Bradykinin-stimulated phosphatidylcholine hydrolysis in airway smooth muscle: the role of Ca\(^{2+}\) and protein kinase C

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The regulation of phosphatidylcholine (PtdCho) hydrolysis by Ca\(^{2+}\) and protein kinase C (PKC) was measured in [\(^3\)H]palmitate-labelled cultured guinea-pig airway smooth-muscle cells as phosphatidylbutanol ([\(^3\)H]PtdBut) and phosphatidate ([\(^3\)H]PtdOH) formation in the presence of butanol. The former is a direct measure of phospholipase D (PLD) activity, whereas the latter, in airway smooth muscle, is indicative of net PtdCho-specific phospholipase C (PLC)-like/diacylglycerol (DG) kinase activity. Bradykinin-stimulated responses exhibited a requirement for extracellular Ca\(^{2+}\) influx, since they were inhibited in the presence of EGTA. This influx was independent of voltage-operated channels, since the L-type channel blocker nifedipine (up to 10 \(\mu\)M) was without effect on bradykinin-stimulated responses. In support of this, membrane depolarization with KCl (30 mM) failed to elicit either response. However, bradykinin-stimulated formation of both [\(^3\)H]PtdBut and [\(^3\)H]PtdOH was partially inhibited by 100 \(\mu\)M SKF96365. Ionomycin, a Ca\(^{2+}\) ionophore, induced PtdCho hydrolysis to a greater extent than bradykinin, also in an extracellular-Ca\(^{2+}\)-dependent manner. Thapsigargin-induced emptying of intracellular Ca\(^{2+}\) pools elicited the formation of both [\(^3\)H]PtdBut and [\(^3\)H]PtdOH and displayed a requirement for extracellular Ca\(^{2+}\). Bradykinin-stimulated PtdCho-specific PLC-like/DG kinase pathway and PLD responses were unaffected by thapsigargin pretreatment, thereby questioning the role of Ins(1,4,5)\(P_3/Ins(1,3,4,5)P_5\)-dependent Ca\(^{2+}\) stores in the receptor stimulation of these activities in airway smooth-muscle cells. In this regard, we have previously demonstrated that the bradykinin-stimulated PLD and PLC-like activities can occur under conditions of apparent complete blockade of bradykinin-stimulated Ins(1,4,5)\(P_3\) formation by receptor antagonist in guinea-pig airway smooth muscle. The PKC inhibitor, Ro31-8220, selectively blocked both bradykinin- and ionomycin-stimulated PLD activity in a concentration-dependent manner (IC\(_{50}\) approx. 1 \(\mu\)M), but was without effect on bradykinin-stimulated PtdCho-PLC-like/DG kinase-derived PtdOH formation. In contrast, an inhibitor of PtdCho-PLC, D609, selectively blocked the formation of [\(^3\)H]PtdOH in the presence of butanol (PtdCho-PLC-like/DG kinase activity), but not [\(^3\)H]PtdBut formation. In conclusion, PtdCho hydrolysis appears to occur via two distinguishable routes which both require extracellular Ca\(^{2+}\), whereas only the PLD route is regulated by PKC.

INTRODUCTION

Agonist-stimulated phosphatidylcholine (PtdCho) hydrolysis can occur via both phospholipase D (PLD) and phospholipase C (PLC) routes [1]. Activation of PLC may involve an ADP-ribosylation factor, ARF, which has been identified as the cytosolic factor required for guanosine 5'-[\(\gamma\)-thio]triphosphate (GTP[S])-stimulated PLD activation in vitro [2,3]. In addition, PtdCho hydrolysis may involve the elevation of intracellular Ca\(^{2+}\), the influx of extracellular Ca\(^{2+}\) and the activation of protein kinase C (PKC) and tyrosine kinases [1,4].

