Bradykinin-stimulated cPLA$_2$ phosphorylation is protein kinase C dependent in rabbit CCD cells

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Bradykinin-stimulated cPLA$_2$ phosphorylation is protein kinase C dependent in rabbit CCD cells. Am. J. Physiol. 273 (Renal Physiol. 42): F907–F915, 1997.—We have used an established cell line of rabbit cortical collecting duct (RCCD) epithelial cells representing a mixed population of principal and intercalated cell types to determine which phospholipase A$_2$ (PLA$_2$) enzyme therein is responsible for bradykinin (BK)-stimulated arachidonic acid (AA) release and how its activation is regulated. BK-stimulated AA release was reduced 92% by arachidonyl trifluoromethyl ketone, an inhibitor of cytosolic PLA$_2$ (cPLA$_2$). Examination of PLA$_2$ activity in vitro demonstrated that BK stimulation resulted in a greater than twofold increase in PLA$_2$ activity and that this activity was dihydrothreitol insensitive and was inhibited by an antibody directed against cPLA$_2$. To determine a possible role for protein kinase C (PKC) in the BK-mediated activation of cPLA$_2$, we used the PKC-specific inhibitor Ro31-8220 and examined its effects on AA release, cPLA$_2$ activity, and phosphorylation. Ro31-8220 reduced BK-stimulated AA release and cPLA$_2$ activity by 51 and 58%, respectively. cPLA$_2$ activity stimulated by phorbol ester [phorbol 12-myristate 13-acetate (PMA)] displayed a similar degree of activation and was associated with an increase in serine phosphorylation identical to that caused by BK. The phosphorylation-induced activation of this enzyme was confirmed by the phosphatase-mediated reversal of both BK- and PMA-stimulated cPLA$_2$ activity. In addition, we have also found that PMA stimulation did not cause a synergistic potentiation of BK-stimulated AA release as did calcium ionophore. This occurred despite membrane PKC activity increasing 93% in response to PMA vs. 42% in response to BK. These data, taken together, indicate that cPLA$_2$ is the enzyme responsible for BK-mediated AA release, and, moreover, they indicate that PKC is involved in the onset responses of cPLA$_2$ to BK.

Availability of the precursor molecule, arachidonic acid (AA), is thought to represent the rate-limiting step for the production of the prostaglandins. This fatty acid is found mainly at the sn-2 position of membrane phospholipids and is readily cleaved off by phospholipase A$_2$ (PLA$_2$) activity known to be exhibited in at least three groups of enzymes: the secretory PLA$_2$ enzymes with a molecular mass of ~14 kDa, dependent on a high concentration of Ca$^{2+}$; the Ca$^{2+}$-independent PLA$_2$ types; and the 85-kDa cytosolic PLA$_2$ (cPLA$_2$), dependent on a low concentration of Ca$^{2+}$. Over the past years, cPLA$_2$ has received a great deal of attention because it is AA specific and appears to represent the enzyme that is distinctively regulated by cell signaling mechanisms downstream of receptor occupancy. Since its purification by Clark et al. (10), cPLA$_2$ has been demonstrated to display both Ca$^{2+}$- and phosphorylation-dependent activation (23). This enzyme possesses a Ca$^{2+}$-lipid binding (CaLB) domain, which, in the presence of micromolar Ca$^{2+}$ concentrations, allows translocation to membranes (9), particularly those of the nuclear envelope and endoplasmic reticulum (18, 35). Furthermore, it has been demonstrated in transfected Chinese hamster ovary cells overexpressing cPLA$_2$ that phosphorylation on serine-505 by mitogen-activated protein kinase, secondary to PKC stimulation, is a necessary component for enzyme activation (24). In contrast to these and other studies (3, 23, 27, 31, 32, 43, 45) that support a role for PKC in mediating cPLA$_2$ phosphorylation in response to various agonists, a number of reports describe a lack of any role for this enzyme (5, 11, 16, 18, 39, 41). It appears that regulation of cPLA$_2$ phosphorylation by PKC may depend on the specific agonist-receptor coupling and the cell type involved. With regard to BK-mediated AA release and cPLA$_2$ involvement in the collecting duct, to date there have been no studies other than extensive ones on Madin-Darby canine kidney (MDCK) cells (19, 20, 38, 42). A role for PKC in mediating BK-stimulated AA release in MDCK cells has been implicated to varying degrees, depending on the method of study (14, 42). Recent evidence obtained in our laboratory has denied a role for PKC in the onset responses of BK stimulation leading to AA release in MDCK cells (unpublished results).

