Bradykinin-stimulated arachidonic acid release from MDCK cells is not protein kinase C dependent

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Summary

Bradykinin-stimulated arachidonic acid release from MDCK cells is not protein kinase C dependent. Am. J. Physiol. 273 (Cell Physiol. 42): C1605–C1612, 1997.—Bradykinin (BK)-induced release of arachidonic acid (AA) from Madin-Darby canine kidney (MDCK) D1 cells was investigated. Phorbol 12-myristate 13-acetate (PMA) caused a synergistic increase in BK- and A-23187-induced release of AA but alone had no effect on this release. Inhibition of protein kinase C (PKC) with bisindolmaleimide I (BIS) abolished the synergistic effects of PMA but did not affect AA release caused by BK or A-23187 alone. Downregulation of PKC with 100 nM PMA resulted in a reduction of AA release induced by BK or A-23187 addition, which corresponded to a decrease in cytoplasmic phospholipase A2 (cPLA2) activity as measured in cell extracts. Although Western blotted revealed no differences in cPLA2 expression as a result of PMA treatment, phosphorylation of the enzyme, as assessed by phosphoserine content, was significantly reduced in PKC-depleted cells. These results imply that, with PKC downregulation, subsequent BK stimulation results in a Ca2+-dependent translocation of a less phosphorylated, less active form of cPLA2. Any stimulation of PKC by BK addition did not appear as a significant event in onset responses leading to AA release. On the other hand, inhibition of the mitogen-activated protein kinase (MAPK) cascade with the MAPK kinase inhibitor, PD-98059, significantly decreased BK-induced release of AA, a finding that, with our other results, points to the existence of a PKC-independent route for stimulation of MAPK and the propagation of onset responses.


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Phorbol ester; downregulation; cytosolic phospholipase A2; phosphorylation; mitogen-activated protein kinase

Release of arachidonic acid (AA) from the sn-2 position of various phospholipids is widely attributed to the 85-kDa form of phospholipase A2, also termed cPLA2 because of its presence in the cytoplasm. Many cell types can be induced to release AA in response to a wide array of agonists. However, the signaling routes taken by each of these extracellular messengers leading to cPLA2 activation and AA release have not all been unequivocally determined. Even so, several activation mechanisms have been elucidated by various groups. Activators such as collagen (6), fibroblast growth factor (33), and epidermal growth factor (8, 12), among others, make use of tyrosine kinase pathways, effectively working through the p21ras-RAF-1-mitogen-activated protein kinase (MAPK) signaling cascade to achieve phosphorylation of cPLA2. In fact, it is well known that cPLA2 can be phosphorylated on Ser505 by MAPK and that this event leads to its enhanced activity (23). Furthermore, there exist many branch points through which effectors can propagate signals originating from seven transmembrane receptor-coupled G proteins. In the case of Madin-Darby canine kidney (MDCK) D1 cells, for example, it has been recently demonstrated that α1-adrenergic agonists mediate the release of AA through the MAPK pathway (43). The ability of epinephrine to activate MAPK was canceled by both sphingosine, a protein kinase C (PKC) inhibitor, and through downregulation of PKC levels by phorbol ester. Either of these two treatments was also sufficient to block epinephrine-stimulated AA release. As had been shown earlier, the activation of PKC can lead to stimulation of the MAPK cascade (24), and it was apparent that the α1-adrenergic agonists in this case proceeded via phospholipase C (PLC) and PKC activation before MAPK activation. Bradykinin (BK) and epinephrine signal through many of the same second messenger cascades. For instance, both these agonists increase PKC activity and cause a mobilization of Ca2+ from both intra- and extracellular sources in MDCK cells (17, 18, 37). However, it was not unequivocally established that AA release induced by BK stimulation of such cells was PKC dependent.