We have previously shown that bradykinin-stimulated PLD activity is dependent on both Ca\(^{2+}\) and PKC in cultured guinea pig airway smooth-muscle cells [5]. However, bradykinin-stimulated PtdIns(4,5)\(P_3\) hydrolysis in airway smooth muscle, mediated by \(B_2\)-bradykinin receptors [6-9], does not appear to account for these signals, since activation of PLD and PtdCho-PLC-like activities persists in the presence of \(B_2\)-bradykinin-receptor antagonists [6,10]. This occurs at concentrations which apparently abolish Ins(1,4,5)\(P_3\) formation [6] and block the translocation of PKCx to the membrane [11]. Thus an atypical \(B_2\)-bradykinin receptor (\(B_2\) receptor), which also mediates Ca\(^{2+}\) efflux [12], may activate PtdCho hydrolysis in guinea-pig airway smooth muscle [6,10].

Agonist-induced elevation of intracellular Ca\(^{2+}\) in airway smooth muscle is derived from both intracellular stores and the extracellular matrix. Multiple intracellular stores, which can be selectively accessed by Ins(1,4,5)\(P_3\), caffeine, ryanodine and GTP[S], have been identified in permeabilized airway smooth-muscle cells [13]. Ins(1,4,5)\(P_3\) itself has also been proposed to be compartmentalized in this tissue [14]. Thapsigargin has been shown to be a selective inhibitor of microsomal Ca\(^{2+}\) pumps [15] and is able to elevate intracellular Ca\(^{2+}\) by inhibiting re-uptake into Ins(1,4,5)\(P_3\)-sensitive and -insensitive stores. In contrast, ionomycin, a Ca\(^{2+}\) ionophore, has been proposed to release Ca\(^{2+}\) from intracellular stores which are refilled by extracellular Ca\(^{2+}\) in ECV304 cells [16]. Influx of extracellular Ca\(^{2+}\) may also occur through voltage-operated channels [17], which are principally of the L-type and involved in the initiation of contraction in smooth-muscle cells [18], and also by receptor-operated channels, which can be regulated by second-messenger-dependent mechanisms [19,20].

In this study, we aimed to assess the role of intracellular and extracellular Ca\(^{2+}\) and PKC in the bradykinin- and ionomycin-stimulated PtdCho hydrolysis. We have previously shown that PtdCho hydrolysis occurs through both PLD and PLC-like routes [10]. For example, both bradykinin and phorbol 12-myristate 13-acetate elicit the formation of [\(^3\)H]phosphatidylbutanol ([\(^3\)H]PtdBut) (PLD activation) in [\(^3\)H]palmitate-labelled airway smooth-muscle cells, whereas [\(^3\)H]diacylglycerol ([\(^3\)H]DG) formation occurs only in response to bradykinin and is not influenced by the presence of butanol. These observations suggest

Abbreviations used: DG, diacylglycerol; GTP[S], guanosine 5'-[\(\gamma\)-thio]triphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PtdOH, phosphatidate; VOC, voltage-operated Ca\(^{2+}\) channels.

*To whom correspondence should be addressed.
that the conversion of phosphatidate (PtdOH) into DG is minimal under conditions of PKC activation and that a distinct bradykinin-stimulated PtdCho-directed PLC-like activity may occur. In the present study, both [3H]PtdBut and [3H]PtdOH (made via PLC-like activity and subsequent DG kinase) exhibit a requirement for extracellular Ca\textsuperscript{2+}, which may enter the cell via a distinct pool from the Ins(1,4,5)\textsubscript{P\textgreek{a}}-sensitive stores and independently of L-type voltage-dependent channels. However, the PLD and PLC-like/DG kinase activities could be distinguished by their differential sensitivities to a PKC inhibitor and a PtdCho-PLC inhibitor. PLD activation appears to be regulated in a manner which displays a conditional requirement for extracellular Ca\textsuperscript{2+} and is independent of PtdCho hydrolysis via the PLC-like route.

