Ca$^{2+}$ Ionophore and Phorbol Ester Stimulate Diacylglycerol Formation and Phosphatidylcholine Hydrolysis in Rat Parotid Acinar Cells

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ABSTRACT—We investigated the effects of A23187 and phorbol 12,13-dibutyrate (PDBu) on sn-1,2-diaclylglycerol (DAG) accumulation and phosphatidylcholine (PC) hydrolysis in rat parotid acinar cells. Both A23187 and PDBu, in concentration ranges of 0.001–0.1 μM, stimulated DAG accumulation and PC hydrolysis in a time- and concentration-dependent manner. Treatment with A23187 and PDBu stimulated the release of $[^{3}$H]$\text{choline}$ and $[^{3}$H]$\text{phosphocholine}$ into the medium, indicating $[^{3}$H]$\text{PC}$ hydrolysis is due to the activation of phospholipases C and D; however, $[^{3}$H]$\text{phosphatidylethanolamine}$ hydrolysis was not indicated. These releases were unaffected by the addition of glucose 6-phosphate, a phosphatase inhibitor. Staurosporine, a protein kinase C inhibitor, significantly inhibited the DAG accumulation and the PC hydrolysis stimulated by these agents. Combinations of A23187 and PDBu potentiated the stimulatory effect which each of these agents alone had on DAG accumulation and PC hydrolysis. This mode of action was additive but not synergistic. These results suggest that DAG accumulation induced by A23187 and PDBu is related to the PC hydrolysis mediated via the activation of phospholipases C and D, and that it is not related to phosphatidylethanolamine hydrolysis.

Keywords: Parotid acinar cells, A23187, Phorbol 12,13-dibutyrate, sn-1,2-Diacylglycerol accumulation, Phosphatidylcholine hydrolysis

Agonist binding to its receptor leads to phosphatidylinositol 4,5-bisphosphate breakdown into sn-1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (1,4,5-IP$_3$) via activation of inositol phospholipid-specific phospholipase C coupled with a GTP binding protein; DAG activates protein kinase C and 1,4,5-IP$_3$ mobilizes Ca$^{2+}$ from the endoplasmic reticulum (1–4). These events are thought to play an important role in stimulus-secretion coupling (5–7).

DAG was shown to be derived from phosphoinositides, phosphatidylcholine (PC), and other lipids (7–10), but the relationship between DAG generation and their source during agonist stimulation is not fully understood. This may be due to the complexity of the kinetics of DAG formation; e.g., the kinetics of DAG formation varies with the agonist and the cell type. Recently, PC hydrolysis has gained much attention, because DAG generated by protein kinase C-dependent PC-specific phospholipase C can provide a sustained activation of protein kinase C (11). In many cell types, Ca$^{2+}$ ionophore or phorbol ester stimulates PC hydrolysis through phospholipase C and/or D and results in DAG accumulation. However, the mode (phospholipase C, phospholipase D) by which these agents elicit DAG formation from PC varies with the cell type (12). As reviewed by Billah and Anthes (12), there are many reports concerning PC hydrolysis induced by Ca$^{2+}$ ionophore and phorbol esters, but the mechanism of PC hydrolysis in this system remains unknown.

We therefore examined the effects of A23187 and phorbol 12,13-dibutyrate (PDBu) on DAG accumulation and PC hydrolysis in rat parotid acinar cells. These results provide evidence that a protein kinase C-dependent PC hydrolysis is closely related to DAG accumulation and that the hydrolysis is mediated through phospholipases C and D.
MATERIALS AND METHODS

Materials

The following chemicals were obtained from the indicated firms: \([\gamma^{-32P}]\)ATP (> 4000 Ci/mmol, ICN Radiochemicals), [methyl-3H]choline chloride (75–85 Ci/mmol) and [L-3H]ethan-1-ol-2-amine hydrochloride (5–30 Ci/mmol, Amersham), DAG kinase (Lipidex), PDBu and staurosporine (Sigma), and A23187 (Calbiochem). All other reagents were of analytical grade.