In the case of BK, different strategies have yielded opposing conclusions in dealing with the question of PKC requirement. For example, ras-transformation of MDCK cells was shown to alter responses to phorbol ester without altering responses to BK (35). Again, the use of inhibitors of PKC, possessing varying degrees of specificity, has undeniably failed to block the actions of BK with respect to AA release in MDCK-D1 (32, 42). In addition, endogenous antagonists of PKC action, such as alkylglycerols, although being entirely capable of inhibiting phorbol ester-mediated AA release, were shown not to affect that mediated by BK (40). With these results at hand, one would be tempted to conclude that the liberation of AA from MDCK elicited through the action of BK occurs independently of PKC. However, in contrast to those making use of PKC inhibitors, other studies have employed long-term incubations with phorbol ester, which effectively downregulate expression levels of those PKC isozymes, including the α- and ε-forms found in MDCK cells. Furthermore, BK stimulation can indeed lead to translocation of a variety of PKC isoforms present in MDCK, including the α- and ε-forms (10, 11). Under these downregulation conditions, the release of AA by either BK or phorbol ester was significantly decreased (10).

The purpose of this study was therefore to try to explain the two findings that have led to opposing conclusions concerning the requirement for PKC activation in the BK-induced AA release from MDCK cells. We observed that bisindolmaleimide I (BIS), a specific PKC inhibitor, was incapable of blocking BK-enhanced release of AA, but it did abrogate the synergistic effect of BK with PMA. This led to a question as to whether the absence of a synergistic effect of BK and PMA on AA release in MDCK cells was due to the presence of endogenous PKC inhibitors or to a specific lack of PKC activation.

Activation of PKC by PMA is thought to influence AA release through various mechanisms. As early as 1977, Haywood et al. (20) demonstrated that PMA caused a rapid increase in cytosolic Ca2+ in a number of cell types, including smooth muscle, adipocytes, and neutrophils. Furthermore, the rise in Ca2+ is thought to be due, at least in part, to stimulation of phospholipase C (PLC), with the subsequent generation of inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 is released from internal stores and causes a mobilization of Ca2+ from intracellular stores, whereas DAG activates PKC, whose activation is thought to be required for the generation of AA from phospholipids (42). Indeed, PMA has been shown to be capable of stimulating AA release from MDCK cells, as well as from a variety of other cell types (17, 42). However, the downregulation of PKC levels by PMA has not unequivocally been established. In MDCK cells, the action of BK occurs independently of PKC. How-
of short-term exposure to phorbol ester on this BK response. Downregulation of PKC levels by long-term incubation with phorbol ester, although resulting in diminished BK-induced AA release, causes a concomitant decrease in cPLA2 activity due to lower steady-state phosphorylation of the enzyme. The results herein strongly suggest that BK does not require at onset the action of PKC for its ability to release AA in MDCK-D1 cells.

EXPERIMENTAL PROCEDURES

Materials. The agents, BK, 1-stearoyl-2-arachidonyl phosphatidylcholine, A-23187, and bovine serum albumin (BSA) were obtained from Sigma Chemical (Mississauga, ON, Canada). Phorbol 12-myristate 13-acetate (PMA) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA), and BIS and PD-98059 were purchased from Calbiochem-Novabiochem International (San Diego, CA). The radiochemicals, [5,6,8,9,11,12,14,15-3H]arachidonyl L-3-phosphatidylcholine, were obtained from Amersham Canada (Oakville, ON) as were the counting scintillants BCS and ACSII, the Western blotting materials, Hyperfilm, enhanced chemiluminescence (ECL) reagents, Hybond nitrocellulose, and anti-rabbit immunoglobulin horseradish peroxidase-linked secondary antibody. Rabbit polyclonal antibody to phosphoserine was purchased from Dimension Laboratories (Toronto, ON), and the antibody to cPLA2 was a kind gift of the Genetics Institute, Boston, MA.

Cell culture. The MDCK-D1 cell line used throughout was originally cloned by Dr. Paul Insel, University of California, La Jolla, CA. Cells were cultured in 150-mm petri dishes containing 20 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% antibiotic-antimycotic solution (GIBCO), 15 mM N-2-hydroxyethylpiperazine-N’-2-hethanesulfonic acid, and 3.7 g/l sodium bicarbonate. Stock cultures were maintained at 37°C in an atmosphere of 5% CO2 and were passaged before reaching confluence. For experiments, cells were diluted either into 12-well dishes (for measurement of AA release) or 100-mm petri dishes (for PLA2 activity assays, Western blotting, or immunoprecipitations) and used before reaching confluence (∼3 days after seeding).