**MATERIALS AND METHODS**

Biochemicals were purchased from Sigma Chemical Co. (U.K.) and Calbiochem-Novabiochem (U.K.). [3H]Palmitate was obtained from Amersham International (U.K.), and cell-culture plasticware and media were from Gibco BRL (U.K.). Male Dunkin–Hartley guinea pigs (200–400 g) were used for the isolation of tracheal smooth muscle. Ro31–8220 was generously given by Dr. G. Lawton (Director of Chemistry, Roche Products, U.K.)

**Cell culture**

The preparation of cultured guinea-pig airway smooth-muscle cells was performed as described previously [5,6]. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-calf serum and 10% (v/v) horse serum and were routinely passaged twice, by using trypsin, before the experiment. Cells were grown to confluence on 24-well plates and used within 14–21 days after the initial isolation.

**[3H]PtdBut and [3H]PtdOH assays**

Confluent airway smooth-muscle cells were preincubated with [3H]palmitate (2 \textmu C/ml) in Dulbecco's modified Eagle's medium containing 1% (v/v) fetal-calf serum and 1% (v/v) horse serum for 24 h. This procedure has previously been shown to result in the preferential incorporation of radioactivity into PtdCho, which contains 82% of the total lipid-associated radioactivity, in comparison with only 6% associated with PtdIns, phosphatidyethanolamine and phosphatidylserine combined [5].

PLD activity was determined by using a transphosphatidylation assay as described previously [5]. Briefly, cells were washed and preincubated in Krebs–Henseleit buffer containing glucose (10 mM), BSA (1%, w/v) and Ca\textsuperscript{2+} (1.5 mM) for 30 min, before preincubation in the presence of 0.3% (v/v) butan-1-ol for 5 min before treatment with bradykinin or ionomycin as required. A lipid extract of the cells was prepared and [3H]PtdBut, which is a direct indicator of PLD activity [21], and [3H]PtdOH were isolated by TLC using the upper phase of ethyl acetate/2,2,4-trimethylpentane (iso-octane)/water/acetic acid (11:5:10:2, by vol.). The \textit{R} \textsubscript{f} values of PtdBut and PtdOH, determined by using internal standards, were approx. 0.35 and 0.17 respectively.

Compounds of interest were excised and quantified by scintillation counting. PtdOH formed in the presence of 0.3% butan-1-ol (a saturating concentration for PtdBut formation [5]) was assumed to be derived by hydrolysis of PtdCho via a PLC-like activity and the concerted action of DG kinase.

In experiments where extracellular Ca\textsuperscript{2+} was absent, the incubation buffer had no added Ca\textsuperscript{2+} and was supplemented with 2.5 mM EGTA. Drugs such as Ro31–8220, nifedipine, SKF96365, thapsigargin or D609 were added to the incubation buffer, at the concentrations required, for 10 min (unless otherwise indicated) before treatment with bradykinin or ionomycin.

**RESULTS**

Dependence of bradykinin- and ionomycin-stimulated PtdCho hydrolysis on extracellular Ca\textsuperscript{2+}

Maximal concentrations of bradykinin (100 nM) elicited activation of PtdCho-specific PLD and net PLC-like/DG kinase activities in intact airway smooth-muscle cells, as evidenced by 3.77±1.13- and 1.77±0.49-fold (n = 17 experiments) increases in [3H]PtdBut and [3H]PtdOH, respectively, in the presence of butanol ([3H]PtdBut: basal, 1047±423, bradykinin (10 min), 3947±1182; [3H]PtdOH: basal, 1864±593, bradykinin (10 min), 3304±909 (d.p.m.; means ± S.D., n = 17 separate experiments)). This is consistent with the magnitude of activation reported by us previously [5]. Depletion of extracellular Ca\textsuperscript{2+}, i.e. by incubation of cells in the absence of added Ca\textsuperscript{2+} and in the presence of 2.5 mM EGTA, was without effect on basal levels of [3H]PtdBut and [3H]PtdOH ([3H]PtdBut: 1029±170 (1.5 mM Ca\textsuperscript{2+}), 785±143 (Ca\textsuperscript{2+}-free); [3H]PtdOH: 1928±800 (1.5 mM Ca\textsuperscript{2+}), 1790±316 (Ca\textsuperscript{2+}-free) (d.p.m.; means ± S.D., combined data from 3 separate experiments and Table 1)). However, the accumulation of both [3H]PtdBut and [3H]PtdOH in response to bradykinin (100 nM) was substantially inhibited ([3H]PtdBut, by 78±7%; [3H]PtdOH, by 77±14%; n = 3 experiments) by removal of extracellular Ca\textsuperscript{2+} (Table 1; [5]).