In the present study, we have used a recently characterized RCCD cell line to study the regulation of AA release by this nephron segment. These transfected RCCD cells represent a mixed population of principal α-intercalated and β-intercalated epithelial cell types as assessed by specific antibody recognition. They have retained both their morphological and hormonal response properties and possess the signaling machinery present downstream of angiotensin signaling (8). When...
RCCD cells were treated with BK, we found that this peptide was able to specifically activate the cPLA₂ isomform and that serine phosphorylation was at least partially involved in this response. Our results reveal that cPLA₂ is the major enzyme responsible for AA release in RCCD cells and that its activity is dependent on PKC recruitment as an onset response to BK stimulation.

**EXPERIMENTAL PROCEDURES**

Materials. BK, the Ca²⁺ ionophore A-23187, potato acid phosphatase, and 1-stearoyl-2-arachidonyl-sn-glycero were purchased from Sigma Chemical (Mississauga, ON, Canada). The agent RHC-80267, phorbol 12-myristate 13-acetate (PMA), and arachidonyl trifluoromethyl ketone (AACOCF₃) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA), whereas Ro31-8220 was supplied by Calbiochem-Novabiochem (La Jolla, CA). Phosphatidylcholine (1-stearoyl-2-α,ω-alkylpolyoxyethylene)-8,1,2,9,11,12,14,15-H₈) phosphatidylcholine was obtained from DuPont NEN (Mississauga, ON). [3H]AA, hybridoxyethylpiperazine-N,N,N',N'-tetraacetic acid (EGTA) and Tris(bis(hydroxymethyl)methylammonium chloride) (Tris) hydrochloride (pH 7.5), 250 mM sucrose, and 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA). They were then scraped off the plates into wash buffer, centrifuged at 1,000 g for 5 min, and resuspended in lysis buffer composed of 50 mM Tris·HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, protease inhibitors (in µg/ml: 100 benzamidine, 20 leupeptin, 2 phenylmethylsulfonyl fluoride (PMSF), 30 bacitracin, 100 aprotinin), phosphatase inhibitors (in mM: 10 sodium vanadate, 10 sodium pyrophosphate, 1 levanoside), and 5 mM dithiothreitol (DTT). The resuspended cells were then sonicated on ice by two pulses of 10 s each, with the use of the same conditions as used for the PLA₂ activity assay described above. Protein concentrations were determined by the Bio-Rad protein assay method with BSA as a standard. For those experiments examining the phosphorylation-dependent activation of cPLA₂, cells were resuspended in the same lysis buffer (without phosphatase inhibitors) as described above, supplemented with 1 U/ml potato acid phosphatase, and adjusted to a final pH of 6.1. After sonication, lysates were incubated at 30°C for 30 min, after which the pH was returned to 7.5.

Total cell lysates were subsequently assayed for PLA₂ activity, according to the protocol described by Leslie (22). Briefly, lysates were incubated in assay buffer [50 mM Tris·HCl (pH 7.5), 250 mM sucrose, 0.05% BSA, 1 mM Ca²⁺] containing 30 µM 1-stearoyl-2-arachidonyl phosphorylcholine and 55,000 dpm 1-stearoyl-2-(arachidonyl-H₈) phosphorylcholine tracer. Incubations were carried out at 37°C and terminated after 1 h by addition of 2.5 ml Dole reagent (2-propanol-heptane-0.5 M H₂SO₄; 20:5:1, vol/vol/vol) (13). This was followed by the addition of 1.5 ml heptane containing 20 µl cold AA. To obtain visibly separate phases, 1 ml of H₂O was added, and an aliquot of the top layer was purified by silicic acid column chromatography. Column fractions were then washed with diethyl ether, and the final collected eluent was dried under nitrogen and analyzed by liquid scintillation spectrometry.