Measurement of AA release. Subconfluent cultures in 12-well cluster dishes were washed three times with serum-free DMEM plus 0.05% BSA and labeled with 0.3 µCi [3H]AA for 24 h in DMEM containing either 0.05% BSA or 0.5% FCS. In some experiments, cells were incubated ~20 h with 100 nM PMA in DMEM containing either 0.05% BSA or 0.5% FCS before the labeling period. In such cases, the labeling medium also contained 100 nM PMA. Cells were washed three times with Hank’s balanced salt solution (HBSS) containing 0.05% BSA. When the effect of inhibiting PKC on AA release was to be tested, the cells were preincubated with 2 µM BIS in HBSS or with HBSS alone for 30 min. Subsequently, the medium was aspirated and replaced with fresh HBSS containing 1 µM BK alone, 10 µM A-23187 alone, 100 nM PMA alone, both PMA and BK together, or both PMA and A-23187 together, with or without BIS. Control conditions without BK, A-23187, PMA, and/or inhibitor were similar. After 10 min of incubation, the medium was removed, centrifuged at 5,000 g for 5 min to pellet dislodged cells, and a 0.5-ml aliquot was taken for counting to determine total [3H]AA released. The remaining 0.5 ml of HBSS was acidified to pH 3.0 with phosphoric acid and extracted three times with an equal volume of ethyl acetate. The combined extracts were dried under a stream of nitrogen and redissolved in 20 µl of ethyl acetate, and the AA metabolites were separated by high-performance thin-layer chromatography using a solvent system consisting of ethyl acetate-acetic acid (95:1 vol/vol) (13). AA was identified by cochromatography with authentic standard. Radioactivity was assessed by counting in 10 ml of ACSII. Throughout, the release values were normalized for the amount of labeled AA incorporated in the lipids of the cell, i.e., calculated as percentages of labeled AA incorporated, and are reported as magnitude of increases compared with control values obtained with cells not treated with an agent.

PLA2 activity assay. To obtain a cell lysate containing PLA2 activity, cultures on 150 × 20-mm plastic dishes were subsequently washed twice with ice-cold 100 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4) containing 250 mM sucrose and 1 mM ethylene glycol-bis(β-aminethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA). Cells were then scraped off the plates and centrifuged at 1,000 g for 5 min, and the pellet was resuspended in sonication buffer (100 mM Tris-HCl (pH 7.4), 250 mM sucrose, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium vanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 5 µM phosphoserine, 1 mM diithiothreitol, 1 mM EGTA, and 5 mM EDTA) (10). The suspension was then sonicated on ice three times for 5 s with 15-s intervals between each pulse to maintain ice-cold conditions. Cell lysates were then centrifuged for 5 min at 1,000 g to pellet unbroken cells. Supernatants were assayed for protein by Bio-Rad protein assay method with BSA as standard.

To determine the effect of long-term incubation with PMA on PLA2 activity, cells were grown as described previously (1, 18) but incubated for 24 h with or without 100 nM PMA in DMEM containing either 0.05% BSA or 0.5% FCS. Incubations were terminated by washing the cells twice with ice-cold Tris buffer. The PLA2 activity of the lysates was determined according to Leslie (21). Briefly, 20 µg of cell lysate were incubated at 37°C for 1 h with 30 µM 1-stearoyl-2-arachidonoyl phosphatidylcholine as substrate (with 55,000 dpm 1-[5,6,8,9,11,12,14,15-3H]arachidonyl L-3-phosphatidylcholine as tracer). The substrate was removed, first by removal of solvent under nitrogen and then by sonication for 3 min on ice, in incubation buffer (100 mM Tris-HCl (pH 7.4), 250 mM sucrose, 0.5 mg/ml BSA, and 5 mM Ca2+). The incubation was terminated with the addition of 2.5 ml of Dole reagent (2-propanol-n-heptane-0.5 M H2SO4, 20:5:1, vol/vol/vol) (9) followed by the addition of 1.5 ml heptane containing 20 µg unlabeled AA as carrier. Separate phases were obtained by the addition of 1 ml of H2O, and the top phase containing free AA was removed and further purified by silicic acid column chromatography. Diethyl ether (2 ml) was used to wash the columns, and the eluant, collected in scintillation vials, was dried under nitrogen and then counted by liquid scintillation spectrometry.