Ionomycin (20 \textmu M), a Ca\textsuperscript{2+} ionophore, also elicited the formation of [3H]PtdBut and [3H]PtdOH in the presence of butanol (Table 1). The ionomycin-stimulated PLD response was substantially greater in magnitude than that induced by bradykinin (8.99±1.07-fold increase above basal; means ± S.D., combined data from 6 separate experiments), whereas ionomycin-stimulated [3H]PtdOH formation was not significantly different from the bradykinin response (1.84±0.53-fold increase above basal; means ± S.D., combined data from 6 separate experiments). As for bradykinin, the effects of ionomycin were largely abolished in the absence of extracellular Ca\textsuperscript{2+} (Table 1). Ionomycin was ineffective at stimulating Ins(1,4,5)\textsubscript{P\textgreek{a}} formation (results not shown).

Since ionomycin elicited a larger PLD response than did

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**Table 1** Dependence of bradykinin- and ionomycin-stimulated [3H]PtdBut and [3H]PtdOH formation on extracellular Ca\textsuperscript{2+}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]PtdBut (d.p.m.)</th>
<th>[3H]PtdOH (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With 1.5 mM Ca\textsuperscript{2+}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>791±91</td>
<td>3060±398</td>
</tr>
<tr>
<td>BK</td>
<td>3571±550</td>
<td>4221±364</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>7482±254</td>
<td>5428±191</td>
</tr>
<tr>
<td>With 2.5 mM EGTA, no added Ca\textsuperscript{2+}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>588±73</td>
<td>2236±110</td>
</tr>
<tr>
<td>BK</td>
<td>1515±38*</td>
<td>2699±287*</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>825±34*</td>
<td>2122±126*</td>
</tr>
</tbody>
</table>
bradykinin, we examined whether ionomycin-stimulated \(^{3}H\)PtdBut formation displayed non-desensitizing kinetics. However, in common with the bradykinin response [5], ionomycin-stimulated \(^{3}H\)PtdBut accumulation was rapid in onset and desensitized within 5–10 min (Figure 1a). The kinetics of ionomycin-stimulated \(^{3}H\)PtdOH formation in the presence of butanol was also similar to those for bradykinin [10], reaching a plateau at 1 min (Figure 1b). Thus, the increased \(^{3}H\)PtdBut response to ionomycin may be due to the magnitude of the Ca\(^{2+}\) signal or to activation of other isoforms of PLD which may not be modulated by bradykinin stimulation, e.g. a cytosolic form [22].

**Nature of the Ca\(^{2+}\) influx involved in bradykinin-stimulated PtdCho hydrolysis**

Pretreatment of airway smooth-muscle cells with nifedipine (0.1–10 \(\mu\)M), an L-type-specific voltage-dependent Ca\(^{2+}\)-channel blocker, was without effect on both basal and bradykinin-stimulated PLD activity (Figure 2a). Similarly, concentrations of 0.1–1 \(\mu\)M nifedipine were ineffective against basal and bradykinin-stimulated \(^{3}H\)PtdOH formation. However, the latter was inhibited by approx. 40\% at concentrations of nifedipine in excess of 10 \(\mu\)M (Figure 2b).