Immuno precipitation. Cells were stimulated under the same conditions as used for the PLA₂ activity assay described above. After stimulation, they were washed and scraped off the plates with ice-cold buffer solution containing 50 mM Tris·HCl (pH 7.5) and 1 mM EGTA. Cells were centrifuged at 1,000 g, resuspended, and then lysed in immunoprecipitation buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 20 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 30 µg/ml bacitracin, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, and 1 mM levanoside]. Protein concentrations were determined and diluted to 50 µg/500 µl of immunoprecipitation buffer in a fresh centrifuge tube. To this aliquot, 25 µl protein A-agarose suspension were added, and the solution was subsequently rocked on a platform for 3 h at 4°C to reduce background that might have been caused by nonspecific adsorption of cellular debris. After a low-speed centrifugation, the resultant supernatant was transferred to a new
cPLA2, we could demonstrate that RCCD cells express this enzyme. Characterization of the enzyme responsible for AA release in RCCD cells was not yet available, so we set out to determine PLA2 activity. ECL reagents according to the manufacturer's specifications. Filters were washed with TTBS, blots were developed with conjugated donkey anti-rabbit Ig (1:2,000) for 1 h. After being incubated with horseradish peroxidase-labeled overnight with 0.3 µCi [3H]AA in Dulbecco's modified Eagle's (DMEM)-F-12 defined medium containing 0.05% (wt/vol) bovine serum albumin (BSA). Cells were subsequently washed twice with Hank's balanced salt solution (HBSS) + 0.05% BSA and then preincubated for 30 min before a 15-min stimulation with indicated concentrations of BK. [3H]AA released into medium and total cell label incorporated into cells were counted. Amount of label released was divided by total incorporation and was expressed as a fold increase in [3H]AA release compared with control (n = 3 experiments).

Statistics. Data are expressed as averages of duplicate determinations from individual experiments and are presented as means ± SE where n ≥ 4 or means ± SD where n = 3 experiments. Statistical significance was accepted at P < 0.05 as determined by Student's t-test.

RESULTS

BK stimulates AA release through cPLA2 activation. On stimulation with BK for 15 min, RCCD cells released AA in a dose-dependent manner (Fig. 1). Previous results from our laboratory with MDCK-D1 cells derived from canine kidney distal tubule/collection duct have revealed that BK-mediated AA release occurs through the action of cPLA2 (20). Because such information was not yet available for RCCD cells, we set out to characterize the enzyme responsible for AA release in these cells.

By Western blotting with a polyclonal antibody to cPLA2, we could demonstrate that RCCD cells express cPLA2 protein (Fig. 2). To examine whether this enzyme is responsible for mediating BK-induced AA release, we employed several strategies. The agent AACOCF3, an analogue of AA that inhibits the 85-kDa cPLA2, was first tested (33). As shown in Fig. 3, BK-stimulated AA release was 92% inhibited by 50 µM AACOCF3, whereas an inhibitor of diacylglycerol lipase (RHC-80267) was without significant effect. The latter result precluded the combined action of phospholipase C (PLC) and diacylglycerol lipase as a major pathway for releasing AA. It has been shown recently that AACOCF3 also inhibits Ca2+-independent PLA2 (2). However, when cells were treated with haloenol lactone suicide substrate, a specific inhibitor of Ca2+-independent PLA2 (15), there was no significant attenuation of BK-stimulated AA release. To demonstrate more definitively that cPLA2 is directly responsible for BK-stimulated AA release, we first measured in vitro PLA2 activity. Results (not shown) from such assays revealed that the PLA2 activity of RCCD cells could be activated by submillimolar Ca2+ concentration and was insensitive to the reducing agent DTT, thus eliminating the
The likelihood of the involvement of 14-kDa PLA₂ as the isoform responsible for mediating the response to BK. Second, we observed (cf. Table 1) that maximal BK (100 nM) stimulation resulted in a 2.0-fold increase in PLA₂ activity from 13.9 ± 1.5 to 27.9 ± 2.5 pmol·min⁻¹·mg⁻¹ as assayed in vitro. This increase in enzyme activity was completely eliminated with the addition of an antibody to cPLA₂. The evidence as a whole demonstrates that cPLA₂ is the major enzyme mediating BK-stimulated AA release.

