Diradylglycerol formation in cholecystokinin-stimulated rabbit pancreatic acini
Assessment of precursor phospholipids by means of molecular species analysis

Jan Joep H. H. M. DE PONT1 and Peter H. G. M. WILLEMS1.

1 Department of Biochemistry, University of Nijmegen, The Netherlands
2 Physiologisches Institut, Universität München, Germany

The aim of the present study was to assess the origin of the 1,2-diradylglycerols produced during prolonged hormonal stimulation of rabbit pancreatic acini by comparison of their relative molecular species composition with that of the major acinar phospholipids. Both phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) consisted of 1,2-diacyl as well as 1-alk-1-enyl-2-acyl species. In contrast, phosphatidylinositol (PtdIns), phosphatidylethanolamine and phosphatidic acid existed only in the 1,2-diacyl form. Acellar cells did not contain detectable amounts of 1-alkyl-2-acyl phospholipids. Similarly, the acinar 1,2-diradylglycerol fraction consisted of 1,2-diacylglycerols and 1-alk-1-enyl-2-acylglycerols. Mass 1,2-diradylglycerol measurements revealed that prolonged stimulation with cholecystokinin resulted in a marked and sustained increase in acinar 1,2-diradylglycerol content. Based on the relative amounts of the 1,2-diacyl species present in both the 1,2-diradylglycerol fraction and the individual phospholipids, it is calculated that under control conditions 60% of the 1,2-diacylglycerols originate from PtdCho and 40% from PtdIns, whereas under stimulatory conditions 53% is calculated to be derived from PtdCho, 46% from PtdIns and 1% from PtdEtn. Likewise, it is calculated that in control as well as stimulated acini 100% of the 1-alk-1-enyl-2-acylglycerols originate from plasmenylcholine. Further evidence in favour of the idea that at least a considerable part of the 1,2-diacylglycerols produced during prolonged hormonal stimulation originate from inositolphospholipids is provided by the observation that labeling of phosphatidylinositol 4,5-bisphosphate with inorganic phosphate reached isotopic equilibrium markedly faster under stimulatory conditions as compared to the control situation, which is in agreement with an elevated turnover rate. The data presented support the idea that PtdCho and inositolphospholipids are the major precursors in basal and stimulated 1,2-diradylglycerol production in rabbit pancreatic acini.

Keywords: pancreatic acini; cholecystokinin; acylglycerol; ether-linked phospholipids.
phorbol ester-stimulated production of 1-alkyl-2-acylglycerols has been observed in neutrophils and MDCK cells [11—15], but not in IIC9 and Swiss 3T3 fibroblasts [16, 17]. Moreover, Dougherty et al. [15] reported that the receptor-evoked elevation of the 1,2-diacylglycerols preceded that of the 1-alkyl-2-acylglycerols. Concerning the physiological relevance of the ether-linked 1,2-diacylglycerols there is some, be it conflicting, evidence favouring a role as endogenous regulators of protein kinase C activity [18—20].

At present, there is no information available regarding the presence and molecular species composition of 1,2-diacylglycerols in unstimulated and stimulated pancreatic acinar cells. This has urged us to analyse the molecular species of the 1,2-diacylglycerols present in control and cholecystokinin-stimulated rabbit pancreatic acini. In order to determine the origin of the 1,2-diacylglycerols, we analysed the molecular species of the major phospholipids. In addition, we performed labeling studies using ortho-32P-phosphate in order to gain insight into phospholipid metabolism under control and stimulatory conditions. The data obtained support the idea that both PtdCho and inositolphospholipids contribute to the increased 1,2-diacylglycerol production observed during long-term hormonal stimulation in rabbit pancreatic acini.

**EXPERIMENTAL PROCEDURES**

**Materials.** Cholecystokinin-(26—33)-peptide amide, phospholipase C (*Bacillus cereus*), dinitrobenzoyl chloride [(NO2)2PhCOCl], diphenylhexatriene, fluorescein, soybean trypsin inhibitor, and bovine serum albumin were purchased from Sigma. Phospholipid standards were obtained both from Sigma and from Avanti Polar Lipids (Birmingham AL). Diacylglycerol kinase was obtained from Calbiochem (La Jolla CA) or from Lipidex (Westfield NJ). Hyaluronidase was purchased from Boehringer, Mannheim; pyridine and HPTLC plates silica gel 60 from Merck (Darmstadt); ortho-32P-phosphate from New England Nuclear (Boston MA) and [γ-32P]ATP from Amersham International. Collagenase was obtained from Worthington (Freehold NJ). All other chemicals were of reagent grade.

**Preparation of rabbit pancreatic acini.** Rabbit pancreatic acini were prepared as described previously [21]. Incubations were performed in a Krebs-Ringer bicarbonate medium (pH 7.4), containing 119 mM NaCl, 3.5 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 1.2 mM CaCl2, 1.2 mM MgCl2, 5.8 mM glucose, 1% (mass/vol.) bovine serum albumin, 0.2 mg/ml soybean trypsin inhibitor and an amino acid mixture according to Eagle, at 37°C. The stimulants were added as indicated in the legends to the figures.