Treatment of airway smooth-muscle cells with 30 mM KCl activates voltage-operated Ca\(^{2+}\) channels (VOC) [17]. However, this failed to elicit the formation of either \(^{3}H\)PtdBut or

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**Table 2 Lack of effect of depolarization by KCl on \(^{3}H\)PtdBut and \(^{3}H\)PtdOH levels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(^{3}H)PtdBut (d.p.m.)</th>
<th>(^{3}H)PtdOH (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1182 ± 147</td>
<td>1345 ± 251</td>
</tr>
<tr>
<td>KCl</td>
<td>3306 ± 42</td>
<td>2505 ± 176</td>
</tr>
<tr>
<td>KCl</td>
<td>1184 ± 58(^{*})</td>
<td>1196 ± 132(^{*})</td>
</tr>
</tbody>
</table>

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**Figure 1 Time-course of ionomycin-stimulated \(^{3}H\)PtdBut and \(^{3}H\)PtdOH formation**

\(^{3}H\)Palmilate-labelled cells were preincubated in the presence of 1.5 mM Ca\(^{2+}\) for 30 min and butan-1-ol (0.3%, v/v) for 5 min before treatment with ionomycin (1 \(\mu\)M) for the times indicated. Lipid extracts were analysed for (a) \(^{3}H\)PtdBut, a direct measure of PLD activity, and (b) \(^{3}H\)PtdOH. Results are in d.p.m. (means ± S.D., \(n = 3\)) for a single representative experiment.

**Figure 2 Lack of effect of nifedipine on basal and bradykinin-stimulated \(^{3}H\)PtdBut and \(^{3}H\)PtdOH formation**

\(^{3}H\)Palmilate-labelled cells were preincubated in the presence of 1.5 mM Ca\(^{2+}\) for 30 min and butan-1-ol (0.3%, v/v) with the indicated concentrations of nifedipine for 10 min before treatment with buffer (○) or bradykinin (●, 100 nM) for 10 min. Lipid extracts were analysed for (a) \(^{3}H\)PtdBut and (b) \(^{3}H\)PtdOH as described in the Materials and methods section. Results are in d.p.m. (means ± S.D., \(n = 3\)) for a single representative experiment.

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Table 3  Effect of thapsigargin on [3H]PtdBut and [3H]PtdOH levels and their dependence on extracellular Ca2+

(a) [3H]Palmite-labelled cells were preincubated in the presence of 1.5 mM Ca2+ for 30 min and butan-1-ol (0.3%, v/v) for 5 min before treatment with bradykinin (BK; 100 nM) or thapsigargin (1 μM) for 10 min. EGTA (2.5 mM) was added 1 min after bradykinin or thapsigargin where indicated. (b) [3H]Palmite-labelled cells were preincubated in the presence of 1.5 mM Ca2+ and thapsigargin (1 μM) as indicated for 30 min before the addition of butan-1-ol (0.3%) for 5 min and bradykinin (100 nM) as indicated for a further 10 min. Lipid extracts were analyzed for [3H]PtdBut and [3H]PtdOH as described in the Materials and methods section. Results are given in d.p.m. (means ± S.D., n = 3) from two representative experiments: (a) *P < 0.005 versus samples +Ca2+; (b) *P < 0.005 and †P < 0.025 versus samples with DMSO.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]PtdBut (d.p.m.)</th>
<th>[3H]PtdOH (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1646 ± 205</td>
<td>2840 ± 171</td>
</tr>
<tr>
<td>BK</td>
<td>5205 ± 102*</td>
<td>4846 ± 188*</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>2856 ± 146*</td>
<td>3404 ± 267</td>
</tr>
<tr>
<td>EGTA at 1 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1135 ± 16</td>
<td>2866 ± 398</td>
</tr>
<tr>
<td>BK</td>
<td>2145 ± 166*</td>
<td>4127 ± 110*</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>1230 ± 91*</td>
<td>3157 ± 43</td>
</tr>
<tr>
<td>(b) DMSO/control</td>
<td>2262 ± 4</td>
<td>3135 ± 267</td>
</tr>
<tr>
<td>DMSO/BK</td>
<td>5736 ± 757</td>
<td>5682 ± 300</td>
</tr>
<tr>
<td>Thapsigargin/control</td>
<td>3840 ± 123*</td>
<td>3776 ± 173†</td>
</tr>
<tr>
<td>Thapsigargin/BK</td>
<td>5663 ± 636</td>
<td>6202 ± 136</td>
</tr>
</tbody>
</table>