BK-mediated AA release is PKC dependent. Taking into consideration the role of cPLA₂ in BK-stimulated AA release, we next tested whether downstream BK-receptor signaling events could be mimicked by PMA activation of PKC and the Ca²⁺ ionophore A-23187. Results summarized in Fig. 4A illustrate that 100 nM PMA alone did not result in an increased release of AA compared with control; however, when PMA was presented to cells together with 100 nM A-23187, there was a clear synergy of release above that caused by A-23187 alone (compare 4.15 ± 0.21-fold for A-23187 with 9.08 ± 0.72-fold for PMA + A-23187). Furthermore, to test whether BK-stimulated AA release was dependent on PKC, we used the bisindolylmaleimide Ro31-8220 as a PKC-selective inhibitor (44). As seen in Fig. 4A, BK-stimulated AA release was inhibited 51% by 5 µM Ro31-8220. Higher concentrations of Ro31-8220 did not result in greater inhibition. Confirming the role for PKC in regulating AA release, the synergy seen with PMA + A-23187 could also be totally blocked.

Table 1. Effect of bradykinin and a cPLA₂ antibody on in vitro PLA₂ activity

<table>
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<tr>
<th>Treatment</th>
<th>PLA₂ Activity, pmol·min⁻¹·mg⁻¹</th>
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<tbody>
<tr>
<td>Control</td>
<td>13.9 ± 1.5</td>
</tr>
<tr>
<td>Control + anti-cPLA₂</td>
<td>3.90 ± 0.29*</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>27.9 ± 2.5*</td>
</tr>
<tr>
<td>Bradykinin + anti-cPLA₂</td>
<td>4.14 ± 1.1†</td>
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Results are means ± SE of 4 individual experiments assayed in duplicate. Rabbit cortical collecting duct cells were serum starved overnight in Dulbecco's modified Eagle's-F-12 defined medium supplemented with 0.05% (wt/vol) bovine serum albumin (BSA). Cells were preincubated with Hanks' balanced salt solution + 0.05% BSA followed by a 2-min stimulation with or without 100 nM bradykinin (BK). PLA₂ activity was determined as described in EXPERIMENTAL PROCEDURES. *P < 0.05 vs. control; †P < 0.001 vs. BK.
by Ro31-8220, returning AA release to levels seen for A-23187 alone (compare 2.59 ± 0.26-fold for A-23187/Ro31-8220 with 2.91 ± 0.05-fold for PMA + A-23187/Ro31-8220). Interestingly, Ro31-8220 was also able to inhibit release by A-23187 alone (Fig. 4A), which suggests that PKC is involved to some extent in mediating this response to ionophore. This result is similar to that reported by Lin et al. (23) and Qiu and Leslie (32).

Slivka and Insel (38) previously reported that BK-stimulated AA release is increased by treatment of MDCK cells together with phorbol ester. Recent studies from our laboratory on these same cells confirm these earlier findings (unpublished observations). However, the present results shown in Fig. 4B reveal that PMA treatment of RCCD cells is unable to potentiate the release of AA caused by BK (compare 4.24 ± 0.57-fold for BK with 3.51 ± 0.51-fold for BK + PMA). It is possible that with our present cells BK engenders a limited Ca²⁺ signal and responses cannot be enhanced by PMA unless ionophore is added (compare 4.24 ± 0.57-fold for BK with 9.48 ± 1.14-fold for BK + A-23187). Interestingly, the inability of PMA to increase BK-stimulated AA release prevails, despite our finding that, with respect to control, PMA caused a 93% increase in membrane PKC activity compared with only 42% for BK (Fig. 5).