Preparation of parotid acinar cells

Parotid glands were removed from sodium pentobarbital (50 mg/kg)-anesthetized male Wistar rats (180–200 g). Acinar cells were prepared by enzymatic digestion with collagenase, as described previously (13). These cells (10⁶ cells/ml) were incubated in a HEPES-buffered Krebs-Henseleit medium containing: 98 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM Na-HEPES, 11 mM dextrose, and 1% (w/v) bovine serum albumin. The medium also contained essential amino acids and was maintained at pH 7.4 under an atmosphere of 95% oxygen and 5% carbon dioxide. The number of cells was determined microscopically in a hemacytometer. All experiments were carried out at 37°C.

DAG assay

DAG levels in the lipid extracts of acinar cells were measured by a highly sensitive assay capable of quantitating the mass of DAG in crude lipid extracts of cells, as previously described by Preiss et al. (14). This assay depends on the measurement of the DAG mass converted from DAG to \([32P]\)phosphatidic acid (\([32P]\)PA) by DAG kinase in the presence of \([\gamma^{-32P}]\)ATP. \([32P]\)PA was separated by thin layer chromatography on a Silica Gel 60 plate. The radioactive spot corresponding to \([32P]\)PA was scraped off and counted by liquid scintillation spectrometry. The DAG content of cells was determined from the sample volume and the specific activity of the ATP.

PC and phosphatidylethanolamine hydrolysis assay

Acinar cells were prelabeled with \([3H]\)choline chloride (10 μCi/ml) or \([3H]\)ethanolamine hydrochloride (10 μCi/ml) for 90 min at 37°C, as described previously by Matozaki and Williams (15). The percentage of tritium incorporated into the cellular PC and phosphatidylethanolamine fractions in the phospholipids was 84% and 99%, respectively, when acinar cells were labeled with \([3H]\)choline or \([3H]\)ethanolamine.

For the hydrolysis assay, acinar cells were incubated with agonists for the indicated time. After centrifugation at 10000 × g for 15 sec, each supernatant (0.8 ml) was extracted with 3 ml of a chloroform/methanol mixture (1 : 2, v/v), and then 1 ml of chloroform and 1 ml of water were added. The aqueous phase was counted to determine the release of \([3H]\)choline or \([3H]\)ethanolamine metabolites.

For the analysis of the released radioactivity, \([3H]\)phosphocholine, \([3H]\)glycerophosphocholine, \([3H]\)choline, and \([3H]\)betaine were separated by thin layer chromatography in a solvent system containing water (0.9% NaCl)/methanol/concentrated NH₄OH (50:50:5, v/v). Each radioactive spot was scraped off and counted by liquid scintillation spectrometry.

Data analyses

All data are expressed as the mean ± S.E. Significance of difference between means was determined using Student’s t-test. P values < 0.05 were considered significant. In each figure where S.E. bars are not shown, they are within the symbols.

RESULTS

Action of A23187 on DAG accumulation induced by PDBu

Figure 1 shows the time course of DAG accumulation in response to A23187 and PDBu. DAG accumulation induced by A23187 and PDBu increased linearly with time. The percentages of DAG accumulation at 30 min were 40% and 38% for stimulation by A23187 and PDBu, respectively. 4 α-Phorbol 12,13-didecanoate, an inactive phorbol ester, did not cause in DAG accumulation (data not shown). As seen in Fig. 2, DAG accumulation induced by A23187 or PDBu alone was dose-dependent. These data also show that combinations of A23187 and PDBu significantly potentiated the stimulatory effect of each agent acting alone; the combinations produced an additive increase.

Analysis of \([3H]\)choline metabolites released into the medium

We evaluated \([3H]\)choline metabolites released into the medium (Fig. 3). Both A23187 and PDBu caused the release of \([3H]\)choline and \([3H]\)phosphocholine into the medium; more \([3H]\)phosphocholine than \([3H]\)choline was released. In contrast, the effects of A23187 and PDBu on the release of \([3H]\)glycerophosphocholine were barely discernible.