[3H]PtdOH (Table 2), confirming the lack of involvement of VOC-accessed Ca2+ in the regulation of PtdCho hydrolysis. SKF96365 has been reported to inhibit store-regulated Ca2+ entry [23]. Pretreatment of airway smooth-muscle cells with SKF96365 (0.1–10 μM, 10 min) was without effect on basal or bradykinin-stimulated formation of [3H]PtdBut and [3H]PtdOH. However, 100 μM SKF96365 partially inhibited bradykinin-stimulated [3H]PtdBut and [3H]PtdOH responses, to 60±9.4% and 62±5.5%, respectively [3H]PtdBut: bradykinin, 3546±137; SKF96365/bradykinin, 2112±335; [3H]PtdOH: bradykinin, 2571±198; SKF96365/bradykinin, 1598±143 (d.p.m. above basal at 10 min stimulation; means ± S.D., combined data for 3 experiments).

Role of Ca2+ in the activation of PtdCho hydrolysis

Thapsigargin was used to deplete intracellular Ca2+ stores and thereby assess Ca2+-dependent Ca2+ refilling in the regulation of the PtdCho hydrolysis.

Incubation of airway smooth-muscle cells in the presence of thapsigargin (1 μM, 10 min) resulted in the formation of [3H]PtdBut and [3H]PtdOH, although to a lesser extent than with bradykinin. The thapsigargin and bradykinin responses were inhibited by the addition of EGTA (2.5 mM) after 1 min, suggesting that the continued presence of extracellular Ca2+ is required for a sustained response (Table 3a). Thus thapsigargin elicits entry of extracellular Ca2+ via an intracellular store to regulate PtdCho hydrolysis.

Pretreatment of airway smooth muscle cells with thapsigargin for 30 min before the addition of butanol for 15 min resulted in an elevated basal level of [3H]PtdBut (Table 3b). However, such treatment had little or no effect on basal levels of [3H]PtdOH, presumably due to the ability of this compound to be metabolized. This chronic thapsigargin pretreatment failed to affect the ability of bradykinin to activate PtdCho hydrolysis (Table 3b).

Differential regulation of [3H]PtdBut and [3H]PtdOH formation

Ro31-8220, a derivative of staurosporine, is a potent and highly selective inhibitor of PKC [24]. Pretreatment of airway smooth-muscle cells with Ro31-8220 resulted in the concentration-dependent inhibition of bradykinin-stimulated PLD activity (Figure 3a; IC50, 1.37±1.21 μM) and was without effect on basal [3H]PtdBut levels at up to 100 μM [control, 1811±809; 100 μM Ro31-8220, 1947±516 (d.p.m., means ± S.D., n = 3 experiments)]. In contrast, Ro31-8220 failed to inhibit bradykinin-stimulated PLC-like/DG kinase-derived [3H]PtdOH formation at concentrations of 10 μM or below (Figure 3a).