We next examined the effects of BK and PMA on cPLA₂ activity to see whether they corresponded to the observed changes in PKC activity. Although the increase in PKC activity for PMA-treated cells was more than double the increase found for BK-treated cells, our results, shown in Fig. 6A, demonstrate that there was no significant difference between the ability of either agonist to stimulate cPLA₂ activity (compare 30.9 ± 2.97 for BK with 36.3 ± 8.08 pmol·min⁻¹·mg⁻¹ for PMA). The specific role of PKC was confirmed by the 58% inhibition in BK-stimulated cPLA₂ activity observed on treatment with Ro31-8220. This was similar to the 51% inhibition seen for AA release (Fig. 4A).

BK-induced cPLA₂ activation occurs through PKC-mediated phosphorylation. The activation of cPLA₂ by BK or PMA was thought to be the result of enhanced enzyme phosphorylation. To determine whether this modification was responsible for the observed increase in cPLA₂ activity, we treated lysates from both stimulated and unstimulated cells with potato acid phosphatase. Our results, depicted in Fig. 6B, illustrate that both BK- and PMA-induced cPLA₂ activities were indeed a result of a phosphorylation event, since phosphatase exposure completely abrogated these responses.

Lin et al. (24) demonstrated that phosphorylation of cPLA₂ on serine-505 is responsible for increased cPLA₂ catalytic activity. In some cases, PKC appears to be
involved either in direct phosphorylation of cPLA₂ (27) or secondarily by activation of a mitogen-activated protein kinase (24, 31). To specifically determine whether BK treatment causes changes in the level of enzyme serine phosphorylation, we immunoprecipitated cPLA₂, performed SDS-PAGE, and subsequently immunoblotted membranes with a rabbit phosphoserine antibody. Results summarized in Fig. 7A show a representative blot, and those in Fig. 7B show the averaged optical density of scans from three such experiments. Both BK and PMA caused an approximately twofold increase in cPLA₂ phosphorylation, and this phosphorylation was completely reduced to control levels by Ro31-8220 treatment. The almost identical responses of both cPLA₂ activation and phosphorylation to BK and PMA, considered together with the differential activation of PKC by either agonist, suggest that PKC need not be fully activated to allow the same result or that specific PKC isozymes are mediating the activation of cPLA₂.

DISCUSSION

AA release and subsequent production of eicosanoids represent key signaling events for salt and water balance in the mammalian kidney. Accordingly, the nonapeptide BK is able to modulate kidney water balance largely through its ability to increase prostaglandin production in the collecting duct segment of the nephron (6). The aim of the present study was to arrive at a better understanding of how BK-mediated signaling events regulate AA release in the collecting duct, and this was achieved with the aid of an immortalized RCCD cell line. Increasing evidence indicates that agonist-stimulated release of AA occurs via activation of cPLA₂. Several mechanisms have been proposed for the activation of cPLA₂ and consequent AA release, namely, direct receptor-G protein coupling to cPLA₂ (4, 21), modulation by products of PLC and/or phospholipase D (20), and dependence on phosphorylation (25, 35, 37) and a rise in cytosolic Ca²⁺ (3, 7, 23, 28).

Results shown in Figs. 1–3 and Table 1 provide substantial evidence in support of the conclusion that cPLA₂ is the enzyme largely responsible for BK-stimulated AA release in RCCD cells. In addition to cPLA₂, the rabbit kidney has been shown to possess secretory PLA₂ enzymes as well as a novel 28-kDa Ca²⁺-independent PLA₂ recently purified by Portilla and Dai (30). A role for the disulfide bond-containing secretory isozymes was eliminated, because BK-stimulated PLA₂ activity was insensitive to the reducing agent DTT and because PLA₂ could be activated by Ca²⁺ in the submillimolar range. The novel 28-kDa Ca²⁺-independent PLA₂ was not responsible for BK-stimulated PLA₂ activity, since we found that omitting Ca²⁺ from the assay buffer completely abolished the stimulated activity. These other PLA₂ isozymes may indeed be present in RCCD cells, but they clearly are not responsible for BK-stimulated AA release.