In order to study whether the formation of \([3H]\)choline by A23187 and PDBu was due to the hydrolysis of \([3H]\)phosphocholine released into the medium by extracellular phosphatases, the phosphatase inhibitor (16) glucose 6-phosphate was added with these
agents for 30 min. Glucose 6-phosphate (20 mM) did not modify the release of [3H]phosphocholine and that of [3H]choline induced by 0.1 μM A23187 and 0.1 μM PDBu: for the basal, basal plus glucose 6-phosphate, A23187, A23187 plus glucose 6-phosphate, PDBu, and PDBu plus glucose 6-phosphate, the levels of [3H]-phosphocholine assayed in the medium were 300 ± 58, 320 ± 41, 509 ± 44, 501 ± 36, 515 ± 33, and 502 ± 44, respectively; and the levels of [3H]choline assayed in the medium were 125 ± 17, 120 ± 16, 237 ± 32, 230 ± 31, 187 ± 28, and 175 ± 11, respectively (dpm/10^6 cells, n = 5).

The sum of the radioactivities of [3H]phosphocholine, [3H]choline, and [3H]glycerophosphocholine released into the medium at 30 min was less than the total activity estimated (Figs. 3 and 5). Since the [3H]choline utilized in this study was labeled in the methyl position, additional labeled metabolites might be generated in the cells, in particular betaine through the action of betaine aldehyde dehydrogenase. We further examined the release of [3H]betaine into the medium. Stimulation by these agents for 30 min had a significant (P < 0.05) effect on the radioactivity associated with this metabolite (dpm/10^6 cells, n = 5, basal, 50 ± 6; 0.1 μM A23187, 188 ± 42; 0.1 μM PDBu, 163 ± 31).

Fig. 1. Time course of DAG formation stimulated with A23187 and PDBu. Acinar cells were incubated with A23187 or PDBu. Each point represents the mean ± S.E. of quadruplicate determinations from two separate experiments. Basal (▲), 0.1 μM A23187 (△), 0.1 μM PDBu (●).

Fig. 2. Effect of A23187 and PDBu alone and in combination on DAG accumulation. Acinar cells were suspended in incubation solution and incubated with various concentrations of A23187 or PDBu for 30 min. Each column represents the mean ± S.E. of quadruplicate determinations from two separate experiments.
Fig. 3. Analysis of \[^{[3]}\text{H}\]choline metabolites released into the medium. Acinar cells were stimulated with 0.1 \(\mu\)M A23187 or 0.1 \(\mu\)M PDBu for 30 min. The amounts of each \[^{[3]}\text{H}\]choline metabolite observed at 0 time were subtracted from each value. Each column represents the mean ± S.E. of quadruplicate determinations from two separate experiments. *\(P < 0.05\), **\(P < 0.01\) vs. basal value. \[^{[3]}\text{H}\]phosphocholine (□), \[^{[3]}\text{H}\]choline (■), \[^{[3]}\text{H}\]glycerophosphocholine (■).

Fig. 4. Time course of the release of \[^{[3]}\text{H}\]choline metabolites induced by A23187 or PDBu. Acinar cells were stimulated with 0.1 \(\mu\)M A23187 or 0.1 \(\mu\)M PDBu for the indicated time. The radioactivity observed at 0 time was subtracted from each value. Each point represents the mean ± S.E. of quadruplicate determinations from two separate experiments. Basal (○), 0.1 \(\mu\)M A23187 (●), 0.1 \(\mu\)M PDBu (△).

Effects of A23187 and PDBu on the release of \[^{[3]}\text{H}\]choline or \[^{[3]}\text{H}\]ethanolamine metabolites

Both A23187 (0.1 \(\mu\)M) and PDBu (0.1 \(\mu\)M) stimulated the release of \[^{[3]}\text{H}\]choline metabolites with time and these effects were dose-dependent (Figs. 4 and 5). Combinations of A23187 and PDBu also significantly potentiated the stimulatory effect of each agent acting alone; the combinations produced an additive increase.