**Lipid extraction.** The lipids were extracted according to a modification of the method of Bligh and Dyer [22]. Briefly, 0.8-ml aliquots of acinar suspension were incubated in the absence and presence of the C-terminal octapeptide of cholecystokinin [cholecystokinin-(26—33)-peptide amide] for the indicated period of time. The incubation was quenched by vigorously mixing with 3 ml chloroform/methanol (1:2, by vol.). Lipids were allowed to extract at room temperature for 30 min. Phase separation was induced by addition of 1 ml chloroform and 1 ml 1 M NaCl. After centrifugation at 1500×g for 5 min, the organic phase was removed. The aqueous phase was extracted again and the combined organic phases were blown to dryness with nitrogen.

**Quantitative determination of phospholipid classes and subclasses.** Aliquots of the acinar lipid extracts were divided over two heat-activated HPTLC plates and chromatographed with chloroform/methanol/acetic acid/water (40:10:10:1, by vol.) until the solvent front reached the top of the plates. Both plates were dried under nitrogen and chromatographed in the same direction with chloroform/methanol/acetic acid/water (120:46:19:3, by vol.) [23]. Spots corresponding to the phospholipid classes PtdCho, PtdEtn, PtdIns, phosphatidylserine (PtdSer), PtdOH and sphingomyelin were scraped off from one plate and used for determination of the phosphate content according to the method described by Fiske and Subba-Roy [24]. The same spots were scraped off from the second plate and used for determination of the phospholipid subclasses. The phospholipids were extracted from the silica with chloroform/methanol (2:1, by vol.); the procedure being repeated twice. In order to determine the plasmalogen content of the phospholipid classes, the extracts were applied to a HPTLC plate and exposed to HCl fumes (37% in water) for 10 min. This procedure results in the selective hydrolysis of the sn-1 ethanolol bond in the 1-alk-1-acyl-2-acyl phospholipids [25]. The plate was dried under nitrogen and developed with either chloroform/methanol/acetic acid/water (90:40:12:2, by vol.) or, in case of PtdCho, with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) [26]. Both systems separate the 1-lyso-2-acyl phospholipids, but not the 1-acyl-2-acyl phospholipids, from the 1-alkyl-2-acyl phospholipids. The spots were scraped off and used for determination of the phosphate content.

**Phospholipid molecular species analysis.** Molecular species analysis of phospholipids was performed essentially as described by Engelmann et al. [27]. After lipid extraction, the phospholipids were separated by two-dimensional TLC according to Bütikofer et al. [28]. The spots were visualized by diphenylhexatriene spray (0.03% in chloroform), scraped off and the lipids were extracted from the silica by the method of Arvidson [29]. During the extraction procedure the lipids were protected against oxidation by butylated hydroxytoluene (50 μg/ml). The phospholipids were sonicated in 2 ml of either 30 mM KH2PO4, 30 mM boric acid, pH 7.0 (PtdCho, PtdEtn, PtdSer, PtdOH), or 30 mM Tris, 30 mM boric acid, pH 7.4 (PtdIns). Phospholipase C from *Bacillus cereus* (P7147 for PtdCho and PtdEtn; P9439 for PtdSer and PtdOH; P8804 for PtdIns) was added followed by 4 ml diethylether. The mixtures were incubated under argon for 8—12 h in a shaking water bath (37°C). After completion of the phospholipase-C-catalysed formation of 1,2-diacylglycerols, which was routinely checked by subjecting an aliquot of the ether phase to one-dimensional TLC using diethyl ether/hexane (3:2, by vol.) as a running solvent, the ether phase was collected. The lower phase was extracted again with diethylether and the combined ether phases were blown to dryness with nitrogen and subsequently dried under vacuum for at least 8 h. After addition of 25 mg 3,5-dinitrobenzoyl chloride [(NO2)2PhCOCl] the mixture was dried under vacuum for another 4 h. Derivatization of the 1,2-diacylglycerols was achieved by addition of 1 ml dry pyridine and subsequent incubation for 15 min at 64°C. The extract was chilled on ice for 15 min and 3 ml ice-cold water was added together with 2 ml hexane. The water phase was extracted twice with 2 ml hexane and the combined hexane phases were dried under nitrogen. The extract was redissolved in 2 ml hexane and successively washed with 2 ml 1 M NaCl and 2 ml water. The 1,2-diacyl-, 1-alk-1-enyl-2-acyl-, and 1-alkyl-2-acyl-glycerol subclasses were separated by HPTLC using hexane/diethylether (7:3, by vol.) as a running solvent. The spots were visualized by dichlorofluorescein (0.001% in 2 mM NaOH) and extracted with diethylether. The extract was washed with double-distilled water in order to remove the water-soluble degradation products. After evaporation of the ether phase, the extract was dissolved in 100 μl acetonitrile/isopropanol (8:2, by vol.) and subjected to reverse-phase HPLC using an ODS Hypersil column (5 μm C18, 200×2.1 mm, Hewlett Packard). The column
was eluted with acetonitrile/isopropanol (8:2, by vol.) at a flow rate of 0.25 ml/min. The elution profile of the single dinitrobenzoyl-diradylglycerols was monitored at 254 nm by means of a Hewlett Packard 1050 UV detector. The molecular species of the derivatized 1,2-diradylglycerols were identified on the basis of the retention times obtained with standard dinitrobenzoyl-diradylglycerols and/or the relative retention times published by Takamura et al. [30].