Western blotting and immunoprecipitations. Cells, grown on 100 × 20-mm petri dishes, were washed three times with serum-free DMEM containing 0.05% BSA and subsequently incubated overnight (~20 h) with or without 100 nM PMA. Immediately afterward, they were washed two times with ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 1 mM EGTA, then scraped off the plates, and pelleted by centrifugation for 5 min at 1,000 g. Pellets were resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), containing 150 mM NaCl, 1 mM EGTA, 1% Nonidet P40, 0.1% sodium deoxycholate, 1 mM PMSF, 10 mM sodium vanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 10 µg/ml leupep-
tin, 10 μg/ml aprotinin, and 5 μM phosphoserine). Suspensions were placed on ice for 20 min and then vortexed vigorously for 2 min to ensure complete lysis of the cells.

For Western blotting, 2 × Laemmli buffer was added until the protein content was 0.5 mg/ml. Samples were run on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels, using the Bio-Rad Mini Protean II apparatus at 200 V, 60 mA for ~45 min, until the Coomasie blue tracking dye ran off the gel. Protein was transferred to Hybond nitrocellulose membranes according to the supplier's instructions. Blots were blocked overnight using 5% skim milk in Tris-buffered saline (TBS) and subsequently incubated for 1 h with rabbit polyclonal cPLA2 antiserum (1:2,000 dilution) dissolved in TBS containing 0.1% Tween-20 (TTBS) and 2% skim milk. After this, the blots were repeatedly washed with TTBS and then incubated for 45 min with an anti-rabbit horseradish peroxidase-linked secondary antibody (1:2,000 dilution). The blots were then washed with TTBS as stated above and developed by means of the ECL system according to the manufacturer's specifications.

For immunoprecipitations, cells were prepared and lysed as described above. In this case, however, 200 μg of cell lysate diluted into 0.5 ml of lysis buffer were preincubated for 3 h with 25 μl of the homogeneous protein A-agarose suspension at 4°C on a rocking platform. Subsequently, 10 μl of the rabbit cPLA2 antiserum were added and allowed to bind antigen for an additional 3 h, after which 25 μl of the protein A-agarose suspension were added. The incubation was continued for ~20 h at 4°C. The immunocomplexes were then collected by centrifugation, washed twice with lysis buffer, twice with lysis buffer containing 500 mM NaCl, 0.1% Nonidet P40, and 0.05% sodium deoxycholate, and finally washed once with lysis buffer containing no NaCl. The last traces of the final wash were removed with strips of filter paper, and 45 μl of gel loading buffer were added to the agarose pellet. The proteins were then denatured by boiling for 5 min in a water bath, and 25 μl of supernatant were run on an SDS-PAGE gel. Western blotting was performed as described above with the exception that a rabbit polyclonal antibody to phosphoserine was used as the primary antibody.

Statistics. Student's t-test for unpaired data was used when only two unrelated treatment groups were compared. To determine the statistical significance of differences between more than two groups, analysis of variance (ANOVA) and the Student-Newman-Keuls multiple comparison test was used. Differences of P < 0.05 were considered statistically significant. Data are averages of duplicate determinations from individual experiments presented as means ± SE for groups of values when n = 4 or means ± SD for groups of values when n = 3.

