis toxin (PTX) and stimulated with LPA or carbachol (Cch) for 15 sec (pmol $Ins(1,4,5)P_3$/mg protein, means ± SE, n = 3). None of the values of untreated and PTX-treated salt gland cells were significantly different ($P > 0.1$).

peared to be similar in peak and plateau values as well as in kinetics (Fig. 3*A*). Upon addition of LPA, the initial $[Ca^{2+}]_i$ peak, usually ascribed to release of calcium from intracellular stores, was followed by a sustained plateau phase depending upon influx of calcium from the extracellular medium (Fig. 3*B*). These observations support the hypothesis that LPA acts as a classical first messenger in salt gland cells, binding to a cell surface receptor and triggering intracellular inositol phosphate production and calcium signals.

Calcium Mobilization by High Concentrations of LPA

Especially upon stimulation of salt gland cells with high concentrations of LPA (>10 μmol/l), the initial calcium spike appeared to be more pronounced compared with the calcium spike induced by maximum concentrations of carbachol (Figs. 3*E* and 4), although the accumulation of $Ins(1,4,5)P_3$ did not increase further at LPA concentrations higher than 1 μmol/l (Table 2). Moreover, in thapsigargin-pretreated salt gland cells, stimulation with high concentrations of LPA (50 μmol/l) elicited a pronounced additional calcium signal whereas lower LPA concentrations (10 μmol/l) were ineffective (Fig. 6). A possible explanation for these observations is that LPA concentrations higher than approximately 10 μmol/l cause calcium influx across the plasma membrane by a direct mechanism that does not involve the production of $Ins(1,4,5)P_3$.

However, as indicated by several experimental approaches, LPA does not permeabilize cells in a general manner. First of all, lysophosphatidic acid did not induce detectable leakiness of cells even when applied at high concentrations (100 μmol/l) as judged by the cell's abilities to keep free indo-1 inside the cytosol (Fig. 3) and to exclude external trypan blue from the cytosol. Lysophosphatidic acid did not allow unlimited access of external calcium to the interior of the cells or complete leakage of dye out of the cells as observed upon addition of the lysophospholipid analogue hexadecylphosphocholine to the cell suspension (Fig. 5*B*).

The mechanism by which high concentrations of LPA mediate the translocation of calcium ions through the plasma membrane is still unknown. It may be speculated, however, that integration of LPA molecules into the lipid bilayer of biological membranes may be instrumental in this process. Such an ability of LPA and other lysophospholipids has been shown previously (Benton et al., 1982; Van der Bend et al., 1992; Weltzien, 1979). Since LPA binds calcium ions with high affinity (Jalink et al., 1990), it may be speculated that LPA, especially when applied in high concentrations to intact cells, crosses the cell membrane, thereby carrying calcium ions into the cytosol.

The translocation of calcium ions mediated by high concentrations of LPA did not result in an equilibration of calcium between the cytosol and the extracellular medium (Fig. 3*E*). A possible explanation for this observation is that LPA may translocate calcium ions at a relatively slow rate, so that activation of calcium pumps can effectively compensate for increased calcium fluxes. The combined actions of such mechanisms may result in only transient overshoots in $[Ca^{2+}]_i$.

LPA Signaling Mediated by a Putative Plasma Membrane Receptor

Since elevation of $[Ca^{2+}]_i$ by treatment of salt gland cells with thapsigargin did not induce any inositol phosphate generation (Fig. 1), it is concluded that the increase in $[Ca^{2+}]_i$ mediated by low concentrations of LPA (up to 10 μmol/l) was the consequence of but not the reason for the activation of phospholipase C and the generation of inositol phosphates (Figs. 1 and 2). This indicates that the LPA-induced inositol phosphate production in avian salt gland cells is most likely mediated by a phospholipase C-coupled plasma membrane receptor.

To compare the efficiencies of LPA and mACh receptors in activating phospholipase C and generating water-soluble inositol phosphates from membrane-bound inositol lipids, the $Ins(1,4,5)P_3$ accumulation rates in cells stimulated with LPA or carbachol were measured. The results show that LPA was less potent in generating $Ins(1,4,5)P_3$ compared with carbachol (Fig. 1). One possible explanation for this observation is that the muscarinic receptors are more tightly coupled to phospholipase C than the LPA-binding receptors. Another possibility is that there are less receptors for LPA on the cell surface of salt gland cells than mAChRs. Nothing is known yet about the binding characteristics of LPA to salt gland cells. Saturation binding assays in other cell types proved unsuccessful due to LPA's ability to interact with the lipid phase of plasma membranes (Van der Bend et al., 1992). Only one study (Shiono et al., 1993) claimed an observation of saturable LPA binding to membranes of PC12 cells, unfortunately without reporting data for

nonspecific binding. On the other hand, Van der Bend et al. (1992) have been able to isolate a 38 kDa protein from plasma membranes of fibroblasts with specific and high-affinity LPA-binding properties. This suggests the existence of LPA-specific receptors in certain types of animal cells.