**Diradylglycerol molecular species analysis.** Diradylglycerols were separated from other lipids by subjecting aliquots of the acinar lipid extract to thin-layer chromatography. Plates were developed with hexane/diethyl ether/formic acid (30:70:2, by vol.) and the spot corresponding to the 1,2-diradylglycerols was scraped off. The 1,2-diradylglycerols were extracted from the silica by means of hexane. The extract was dried under vacuum, derivatized with (N0 2)2PhC0Cl and separated into its molecular species as described above.

**Mass 1,2-diradylglycerol measurement.** Diradylglycerol levels in acinar extracts were determined according to the method described by Press et al. [31] and modified by Paterson et al. [32]. Briefly, the reaction mixture contained 50 mM imidazole/HC1 pH 6.6, 50 mM NaCl, 12.5 mM MgCl2, 0.5 mM EGTA, 10 mM dithiothreitol, 0.3% Triton X-100, 0.5 mM [7-3H]ATP (specific activity 0.02 Ci/mmole), 144 µM PuSer and 15 mU diacylglycerol kinase (Escherichia coli) at a final volume of 100 µl. The reaction was started by the addition of ATP and allowed to proceed for 30 min at 30°C. The incubation was stopped by the addition of 470 µl chloroform/methanol/2% acetic acid (15:30:2, by vol.). In order to prevent hydrolysis of endotherm bonds, no strong acid was used in the latter mixture. Phase separation was obtained by the addition of 300 µl chloroform and 1 ml 2% acetic acid. After centrifugation at 1500×g for 5 min, the organic phase was washed with 1 ml 2% acetic acid and dried under nitrogen. The lipids were separated by thin-layer chromatography using silica gel 60 HPTLC plates. The plates were developed with chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, by vol.) followed by autoradiography. The radioactive spot corresponding with PuOH was scraped off and 32P radioactivity was measured using a liquid scintillation analyzer. The 1,2-diradylglycerol content of the original sample was calculated from the amount of [32P]PuOH produced. The data presented are expressed as molar amount/mass of acinar protein. Protein was determined with a commercial Coomassie blue kit (Bio-Rad) [33]. Bovine serum albumin was used as a standard.

**Analysis of 32P-labeled phospholipids.** Rabbit pancreatic acini, resuspended in Krebs-Ringer bicarbonate medium, pH 7.4, were labeled with ortho[32P]phosphate (25 µCi/ml) in the absence or presence of cholecystokinin-(26–33)-peptide amide (10 nM) for 70 min at 37°C. After 60 min of labeling, control acini were stimulated with cholecystokinin-(26–33)-peptide amide (10 nM). At appropriate times, 200-µl samples were removed in duplicate and vigorously mixed with 0.9 ml ice-cold extraction medium containing dichloromethane/methanol/concentrated HCl (20:40:1, by vol.). Phase separation was obtained by adding 200 µl dichloromethane and 200 µl water, followed by centrifugation (5 min, 7000×g). After collection of the organic phase, the aqueous phase was extracted again and the combined organic phases were blown to dryness with nitrogen. The extracts were dissolved in dichloromethane/methanol/water (75:25:2, by vol.) and divided over two potassium-oxalate-treated HPTLC plates. One plate was developed with dichloromethane/methanol/20% acetic acid (60:36:10, by vol.), separating PuInsls(4,5)P2, phosphatidilylinositol 4-phosphate [PuInsls(4)P], PuCho and PuEtN, but not PuIns from PuOH; the other plate was developed with dichloromethane/acetone/methanol/acetic acid/water (40:15:12:8, by vol.), separating PtdIns(4,5)P2, PtdIns(4)P, PtdOH and PtdEtN, but not PtdIns from PtdCho. Radioactive spots, visualized by means of autoradiography, were scraped off and 32P measured in a liquid scintillation analyzer. Under the above conditions, PuSer was not labeled, whereas labeling of PuEtN was very weak. In each experiment, for each individual phospholipid, the amount of labeling reached in control acini at 60 min was set at 100%, to which all other values were related.

**RESULTS**

**Time-dependence of cholecystokinin-(26–33)-peptide amide-stimulated 1,2-diradylglycerol formation in rabbit pancreatic acini.** Cholecystokinin has been reported in the literature to evoke a biphasic increase in pancreatic acinar 1,2-diacylglycerol content in the rat [34]. However, preliminary experiments revealed that the diacylglycerol kinase used in this particular assay does not discriminate between alk-1-ethyl-2-acylglycerols and 1,2-diacylglycerols and that therefore acinar 1,2-diradylglycerol rather than 1,2-diacylglycerol levels are determined. In the present study, a biphasic increase in mass 1,2-diradylglycerol was observed in rabbit pancreatic acini following stimulation with 10 nM cholecystokinin-(26–33)-peptide amide, which is the maximally stimulatory concentration for amylase secretion in these cells (Fig. 1). During the early phase of the response, the acinar diradylglycerol content only slightly increased by a factor of 1.16; the basal 1,2-diradylglycerol content amounting to 1.84 nmol/mg protein (SE 0.06; n = 7). This early rise in mass 1,2-diradylglycerol was clearly transient with a peak value reached at 10 s following the onset of stimulation. After having returned to basal levels, a second more pronounced increase in acinar 1,2-diradylglycerol content was observed. This late rise in mass 1,2-diradylglycerol was clearly long-lasting with a maximum of 3.85 nmol/mg protein (SE 0.39; n = 7) reached at 30 min following the onset of stimulation. Analysis of the 1,2-diradylglycerol species after 10 s did not show any significant difference whether or not the acini were stimulated (not shown).
Table 1. Relative phospholipid composition in rabbit pancreatic acini. Pancreatic acinar phospholipids were separated into subclasses by means of HPTLC and phosphate analysis as described by Fieske and Subba-Rao [24]. The values presented are the means ± SE of six independent determinations.