RESULTS

Effects of PKC inhibitor, BIS, on AA release in MDCK-D1 cells. Experiments were performed to determine whether inhibition of PKC would cause a concomitant decrease in agonist-stimulated AA release in MDCK-D1 cells. Thirty minutes before stimulation with 10 μM A-23187, 1 μM BK, 100 nM PMA, both PMA and A-23187, or both PMA and BK, cells in DMEM containing 0.05% BSA were preincubated with or without 2 μM BIS, a potent and very specific inhibitor of PKC (38). After stimulation for 10 min with the above-mentioned agents, sample media were collected and processed for [3H]AA released. As shown in Fig. 1A, Ca2+ ionophore was able to induce a 4.8 ± 0.4-fold increase in AA released, which was not significantly altered by PKC inhibition in the presence of 2 μM BIS (5.6 ± 0.7-fold increase). This result agrees with those observed in other cell types (2, 6, 29) and indicates that, under our conditions, the ability of ionophore to cause a release of AA does not depend on PKC but predominantly on the elevation of intracellular Ca2+, which enables cPLA2 to translocate from the cytosol to membranes where it can access its substrate (26). On the other hand, activation of PKC by phorbol ester has been shown to lead to increased cPLA2 activity (43) and AA

Fig. 1. Bisindolmaleimide I (BIS) inhibits phorbol ester-induced synergistic effect on arachidonic acid (AA) release but blocks neither A-23187- nor bradykinin (BK)-stimulated AA release. Cells were labeled overnight with [3H]AA in DMEM containing 0.05% bovine serum albumin (BSA; wt/vol) and washed with Hanks' balanced salt solution (HBSS) + 0.05% BSA to remove unincorporated label. Cells were then preincubated with or without 2 μM BIS for 30 min followed by a 10-min stimulation with or without 100 nM PMA. Finally, cells were stimulated at 37°C in A with or without 1 μM BK, 10 μM A-23187, 100 nM phorbol 12-myristate 13-acetate (PMA), or both PMA and A-23187 together. For experiments depicted in B, cells were treated as mentioned above with exception that both PMA and A-23187 were used together for 10-min stimulation period. Medium was removed and counted for labeled AA released by cells. Cells were solubilized with 5% SDS, and an aliquot was taken to determine total AA incorporated. Amount of AA released is normalized for total label incorporated into cells and expressed as magnitude of increase in release compared with controls, i.e., containing no BK, A-23187, or PMA. Mean control value expressed as percentage of total AA incorporated = 0.63 ± 0.10% (n = 5). Other values are means of at least 4 independent experiments performed in duplicate ± SE. *P < 0.05 vs. no BIS pretreatment + PMA + A-23187, or + PMA + BK.
release in MDCK cells after prolonged incubations of 30–60 min (29, 32). However, after 10 min of incubation with PMA alone as in our case, no significant enhancement of AA release occurs (1.1 ± 0.2-fold the control value). This lack of effect could be explained on the basis that PMA fails to elicit any Ca²⁺ mobilization (1, 15) and therefore would not provoke translocation of cPLA₂ to membranes. One could picture that, even in resting cells, there is a limited pool of membrane cPLA₂ which turns over and is replaced by new cytosolic enzyme. If, concurrently, the cytosolic enzyme becomes phosphorylated by a PKC-dependent mechanism, this would result in a slow increase in membrane activity and AA release and would not be noticeable within 10 min. The idea that rapid release of AA requires Ca²⁺ influx is demonstrated when both PMA and A-23187 are incubated together. The combination of these two agents causes a synergistic release of AA over and above that seen with ionophore alone, increasing from 4.8 ± 0.4- to 10.0 ± 0.8-fold the control level on addition of 100 nM PMA. The PMA-stimulated portion of the AA release was virtually eliminated by BIS, as seen by a return to levels similar to those obtained with ionophore alone (cf. 5.6 ± 0.7-fold for A-23187 + BIS with 6.3 ± 0.9-fold for BIS + ionophore + PMA), a result consistent with a decrease in PKC activity. In direct contrast, BIS was unable to exert any effect on the AA liberated by BK alone as shown in Fig. 1 (i.e., 2.6 ± 0.1-fold with BK vs. 2.6 ± 0.1-fold with BK + BIS). In addition, the synergistic effect observed with PMA and BK was eliminated by treatment with BIS (cf. 5.2 ± 1.1-fold for BK + PMA and 3.2 ± 0.9-fold for BIS + BK + PMA). Therefore, despite being able to eliminate the PMA-induced synergistic release of AA in combination with ionophore or BK, BIS was incapable of blocking that observed with either BK or A-23187 alone.