In Rat-1 cells, LPA stimulates inositol phosphate generation and mitogenesis (Van Corven et al., 1992). Both effects were attenuated by suramin, a polyanionic compound interfering with growth factor receptor binding in many cell types (Coffey et al., 1987). The inhibitory mechanism of suramin is not known. It could either involve an inhibitory mechanism at the receptor level or direct interaction of suramin with LPA. Preincubation of salt gland cells with 100 μmol/l suramin, a concentration that has been shown to be inhibitory for LPA effects in several cell types (Coffey et al., 1987; Van Corven et al., 1992; Van der Bend et al., 1992), failed to attenuate the Ins(1,4,5)P_3 accumulation in LPA-stimulated salt gland cells (Fig. 1). A conclusion from this observation is that suramin effects on LPA signaling are not due to LPA binding in the extracellular medium but probably a consequence of interaction of suramin with some types of receptors. This, in turn, indicates that epithelial cells like avian salt gland cells may express a suramin-insensitive type of LPA-binding receptor.

Relationship of LPA- and ACh-signaling Pathways

To clarify the relationship of muscarinic and LPA-signaling pathways, the ability of either agonist to affect calcium signals evoked by the other was tested. Indo-1-loaded salt gland cells resuspended in low-Ca^{2+} saline showed the same sequences of $[Ca^{2+}]_i$ signals when stimulated with LPA and carbachol, regardless of the order of the agonist application (Fig. 3*D*). Together with the observation that simultaneous application of carbachol and LPA did not produce a larger calcium peak than application of only one compound at a time (Fig. 3*C*), this indicates that both agonists deplete the same intracellular calcium pool. In cells resuspended in saline containing 1.3 mmol/l calcium, however, carbachol was able to further enhance the $[Ca^{2+}]_i$ signal elicited by LPA 2 min before (Fig. 3*D*) and vice versa. A possible explanation for this observation is that the receptor activated first partially desensitized during this 2 min-period, so that stimulation of cells with the second agonist again maximally activated PI-turnover (Table 1) and the mobilization of calcium ions (Fig. 3*D*).

Although a thorough investigation of the interactions of LPA- and mAChR-signaling pathways is still ahead, this suggests that both agonists, although having different receptors, may utilize the same intracellular signaling mechanism. Since the muscarinic receptor in salt gland cells is known to be coupled to a pertussis toxin (PTX)-insensitive G protein of the G_q type (Hildebrandt & Shuttleworth, 1993), we tested whether the inositol phosphate production in LPA-stimulated cells was sensitive to PTX or not. Salt gland cells in which more than 80% of the ribosylatable G proteins were dysfunctional due to modification by ADP ribosylation (*cf.* Hildebrandt & Shuttleworth, 1993) showed no difference in the accumulation of Ins(1,4,5)P_3 upon stimulation with LPA compared with untreated controls (Table 3). This finding is consistent with previous reports in fibroblasts demonstrating that LPA-mediated inositol phosphate production is not sensitive to PTX pretreatment of cells (Van Corven et al., 1989). It indicates that, at least in salt gland cells, LPA-binding receptors and mAChR share the same pool of G_q proteins coupling both types of receptors to phospholipase C.

Conclusion

The biological significance of lysophosphatidic acid effects on different cell types has not yet been fully understood. There is some evidence that LPA has mitogenic properties in fibroblasts, a mechanism requiring high concentrations of LPA (>10 μmol/l, Van Corven et al. 1992) and mediated by a pertussis toxin-sensitive G protein (Van Corven et al., 1989). Whether other actions of LPA (including its metabolic conversion to phosphatidic acid or diacylglycerol inside the cells) play an additional role in the Ras/MAP-kinase signaling pathway involved in the mitogenic effects in fibroblasts has not been investigated in detail (Moolenaar et al., 1992). LPA-induced inositol phosphate generation, however, occurs at much lower concentrations and reaches maximal values at 1 μmol/l (Table 2).

Since phospholipase C activation in salt gland cells by LPA was less pronounced compared with carbachol (Fig. 1), it may be speculated that, in the intact avian salt gland in vivo, LPA may modulate the basal turnover rate in the phosphatidylinositide system rather than providing an acute stimulus for secretion. A possible implication of these results is that changes in the basal inositol lipid turnover may regulate long-term processes such as inducing or maintaining cellular differentiation.

I would like to thank Prof. Dr. I. Schulz for providing lab space and some of the equipment used in this study and Prof. Dr. T.J. Shuttleworth for his helpful comments on a draft of the manuscript. This study was supported by the Deutsche Forschungsgemeinschaft (Hi 448/2-1 and 2-2).

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