<table>
<thead>
<tr>
<th>Phospholipid class</th>
<th>Proportion</th>
<th>1,2-Diacyl subclass</th>
<th>1-Alk-1-ethenyl-2-acyl subclass</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>12.8 ± 0.6</td>
<td>40.0</td>
<td>18.3 ± 1.1</td>
<td>18.4 ± 1.1</td>
</tr>
<tr>
<td>PtdOH</td>
<td>5.0 ± 0.6</td>
<td>100</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>PtdSer</td>
<td>9.7 ± 0.7</td>
<td>100</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>PtdIns</td>
<td>8.6 ± 0.2</td>
<td>100</td>
<td>0</td>
<td>100.0</td>
</tr>
<tr>
<td>PtdCho</td>
<td>43.0 ± 1.3</td>
<td>81.7 ± 1.1</td>
<td>18.3 ± 1.1</td>
<td>18.4 ± 1.1</td>
</tr>
<tr>
<td>PtdEtn</td>
<td>22.0 ± 0.5</td>
<td>43.1 ± 2.2</td>
<td>56.9 ± 2.2</td>
<td>100.0</td>
</tr>
</tbody>
</table>

In order to elucidate the source of the 1,2-diradylglycerols formed during prolonged stimulation, we analysed the molecular species of the 1,2-diradylglycerols present at 30 min following the onset of stimulation and compared these with the molecular species of the major acinar phospholipids.

Quantification of the major phospholipid classes and subclasses in rabbit pancreatic acini. The major acinar phospholipids were separated by thin-layer chromatography and subsequently quantified by lipid phosphorus analysis as described in Experimental Procedures. As shown in Table 1, PtdCho (43%) and PtdEtn (22%) formed the predominant acinar phospholipid classes. In addition, significant amounts of sphingomyelin (13%), PtdIns (9%), PtdSer (10%) and PtdOH (5%) were detected. Concerning the different phospholipid subclasses, the PtdEtn fraction of rabbit pancreatic acini contained both plasmenylethanolamine (57%) and the 1,2-diacyl form of PtdEtn (43%). Similarly, acinar PtdCho contained appreciable amounts of plasmenecholine (18%) and the 1,2-diacyl form (82%). In contrast, none of the other major phospholipids contained detectable amounts of the 1-alk-1-ethyl-2-acyl subclass. It should be noted that the method used to separate the phospholipid subclasses does not discriminate between 1,2-diacyl and 1-alkyl-2-acyl phospholipids. However, the experimental data described in the next paragraph clearly demonstrate that pancreatic acinar cells do not contain detectable amounts of 1-alkyl-2-acyl phospholipids.

Molecular species analysis of the major phospholipid subclasses in rabbit pancreatic acini. In order to determine the molecular species composition of the major phospholipid subclasses, the acinar phospholipids, separated by means of thin-layer chromatography, were treated with phospholipase C to yield the corresponding 1,2-diradylglycerol fractions. Subsequently, each 1,2-diradylglycerol fraction was derivatized with (NO2)2PhC0Cl and separated into its subclasses. It was at this stage, that it could be demonstrated that none of the acinar phospholipids contained detectable amounts of the 1-alkyl-2-acyl subclass. Finally, each of the subclasses was subjected to molecular species analysis by means of the HPLC technique. The molecular species composition of the major 1,2-diacyl and 1-alkyl-1-ethyl-2-acyl phospholipids are listed in Tables 2 and 3, respectively.

Separation of the 1,2-diacyl form of PtdCho yielded mainly molecular species with palmitic acid (16:0) and possibly also stearic acid (18:0) at the sn-1 position and linoleic acid (18:2) and possibly also oleic acid (18:1) at the sn-2 position. Similarly, the 1,2-diacyl form of PtdEtn contained mainly palmitic acid and possibly also stearic acid at the sn-1 position and linoleic acid and possibly also oleic acid at the sn-2 position. In addition, approximately 10% of the 1,2-diacyl species of PtdEtn