In several cell types, phosphatidylethanolamine hydrolysis was shown to be related to DAG formation (17). To further investigate this possibility in the present study, we next examined the effect of 0.1 \(\mu\)M A23187 or 0.1 \(\mu\)M PDBu on the release of \[^{[3]}\text{H}\]ethanolamine metabolites into the medium at 30 min. These agents had no effect on the release of such metabolites (dpm/10^6 cells, \(n = 5\), basal, 897 ± 25; 0.1 \(\mu\)M A23187, 885 ± 30; 0.1 \(\mu\)M PDBu, 900 ± 21).
Effects of staurosporine on DAG accumulation and the release of \[^{3}H\]choline metabolites induced by A23187 or PDBu

We next evaluated the effect of staurosporine (18), a putative protein kinase C inhibitor, on DAG formation and the release of \[^{3}H\]choline metabolites induced by A23187 or PDBu. The data given in Table 1 revealed that staurosporine significantly inhibited DAG accumulation and the release of \[^{3}H\]choline metabolites induced by A23187 or PDBu; however, it did not alter basal responses in control cells. In addition, the inhibitory effect of staurosporine on DAG formation and the release of \[^{3}H\]choline metabolites was observed 5 min after the addition and continued for at least 30 min (data not shown).

**DISCUSSION**

Activation of protein kinase C has been shown to be important in various physiological processes. In exocrine glands, protein kinase C activation is involved in the secretory response and seems to modulate the action of various substances. As already indicated, carbachol, a Ca\(^{2+}\)-mobilizing receptor agonist, stimulates

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**Table 1.** Effects of staurosporine on DAG accumulation and the release of \[^{3}H\]choline metabolites induced by A23187 or PDBu

<table>
<thead>
<tr>
<th>Conditions</th>
<th>DAG accumulation (nmol/10(^6) cells)</th>
<th>Release of [^{3}H]choline metabolites (dpm/10(^6) cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.43 ± 0.01 (4)</td>
<td>882 ± 23 (6)</td>
</tr>
<tr>
<td>+ 0.1 (\mu)M Staurosporine</td>
<td>0.44 ± 0.02 (4)</td>
<td>881 ± 27 (6)</td>
</tr>
<tr>
<td>+ 0.1 (\mu)M A23187</td>
<td>0.64 ± 0.01 (4)</td>
<td>1691 ± 11 (6)</td>
</tr>
<tr>
<td>+ 0.01 (\mu)M Staurosporine</td>
<td>0.51 ± 0.02(^*) (4) (62%)</td>
<td>1567 ± 24(^*) (6) (15%)</td>
</tr>
<tr>
<td>+ 0.1 (\mu)M Staurosporine</td>
<td>0.42 ± 0.01(^**) (4) (100%)</td>
<td>888 ± 26(^**) (6) (99%)</td>
</tr>
<tr>
<td>0.1 (\mu)M PDBu</td>
<td>0.64 ± 0.03 (4)</td>
<td>1573 ± 31 (6)</td>
</tr>
<tr>
<td>+ 0.01 (\mu)M Staurosporine</td>
<td>0.57 ± 0.04 (4) (33%)</td>
<td>1499 ± 38 (6) (11%)</td>
</tr>
<tr>
<td>+ 0.1 (\mu)M Staurosporine</td>
<td>0.47 ± 0.05(^*) (4) (81%)</td>
<td>894 ± 30(^*) (6) (83%)</td>
</tr>
</tbody>
</table>