Similarly, ionomycin-stimulated PLD activity, but not PLC-like/DG kinase-derived [3H]PtdOH formation, was inhibited in a concentration-dependent manner by Ro31-8220 (Figure 3b;
Table 4  Differential effect of D609 on bradykinin-stimulated \(^{3}H\)PtdBut and \(^{3}H\)PtdOH formation

<table>
<thead>
<tr>
<th>[D609] ((\mu M))</th>
<th>(^{3}H)PtdBut (d.p.m.)</th>
<th>(^{3}H)PtdOH (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>BK</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>608 ± 36</td>
<td>1663 ± 142</td>
</tr>
<tr>
<td>1</td>
<td>634 ± 33</td>
<td>1792 ± 156</td>
</tr>
<tr>
<td>10</td>
<td>784 ± 46</td>
<td>1560 ± 156</td>
</tr>
</tbody>
</table>

DISCUSSION

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The role of PKC

Bradykinin-stimulated PtdCho hydrolysis exhibited a requirement for PKC, whereas the PLC-like/DG kinase route of \(^{3}H\)PtdOH formation did not. Similarly, ionomycin, which was a more effective agent, stimulated PtdCho activity in a PKC-dependent manner. The latter may suggest that Ca\(^{2+}\) acts upstream of PKC in the activation of PtdCho. It is perhaps noteworthy that, under these conditions, the sensitivity of the inhibition of PtdCho activity by Ro31-8220 is similar to that of bradykinin. Furthermore, ionomycin does not apparently activate PtdIns(4,5)\(\mathcal{P}_s\) hydrolysis, and therefore further supports the proposal that PKC-dependent stimulation of PLD is not downstream of this phospholipid pathway.

Furthermore, the ability of D609 to inhibit selectively the PLC-like/DG kinase activity suggests that this is not a requirement for PLD activation. In this regard, the rapidity of PLD activation may indicate that the relevant PKC isoform is already localized in the plasma membrane. Indeed, this appears to be the case for the \(\xi\) and \(\epsilon\) isozymes, which are present in membranes prepared from unstimulated airway smooth muscle cells (results not shown), the latter of which has been implicated in the regulation of PLD in mesangial cells [27].

In conclusion, these studies support the notion that bradykinin is capable of activating an ion channel, which elicits specific Ca\(^{2+}\) influx, in airway smooth-muscle cells, and this regulates both IC\(_{50}\) 1 \(\mu M\), with complete inhibition being observed at 20–100 \(\mu M\).

Basal \(^{3}H\)PtdOH was significantly increased by incubation of airway smooth-muscle cells in the presence of 100 \(\mu M\) Ro31-8220 [control, 1634 ± 199; 100 \(\mu M\) Ro31-8220, 3292 ± 205 (d.p.m., means ± S.D., \(n = 3\) experiments)] and this probably accounts for the apparent increase in ionomycin-stimulated \(^{3}H\)PtdOH levels at 100 \(\mu M\) Ro31-8220. This latter effect does not appear to be due to inhibition of phosphatidate phosphohydrolase activity (D. Tolan and S. Pyne, unpublished work). However, we have previously shown that PCK down-regulation of airway smooth-muscle cells results in elevated basal \(^{3}H\)PtdOH levels [10]. These observations would suggest an inhibitory role for PCK in the regulation of either PtdCho-PLC-like activity or DG kinase, or both, under resting conditions.

PLC-catalysed hydrolysis of PtdCho has been shown to be inhibited by D609 in Jurkat T cells [25]. Pretreatment of airway smooth muscle cells with D609 resulted in the concentration-dependent inhibition of bradykinin-stimulated \(^{3}H\)PtdOH formation in the presence of butanol, but was without effect on bradykinin-stimulated PLD activity (Table 4). This suggests that the activation of PLD occurs independently of agonist-stimulated PtdCho PLC-like activity in these cells.

Ca\(^{2+}\) regulation of PtdCho hydrolysis

Thapsigargin inhibits microsomal ATPases [15] and thereby progressively empties the Ins(1,4,5)\(\mathcal{P}_s\)-sensitive Ca\(^{2+}\) pool by blocking Ca\(^{2+}\) re-uptake. This will convert the empty Ins(1,3,4,5)\(\mathcal{P}_r\) receptor into a low-conductance state that allows extracellular Ca\(^{2+}\) entry in proportion to the rate of blockade of uptake of Ca\(^{2+}\) into the Ins(1,4,5)\(\mathcal{P}_s\)-sensitive Ca\(^{2+}\) pool [19]. Under these conditions, thapsigargin elicited the activation of PtdCho-specific PLD and PLC-like/DG kinase responses, and this was entirely dependent on the continued presence of extracellular Ca\(^{2+}\). Thus these data preclude a direct role for the Ins(1,4,5)\(\mathcal{P}_s\)-sensitive Ca\(^{2+}\) pool in the activation of PtdCho hydrolysis.