<table>
<thead>
<tr>
<th>Proportion</th>
<th>1,2-Diacyl subclass</th>
<th>1-Alk-1-ethenyl-2-acyl subclass</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.3 ± 0.3</td>
<td>81.7 ± 1.1</td>
<td>18.3 ± 1.1</td>
<td>18.4 ± 1.1</td>
</tr>
<tr>
<td>22.0 ± 0.5</td>
<td>43.1 ± 2.2</td>
<td>56.9 ± 2.2</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 2. Relative distribution of 1,2-diacyl phospholipid molecular species in rabbit pancreatic acini. Acinar phospholipids were separated and hydrolysed with phospholipase C. The resulting 1,2-diradylglycerol fractions were di-tritohexanoylated and separated into subclasses. Molecular species of the 1,2-diacylglycerol fractions were analysed by means of reverse-phase HPLC. The values presented for PtdCho and PtdIns are the means ± SE of three independent experiments, whereas those presented for PtdEtn are the means of two independent experiments. The values presented for PtdSer and PtdOH are from a single experiment; n.d., not detected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amount in mol/100 mol</th>
<th>PtdCho</th>
<th>PtdEtn</th>
<th>PtdIns</th>
<th>PtdSer</th>
<th>PtdOH</th>
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<tr>
<td>16:0/20:5 + 18:1/20:5</td>
<td>0.1 ± 0.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>16:0/20:5 + 18:1/20:5</td>
<td>0.1 ± 0.1</td>
<td>0.8</td>
<td>5.3 ± 1.3</td>
<td>8.0 ± 0.6</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>18:0/22:6</td>
<td>1.8 ± 0.2</td>
<td>4.0</td>
<td>2.3</td>
<td>n.d.</td>
<td>n.d.</td>
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</tr>
<tr>
<td>16:0/22:6</td>
<td>2.9 ± 0.8</td>
<td>0.8</td>
<td>2.7 ± 0.8</td>
<td>9.3</td>
<td>13.4</td>
<td>13.5</td>
</tr>
<tr>
<td>16:0/22:6</td>
<td>0.4 ± 0.4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>16:0/22:6</td>
<td>0.1 ± 0.1</td>
<td>0.8</td>
<td>5.3 ± 1.3</td>
<td>8.0 ± 0.6</td>
<td>n.d.</td>
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<tr>
<td>18:0/20:4</td>
<td>1.2 ± 1.2</td>
<td>1.2 ± 1.2</td>
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<tr>
<td>18:0/20:4</td>
<td>36.4 ± 1.4</td>
<td>45.5</td>
<td>28.3 ± 2.7</td>
<td>31.7</td>
<td>26.0</td>
<td>43.0 ± 1.3</td>
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</table>
Table 3. Relative distribution of 1-alk-1-enyl-2-acyl phospholipid molecular species in rabbit pancreatic acini. Acinar phospholipids were separated and hydrolysed with phospholipase C. The resulting 1,2-diacylglycerol fractions were dinitrobenzoylated and separated into subclasses. Molecular species of the 1-alk-1-enyl-2-acylglycerol fractions were analysed by means of reverse-phase HPLC. The values presented are from a single experiment; n.d., not detected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amount in mol/100 mol</th>
<th>plasmerylencholesterol</th>
<th>plasmerylen-ethanolamine</th>
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<tr>
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<tr>
<td>16:0/20:4</td>
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<td>18:1/20:4</td>
<td>9.6</td>
<td>1.3</td>
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<tr>
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<td>0.1</td>
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<td>2.8</td>
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<td>n.d.</td>
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<tr>
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<td>n.d.</td>
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<tr>
<td>20:0/20:4</td>
<td>n.d.</td>
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</tbody>
</table>

Molecular species analysis of the 1,2-diradylglycerols in unstimulated acini. Diradylglycerols, extracted from freshly isolated acini, were derivatized with (NO₂)₂PhCOCI and subjected to TLC in order to separate the 1,2-diradylglycerols from the 1-alkyl-2-acylglycerols and the 1-alk-1-enyl-2-acylglycerols. In doing so, it could be demonstrated that the acinar 1,2-diradylglycerol fraction did not contain detectable amounts of the 1-alkyl-2-acylglycerol subclass. Subsequently, each subclass was subjected to reverse-phase HPLC in order to separate its molecular species. Table 4 lists the molecular species of the 1,2-diglycerol subclass. The major acyl groups at the sn-1 position of the 1,2-diradylglycerols were palmitic acid, oleic acid and possibly also arachidonic acid at the sn-1 position. The predominant molecular 1,2-diacyl species in both PtdCho and PtdEtn were 16:0/18:2 (50% and 26% in PtdCho and PtdEtn, respectively) and 16:0/18:1 and/or 18:0/18:2 (36% and 45% in PtdCho and PtdEtn, respectively). A relatively small amount of 18:0/20:4 was detected in 1,2-diacyl species of PtdEtn. In PtdIns the predominant 1,2-diacyl species contained stearic acid, oleic acid and possibly also palmitic acid at the sn-1 position. Arachidonic acid, docosahexaenoic acid (22:6), oleic acid and possibly also linoleic acid were present in high amounts at the sn-2 position in 1,2-diacyl PtdIns species. In contrast to the 1,2-diacyl species of PtdCho and PtdEtn, that of PtdIns contained significant amounts of 18:1/18:1, 18:0/20:4 and 18:0/22:6, whereas the amount of 16:0/18:2 was relatively small in this PtdIns subclass. Separation of 1,2-diacyl PtdSer species yielded mainly those containing stearic acid, oleic acid and palmitic acid at the sn-1 position and arachidonic acid, docosahexaenoic acid, linoleic acid and possibly also oleic acid at the sn-2 position. In contrast to the 1,2-diacyl species of PtdCho, PtdEtn and PtdIns, PtdSer contained significant amounts of 18:1/18:2. Finally, 1,2-diacyl species of PtdOH consisted mainly of palmitic acid, stearic acid and oleic acid at the sn-1 position, while at the sn-2 position, arachidonic acid, docosahexaenoic acid, linoleic acid and possibly also oleic acid predominated. In contrast to the other 1,2-diacyl phospholipids, that of PtdOH contained significant amounts of 18:1/22:6. In conclusion, the 1,2-diacyl species 18:0/22:6 and 18:1/18:1 proved to be relatively specific for PtdIns. Similarly, 1,2-diacyl species 18:1/22:6 proved to relatively specific for PtdOH.