Cells were stimulated with 0.1 \(\mu\)M A23187 or 0.1 \(\mu\)M PDBu in the presence of 0.1 \(\mu\)M staurosporine for 30 min. The radioactivity of \[^{3}H\]choline observed at time 0 was subtracted from each value. Each value represents the mean ± S.E. \(^*\)\(P < 0.05\), \(^**\)\(P < 0.001\) vs. value with A23187 or PDBu alone.
DAG formation in part through a protein kinase C in rat parotid acinar cells (13). In many cell types, Ca^{2+} ionophores and phorbol esters are now known to increase DAG levels through PC, but not phosphoinositide, hydrolysis (12, 15, 19–21). In this study, we showed that both A23187 and PDBu stimulated DAG generation (Figs. 1 and 2) and the release of [3H]choline metabolites into the medium (Figs. 4 and 5), and that their effects were significantly inhibited by staurosporine, a protein kinase C inhibitor, which is equivalent to H-7 in inhibiting DAG accumulation induced by carbachol (13) (Table 1). The dose-dependence of the inhibitory influence of staurosporine was similar for both responses. These results show that PC metabolism in parotid acinar cells is regulated by a protein kinase C, and are in agreement with those reported by others in different cell types. Preliminary experiments had shown that neither A23187 nor PDBu stimulated IP_3 production in rat parotid acinar cells. The present study also shows that neither A23187 nor PDBu stimulated phosphatidylethanolamine hydrolysis. These observations indicate that DAG generated by A23187 and PDBu is derived from PC.

Staurosporine also inhibits cyclic AMP- and cyclic GMP-dependent kinases, although with much less potency. McAtee and Dawson (22) have reported that activation of protein kinase A decreases DAG generation through the inhibition of phospholipase C in the neurotumor cell line NCB-20. If staurosporine had an effect on protein kinase A, DAG generation should increase. Therefore, it does not seem that the inhibitory effect of staurosporine is related to protein kinase A.

PC hydrolysis by a phospholipase C produces phosphocholine and DAG. In contrast, a phospholipase D would catalyze the formation of choline and phosphatidate from PC. Phosphatidate releases DAG by the subsequent action of phosphatidate phosphohydrolase (23). Thus, PC hydrolysis is mediated through phospholipase C and/or D (12); however, no conclusions regarding phospholipase stimulation were reached in this study on parotid acinar cells. We have observed that carbachol-stimulated PC hydrolysis is related to the activation of phospholipase C in this system (24). In the present study, both A23187 and PDBu stimulated the release of [3H]phosphocholine and [3H]choline into the medium, indicating the activation of phospholipases C and D (Fig. 3). The contribution of a phospholipase D to PC hydrolysis stimulated by these agents is further supported by evidence that [3H]choline released into the medium is not due to the degradation of [3H]phosphocholine by phosphatase. These results are similar to those shown by Huang and Cabot (25) in rat aorta smooth muscle cells, but is not in agreement with results that PC hydrolysis induced by phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, is mediated through a phospholipase D in REF52 cells (23) and a phospholipase C in myoblast cells (26), respectively. From these observations it seems likely that the mode of PC hydrolysis varies among cell and agonist types.

The synergistic action of phorbol ester and Ca^{2+} ionophore has been observed in several cell types (27–31). This action has been ascribed to the interaction of the Ca^{2+}/calmodulin-dependent pathway and the protein kinase C-dependent pathway, but the detailed mechanism underlying the synergistic action is unclear. Takuma and Ichida (32) reported that the combination of A23187 (1 μM) and phorbol 12-myristate 13-acetate (1 μM) induced a weak synergistic effect on amylase release (about 5%). This synergistic effect is much smaller than that seen in other cell types (27–31). Our previous report (33) showed that an increase in DAG accumulation was involved in the potentiating effect of A23187 on carbachol-stimulated amylase release in parotid acinar cells and that this effect was additive. Furthermore, the present study shows that the combination of A23187 and PDBu evoked an additive increase in DAG accumulation and PC hydrolysis (Figs. 2 and 5). These data cannot completely account for the synergistic effect of Ca^{2+} ionophore and phorbol ester on amylase release, but DAG, generated from PC, may be closely related to the increase in amylase release induced by the combination of Ca^{2+} ionophore and phorbol ester.

In summary, the present results demonstrate that A23187- and PDBu-stimulated DAG accumulations are associated with PC hydrolysis mediated through phospholipases C and D, and it extends the hypothesis of a role of DAG in stimulus-response coupling to the enzyme secretion process in parotid glands.

REFERENCES