To determine whether PKC was involved in the BK-stimulated increase in AA release and PLA₂ activity, we used PMA to activate and Ro31-8220 to inhibit PKC activity. The agent, Ro31-8220, a member of the bisindolylmaleimide class of PKC inhibitors, blunts the activity of a partially purified rat brain preparation containing PKC-α, -β₁, -β₂, -γ, -δ, -ε, and -ζ, with a 50% inhibitory concentration of 23 nM (44). In RCCD cells, BK-stimulated AA release was partly dependent on PKC, since treatment with Ro31-8220 resulted in >50% inhibition of this response. Activation of PKC by PMA alone did not mimic BK in its ability to release AA. However, when cells were treated with PMA + A-23187 together, there was a synergistic increase in AA release. The inability of PMA to induce AA release on its own is not surprising, since it does not generate a Ca²⁺ signal, a result that has been reported for other systems (5, 23, 38, 40, 45). Indeed, when cPLA₂ activity was determined in the presence of an assay buffer containing Ca²⁺, PMA could be seen to increase the activity of this enzyme. The mechanism of the PKC- and Ca²⁺-dependent synergy of AA release is probably a result of PKC-mediated phosphorylation of cPLA₂ and its Ca²⁺-dependent translocation to membrane substrates (23).

For the purpose of determining which of these two signals limits BK-stimulated AA release, we incubated BK in the presence of either PMA or A-23187. In contrast to results on MDCK cells (38), simultaneous

Fig. 7. Serine phosphorylation of cPLA₂ with BK or PMA stimulation is reversed by Ro31-8220. RCCD cells were incubated overnight in DMEM-F-12 defined medium containing 0.05% (wt/vol) BSA. The next day, cells were preincubated with or without 5 μM Ro31-8220 for 30 min before stimulation with 100 nM BK or 100 nM PMA for 2 min. cPLA₂ was immunoprecipitated, using a polyclonal cPLA₂ antibody, and was resolved on SDS-PAGE (7.5%). C, control: A: serine phosphorylation was detected, using a rabbit polyclonal antibody to phosphoserine residues (top). Total cPLA₂ present in each lane was similar, as detected by stripping membrane and reblotting membrane with cPLA₂ antibody (bottom). Position of molecular mass markers is indicated to the left of each blot. B: densitometry analysis of 3 such experiments.
addition of BK + PMA did not result in a potentiation of AA release. It appears that PKC activation does not limit the ability of BK to cause AA release but rather that Ca\(^{2+}\) may be limiting, since BK + A-23187 treatment resulted in a potentiation of BK-stimulated AA release that was similar to that resulting from PMA + A-23187 treatment (cf. Fig. 4, A and B). Although Ca\(^{2+}\) is critically important to AA hydrolysis by cPLA\(_{2}\), it should also be appreciated that A-23187 treatment does not likely mimic the physiological release of Ca\(^{2+}\) by agonists such as BK, since, in the prior case, both extracellular and intracellular Ca\(^{2+}\) stores would indiscriminately raise intracellular Ca\(^{2+}\) concentration. The ionophore, in addition to its ability to recruit PKC activity (Fig. 4A) and mediate Ca\(^{2+}\) influx, may also modify the integrity of the phospholipid bilayer, thereby altering cPLA\(_{2}\) binding to its substrate. It is therefore difficult to firmly establish whether ionophore alters cPLA\(_{2}\) activity in ways other than via its effect on Ca\(^{2+}\) influx.