At the sn-1 position of both plasmerylencholesterol and plasmerylen-ethanolamine mainly C₁₆ and C₁₈ aliphatic chains were found (Table 3). Plasmerylencholesterol contained more arachidonic acid at the sn-2 position than plasmerylencholesterol. Moreover, the molecular species of plasmalogenes contained relatively more arachidonic acid and docosahexaenoic acid at the sn-2 position than the corresponding 1,2-diacyl phospholipids.
The molecular species profiles of the control and stimulated 2-0-1,2-diacylglycerol production. In stimulated acini, 53% of the 1,2-diacylglycerols were calculated to derive from PtdCho, 46% from Ptdlns, and 1% from PtdEtn. None of the 1,2-diacylglycerols were derived from PtdSer or PtdOH. Likewise, we compared the molecular species profiles of the control and stimulated 1-alk-1-enyl-2-acylglycerol fractions (Table 5) with those of combinations of plasmenylethanolamine and plasmenylcholine (Table 3). Under control as well as stimulated conditions 100% of the 1-alk-1-enyl-2-acylglycerols were calculated to originate from plasmenylethanolamine.

Effects of cholecystokinin-(26-33)-peptide amide on phospholipid labeling. A second approach to investigate the effect of hormonal stimulation on acinar phospholipid metabolism was to stimulate acini with cholecystokinin-(26-33)-peptide amide in the presence of ortho[32P]phosphate. Fig. 2 demonstrates that the incorporation of label into PtdOH (Fig. 2A) and PtdIns (Fig. 2B) was stimulated by factors of 2.6 and 3.6, respectively, in acini incubated in the presence of 10 nM cholecystokinin-(26-33)-peptide amide for 60 min. The hormone did not affect the labeling of PtdCho (not shown) and only slightly decreased the incorporation of label into PtdIns(4)P (Fig. 2C). However, labeling of PtdIns(4,5)P2 was reduced by 50% in acini labeled in the presence of hormone for 60 min (Fig. 2D). Moreover, under stimulatory conditions, the incorporation of label into PtdIns(4,5)P2 reached equilibrium within 40 min following the onset of labeling, whereas in control acini labeling of PtdIns(4,5)P2 was nearly linear with time for at least 60 min. At 60 min, stimulation of control acini with 10 nM cholecystokinin-(26-33)-peptide amide resulted in a rapid increase in [32P]PtdOH (Fig. 2A), followed by a more gradual increase in [32P]PtdIns (Fig. 2B). In contrast, the amount of [32P]PtdIns(4,5)P2 (Fig. 2D) immediately decreased followed by a more gradual decrease in [32P]PtdIns(4)P (Fig. 2C).

DISCUSSION

The aim of the present study was to assess the origin of the 1,2-diacylglycerols produced during prolonged hormonal stimulation of rabbit pancreatic acini by comparison of their relative molecular species composition with that of the major acinar phospholipids. In doing so, it was found that the acinar 1,2-diacylglycerol fraction consisted of two subclasses, namely the 1,2-diacylglycerols and the 1-alk-1-enyl-2-acylglycerols. Conversely, pancreatic acini did not contain detectable amounts of the 1-alkyl-2-acylglycerol subclass. Although we did not determine the exact amounts of the two subclasses this observation demonstrates that a not inconsiderable part of the acinar 1,2-diacylglycerol fraction may consist of a subclass, the 1-alk-1-enyl-2-acylglycerols, the effect of which on protein kinase C is questionable [18-20]. Thus far, it is unknown whether the marked increase in mass acinar 1,2-diacylglycerol, observed during prolonged hormonal stimulation (see also [34]), involves the increased formation of both the 1,2-diacylglycerols and the 1-alk-1-enyl-2-acylglycerols.