When the Ins(1,4,5)\(\mathcal{P}_s\)-sensitive Ca\(^{2+}\) pool is emptied by thapsigargin, extracellular Ca\(^{2+}\) entry will be maintained at a slow rate, even in the presence of Ins(1,3,4,5)\(\mathcal{P}_s\), produced, for example, in response to bradykinin, which we have previously shown to stimulate PtdIns(4,5)\(\mathcal{P}_s\) hydrolysis [6]. This model suggests that Ins(1,3,4,5)\(\mathcal{P}_s\)-sensitive Ca\(^{2+}\) entry may not be involved in the activation of PtdCho hydrolysis, since chronic pretreatment of the cells with thapsigargin was without effect on the subsequent bradykinin-stimulated PtdCho-specific PLD and PLC-like/DG kinase responses.

Further support of this proposal is evident from our studies demonstrating that B\(_{\text{2}}\)-bradykinin-receptor antagonists fail to inhibit bradykinin-stimulated PtdCho-specific phospholipase responses under conditions where Ins(1,4,5)\(\mathcal{P}_s\) formation is abolished [6,10]. The apparent complete blockade of Ins(1,4,5)\(\mathcal{P}_s\) formation occurs with an antagonist concentration of 10 \(\mu M\), which has also been shown, in bovine airway smooth muscle, to abolish bradykinin-stimulated Ca\(^{2+}\) release from Ins(1,4,5)\(\mathcal{P}_s\)-sensitive stores [8]. In addition, B\(_{\text{2}}\)-bradykinin-receptor antagonists have been shown to block bradykinin-stimulated Ca\(^{2+}\) efflux in guinea-pig airway smooth muscle, which supports a role for an alternative route of Ca\(^{2+}\) entry [12].

A novel receptor-activated Ca\(^{2+}\)-specific influx pathway has been observed for histamine in airway smooth muscle [26]. Histamine elicits a substantial Ca\(^{2+}\) influx that appears to be dependent on the holding potential, i.e. depolarization actually inhibits Ca\(^{2+}\) entry, whereas hyperpolarization enhances it. A similar mechanism of Ca\(^{2+}\) entry may account for the bradykinin-stimulated activation of PtdCho hydrolysis observed in the present study. The lack of effect of nifedipine on bradykinin-stimulated PtdCho-specific PLD suggests that L-type Ca\(^{2+}\) channels are not involved, although these can not be entirely excluded for the PLC-like/DG kinase route of PtdOH formation. Alternatively, bradykinin may activate the phospholipases via a Ca\(^{2+}\)-dependent mechanism that involves Ca\(^{2+}\) entry through a receptor-operated Ca\(^{2+}\) channel. The latter would require invoking a model which includes a compartmentalized pool of PLD/PLC-like activities that is accessible to extracellular Ca\(^{2+}\), but not to Ins(1,4,5)\(\mathcal{P}_s\)-sensitive Ca\(^{2+}\) stores.

Ionomycin-stimulated PtdCho hydrolysis by PLD and PLC-like/DG kinase routes was also dependent on extracellular Ca\(^{2+}\). Ionomycin has been shown to trigger emptying of intracellular Ca\(^{2+}\) stores, a process that may stimulate its refilling by extracellular Ca\(^{2+}\) and which, in ECV304 endothelial cells, is blocked by SKF96365 [16].
PtdCho-specific PLD and net PLC/DG kinase activities, the former of which requires a PKC-dependent component.

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REFERENCES

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