In view of previous reports that cPLA\(_{2}\) phosphorylation on serine residues is critical for its catalytic activity and AA release (23, 24, 31) and our determination that phosphatase was able to blunt this enzyme’s activation, we sought to verify by Western blot whether serine phosphorylation occurred in BK- and PMA-stimulated RCCD cells. Our results show that BK, like PMA, caused an almost twofold increase in cPLA\(_{2}\) serine phosphorylation compared with control and that these responses could be completely blocked by Ro31-8220. Consideration of this and our other results on AA release and cPLA\(_{2}\) activity brings to mind interesting possibilities regarding the regulation of this enzyme. Although Ro31-8220 completely blocked BK-induced cPLA\(_{2}\) phosphorylation, it was unable to completely block cPLA\(_{2}\) activity. Therefore, in addition to serine phosphorylation, BK may activate cPLA\(_{2}\) by other mechanisms, possibly via nonserine phosphorylations of cPLA\(_{2}\) or by effects on a PLA\(_{2}\)-activating protein or PLA\(_{2}\)-inhibitory protein (26). Another explanation perhaps may be more likely, given the results of the serine phosphorylation. Previous studies based on gel-shift mobility and phosphatase treatment of cPLA\(_{2}\) have demonstrated that basal cPLA\(_{2}\) can occur with varying degrees of phosphorylation (3, 5, 23, 31). Under basal conditions (i.e., in the absence of BK stimulation), as seen in Fig. 7, RCCD cPLA\(_{2}\) appears to display a significant amount of phosphorylation. Thus some of the RCCD cPLA\(_{2}\) could actually be primed for activation, just needing a Ca\(^{2+}\) signal for translocation to the membrane substrate. In fact, cPLA\(_{2}\) appears to be at least partially active under basal conditions, given the modest yet statistically insignificant decrease in activity on phosphatase treatment. Bradykinin stimulation, however, is able to enhance cPLA\(_{2}\) activity via its activation of PKC, thereby causing recruitment of basally phosphorylated enzyme as well as amounts of freshly phosphorylated enzyme to the membrane.

On the basis of the similarities of cPLA\(_{2}\) activation and phosphorylation and the AA release by BK and PMA, it would seem reasonable that these agonists would increase PKC activity by the same amount. However, we found that PMA caused significantly greater membrane-associated PKC activity than BK. The question then arises as to why further PKC activation by PMA does not result in greater activation and phosphorylation of cPLA\(_{2}\). It is possible that cPLA\(_{2}\), which already exists in a partially phosphorylated state, becomes further maximally phosphorylated when only a portion of the PKC pool is activated. Another attractive possibility is that specific PKC isozymes may be responsible for regulating cPLA\(_{2}\) activation. At least 13 PKC isozymes have been identified and characterized with respect to their structure and cofactor regulation (29), while evidence exists for isozyme-selective activation, substrate preference, and localization (17). In our scenario, BK may specifically activate only a subset of PKC isozymes, whereas PMA activates a more complete selection as reflected by its activation of PKC. However, these additional PKC isozymes would not contribute to cPLA\(_{2}\) activation. Support for the involvement of specific PKC isozymes in regulating cPLA\(_{2}\) function is provided by Godson et al. (14) and Wang et al. (40), who have demonstrated that PKC-\(\alpha\) is the main PKC isozyme involved in phorbol ester-mediated arachidonate release in MDCK cells and \(\alpha_{1}\)-adrenergic activation of cPLA\(_{2}\) in FRTL-5 thyroid cells, respectively. Which PKC isozymes are present and involved after BK stimulation of RCCD cells is unknown but is the subject of current investigation.

In summary, the results of this research demonstrate that BK-stimulated AA release in an RCCD cell line is mediated through the activation of cPLA\(_{2}\) and that this process is largely dependent on cPLA\(_{2}\) phosphorylation via a PKC-dependent route. Interestingly, despite evidence from our laboratory that cPLA\(_{2}\) is also the enzyme responsible for BK-mediated AA release in MDCK cells, PKC in this case is not involved in onset responses to this hormone (unpublished observations). However, a role for PKC in regulating cPLA\(_{2}\) can be seen with PKC downregulation in MDCK cells, which results in reduced AA release, cPLA\(_{2}\) activity, and cPLA\(_{2}\) serine phosphorylation (19). Thus it appears that BK signaling in MDCK and RCCD cells, although activating cPLA\(_{2}\), has a different dependence on PKC with respect to the onset response. The reason for such differences in BK-dependent signaling may be a result of variable PKC isoform involvement, a possibility that also is being currently examined.

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