The main observation of the present study is that prolonged hormonal stimulation did not significantly change the relative amounts of the molecular species in the two 1,2-diacylglycerol subclasses. In view of the large (twofold) increase in acinar 1,2-diacylglycerol content (see also [34]), this suggests that in both cases the same precursor phospholipids are used during basal and stimulated 1,2-diacylglycerol production. This conclusion is supported by the outcome of the precursor profile calculations. Thus, based on the relative molecular species composition of the 1,2-diacylglycerol fraction, it was calculated that, under unstimulated conditions, 60% of the 1,2-diacylglycerols originated from PtdCho and 40% from PtdIns, whereas under stimulatory conditions 53% of the 1,2-diacylglycerols were calculated to be derived from PtdCho, 46% from PtdIns and 1% from PtdEtn. Likewise, it was calculated that, under both control
Fig. 2. Effect of cholecystokinin-(26–33)-peptide amide on 32P-labeling of pancreatic acinar cell phospholipids. Rabbit pancreatic acini were incubated in the presence of ortho [32P]phosphate and the absence (C) or presence of 10 nM cholecystokinin-(26–33)-peptide amide (CCK-8) (●) for 70 min at 37°C. Samples for extraction and analysis of 32P-labeled phospholipids were removed at the indicated times. After 60 min of labeling, control acini were stimulated with cholecystokinin-(26–33)-peptide amide (10 nM). In each experiment, for each phospholipid, the amount of label incorporated in control acini at 60 min is set at 100%, to which all other values are related. The values presented are the means ± SE of three experiments. (A) PtdOH; (B) PtdIns; (C) PtdIns(4)P; (D) PtdIns(4,5)P2.

and stimulatory conditions, 100% of the 1-alk-1-enyl-2-acyl-
glycerols was derived from plasmenylcholine. Since the relative
species distribution for both phospholipids and 1,2-diradylglycer-
ol was obtained from analysis of total cell extract, the
following possibilities concerning the cellular localization of the
precursor phospholipids occur. First, on the assumption that hor-
monal stimulation affects plasma membrane phospholipids ex-
clusively (agonist-sensitive pool) the present findings suggest that plasma membrane phospholipids function also as the major
source of 1,2-diradylglycerols in the unstimulated cell (biosyn-
thetic pool). Second, under the same assumption the present
findings also suggest that phospholipids present in plasma mem-
bane and intracellular membranes may not differ markedly in
molecular species composition. Third, since the biosynthetic
pool consists of 1,2-diradylglycerols derived from both the
plasma membrane and intracellular membranes, the data pre-
sented suggest that phospholipids present in intracellular mem-
branes may also be affected during prolonged stimulation. Some
evidence that this might be the case will be discussed below.

An important conclusion to be drawn from the above obser-
vations is that a considerable amount of the 1,2-diacylglycerols
produced during the second phase of the biphasic increase in
mass 1,2-diradylglycerol is derived from PtdIns and not, as has
been suggested in the literature [35], more or less exclusively
from PtdCho. Further evidence in favour of this conclusion is
provided by the finding that labeling of PtdIns(4,5)P2 with
ortho [32P]phosphate reached isotopic equilibrium considerably
faster under stimulatory conditions. Together with the observa-
tion that the amount of [32P]PtdIns(4,5)P2 was markedly (50%)
decreased in stimulated acini this suggests that the phospholi-
pase-C-catalysed hydrolysis of PtdIns(4,5)P2 is not transient but
remains elevated during prolonged hormonal stimulation. Simi-
larly, in a recent study by Allan et al. [36], it was found that
vasopressin evoked a sustained decrease in the amount of
[3H]PtdIns(1,3,4)P3 in hepatocytes. These observations demon-
strate that, in contrast to suggestions in the literature [37], the
cellular PtdIns(4,5)P2 content does not increase to prestimula-
tory levels but remains lowered during prolonged hormonal
stimulation. Additional support in favour of the above conclu-
sion that PtdIns(4,5)P2 hydrolysis remains increased during pro-
longed stimulation comes from the literature finding that, under
stimulatory conditions, the production rate of [3H]Ins(1,3,4)P3,
which reflects the production rate of [3H]Ins(1,4,5)P₃, remains increased for prolonged periods of time [38]. This finding might also explain the observation by Bansbach et al. [39] that the fatty acid composition of the acinar 1,2-diacylglycerol fraction was not comparable to that of the inositol phospholipids. The observation that labeling of PtdIns did not reach equilibrium despite the fact that it was markedly increased by the action of cholecystokinin-(26–33)-peptide amide demonstrates that the size of the PtdIns pool is considerably larger than that of PtdIns(4,5)P₂. In addition, the observation that the labeling of PtdIns(4,5)P did not reach equilibrium despite the fact that it was slightly reduced as compared to unstimulated acini demonstrates that the size of its pool, although markedly smaller than that of PtdIns, is also considerably larger than that of PtdIns(4,5)P₂.

Biphasic increases in cellular 1,2-diacylglycerol content, usually referred to as cellular 1,2-diacylglycerol content, have been observed in many cell types in response to hormonal stimulation [4, 34, 40, 41]. It should be noted, however, that monophasic response patterns have been observed as well. In the latter case, the stimulants involved included phorbol esters [11, 42], certain growth factors [35, 43] and some interleukins [44]. In contrast, biphasic response patterns are observed with hormones or neurotransmitters stimulating the phospholipase-C-catalysed hydrolysis of PtdIns(4,5)P₂. In the exocrine pancreas, the observation that the rapid increase in mass 1,2-diacylglycerol, which is transient by nature and which occurs immediately following the onset of stimulation, coincides with the rapid and transient increase in mass Ins(1,4,5)P₃ has led to the idea that PtdIns(4,5)P₂ is the major source of 1,2-diacylglycerols produced during the early phase of hormonal stimulation [34]. But, whereas in the continuous presence of the hormone the acinar 1,2-diacylglycerol content increases for a second time, the acinar Ins(1,4,5)P₃ content remains near basal levels. The latter observation has been explained by assuming that during the second phase of the response, PtdCho is the major source for the 1,2-diacylglycerols produced. However, as already discussed above, the data presented in this study suggest that this is not entirely the case and that at least part of the 1,2-diacylglycerols produced during prolonged stimulation are derived from PtdIns(4,5)P₂. The fact that the cellular Ins(1,4,5)P₃ content is only transiently increased is best explained by assuming that it is rapidly phosphorylated to Ins(1,3,4,5)P₄. Indeed, in hepatocytes, hormonal activation has been demonstrated to result in an increase in activity of the kinase involved in this phosphorylation reaction [36]. Interestingly, recent studies have demonstrated that the receptor-stimulated hydrolysis of acinar PtdIns involves the action of phospholipase D yielding PtdOH, which is subsequently hydrolysed to 1,2-diacylglycerol by the action of phosphatidic acid phosphohydrolase [6].

The present study demonstrates that choline and ethanolamine phospholipids exist in two forms in pancreatic acini, namely the diacyl form (82% and 43% in PtdCho and PtdEtn, respectively) and the 1-alk-1-enyl-2-acyl form (18% and 57% in plasmenylcholine and plasmenylethanolamine, respectively). In contrast, no 1-alk-1-enyl-2-acyl forms of PtdIns, PtdSer or PtdOH were found. The acinar cells did not contain detectable amounts of the 1-alk-1-acyl form of any of the major phospholipids. In this respect acinar cells differ from other cell types such as human neutrophils (5% 1-alk-1-enyl-2-acyl, 45% 1-alkyl-2-acyl and 50% 1,2-diacyl for PtdCho [11]) and the human leukemia cell lines HL-60 (57% 1-alk-1-enyl-2-acyl, 7% 1-alkyl-2-acyl and 36% 1,2-diacyl for PtdCho, 7% 1-alkyl-1-enyl-2-acyl, 28% 1-alk-1-enyl-2-acyl and 66% 1,2-diacyl for PtdCho [45]) and K562 (38% 1-alk-1-enyl-2-acyl, 4% 1-alkyl-2-acyl and 58% 1,2-diacyl for PtdEtn; 4% 1-alkyl-1-enyl-2-acyl, 10% 1-alk-1-enyl-2-acyl and 86% 1,2-diacyl for PtdCho [45]). Similarly, the acinar 1,2-diacylglycerol fraction contained 1,2-diacylglycerols and 1-alk-1-enyl-2-acylglycerols, but no 1-alkyl-2-acylglycerols.

Molecular species analysis, performed according to the method described by Engelmann et al. [27], revealed that the fatty acids at the sn-1 and sn-2 position of the acinar phospholipids occurred in at least 18 different combinations. After separation of the individual phospholipids, the relative amounts of the molecular species were determined and used to calculate the precursor profile for the 1,2-diacylglycerols present in control and stimulated acini as already discussed. The 1,2-diacyl species 18:1/18:1 is relatively abundant in PtdIns. However, there is some evidence that this particular PtdIns species is solely present in the nuclear envelope [46]. Interestingly, its relative amount in the 1,2-diacylglycerol fraction of stimulated acini was increased rather than decreased, suggesting an increased production rate during prolonged hormonal stimulation. The 1,2-diacyl fraction of PtdIns consists for 25% of the molecular species 18:0/20:4. However, this species was not detected in the 1,2-diacylglycerol fraction of the 1,2-diacylglycerols. A possible explanation might be that this 1,2-diacylglycerol species is effectively phosphorylated by a specific 1,2-diacylglycerol kinase first described in Swiss 3T3 cells [47]. Indeed, the 1,2-diacyl fraction of acinar PtdOH was found to contain a high percentage of the molecular species 18:0/20:4. However, it was not investigated in this study whether the relative amount of 18:0/20:4 PtdOH was actually increased under stimulatory conditions. Another explanation might be that the 1,2-diacylglycerol molecular species 18:0/20:4 serves as a precursor in arachidonic acid formation. It should be noted that the absence of 18:0/20:4 1,2-diacylglycerol in the acinar 1,2-diacylglycerol fraction will lead to underestimation of the relative contribution of PtdIns as a precursor for 1,2-diacylglycerol.

In conclusion, the data presented are in agreement with the idea that the 1,2-diacylglycerols produced during sustained hormonal stimulation of pancreatic acini are derived mainly from PtdIns and PtdCho. In addition, the present study demonstrates that the acinar 1,2-diacylglycerol fraction contains significant amounts of 1-alk-1-enyl-2-acylglycerols, the function of which is unknown. Most probably, these 1-alk-1-enyl-2-acylglycerols are derived from plasmencylcholine. However, it remains to be elucidated whether the production of the 1-alk-1-enyl-2-acylglycerols also occurs in a stimulated manner.

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