Determination of effects of long-term downregulation of PKC by phorbol ester on basal and A-23187-induced AA release. In contrast to the lack of effect by chemical inhibitors on BK-mediated AA release, results obtained by downregulation of PKC via long-term exposure to phorbol ester have argued for the involvement of this serine-threonine kinase in the signaling mechanism used by BK to liberate AA (10). We therefore decided to test the effects of long-term pretreatment with 100 nM PMA on AA release by cells that had been deprived of serum during the PMA treatment and radioactive AA labeling periods and cells that were kept in 0.05% serum during these operations. It was reasoned that serum contained various agonists, which, enhancing the activities of signaling pathways, could possibly lead to increased cPLA₂ activity and AA release. The effect of PKC downregulation on such responses to serum, if any, remained to be determined. The phorbol ester treatment chosen was similar to that reported to effectively downregulate PKC in MDCK-D1 cells (10). The action of Ca²⁺ ionophore and the onset responses to BK as demonstrated earlier do not require PKC mediation but rely instead on their ability to increase intracellular Ca²⁺. It was therefore expected that downregulation of PKC levels would not alter AA release when these agents were used. In the absence of Ca²⁺-mobilizing agents such as BK and ionophore, the basal release of AA expressed as a percentage of labeled AA incorporated was not significantly changed by exposure of cells to serum (cf. legend to Fig. 2); however, serum enhanced the responses of cells to ionophore and BK stimulation. This was apparent when values were expressed as magnitude of increases compared with control as illustrated in Fig. 2. Qualitatively similar results were obtained when the cells were serum starved and PKC downregulated (i.e., from 7.2 ± 0.6- to 4.4 ± 1.0-fold of control value for A-23187 and from 3.4 ± 0.8- to 1.6 ± 0.3-fold of control value for BK). Furthermore, serum enhancement effects were all but completely abolished, and the effects of BK and ionophore were again largely decreased. Although other possibilities could not be precluded, it was hypothesized that downregulation might target cPLA₂ itself, the activity of which somehow depended on PKC. The results did in fact support our supposition that serum contains agonists which cause responses at the level of cPLA₂, and these appeared to be PKC mediated. This point was further investigated (see below).

Examination of effects of long-term downregulation of PKC by phorbol ester on basal cPLA₂ activity. To determine whether cPLA₂ itself was being affected by downregulation of PKC, cPLA₂ activity was measured in cell lysates obtained from cells grown in the presence and absence of serum but not stimulated by BK. Growth factors and other agonists in the serum-
containing medium, by activating signaling systems, were expected to affect the levels of expression and/or the levels of active phosphorylated state of PLA2, since these factors enhanced AA release. As shown in Fig. 3, preparations from cells that had been serum starved (i.e., incubated in DMEM containing 0.05% BSA) to minimize basal cPLA2 phosphorylation displayed a much lower degree of activity compared with lysates derived from cells exposed overnight to serum (i.e., 54.4 ± 7.9 pmol·min⁻¹·mg⁻¹ for 0.5% FCS compared with 31.2 ± 5.5 pmol·min⁻¹·mg⁻¹ for 0.05% BSA). This result agrees with that observed in the arachidonate release studies in which an enhancing effect of serum on ionophore- and BK-induced release could be observed (Fig. 2). Again, pretreatment for 24 h with 100 nM PMA caused a significant decrease in PLA2 activity of cell lysates, reaching levels which were very comparable in serum-starved and serum-treated cells (i.e., 22.5 ± 2.9 pmol·min⁻¹·mg⁻¹ for PMA + 0.5% FCS vs. 17.1 ± 2.1 pmol·min⁻¹·mg⁻¹ for PMA + 0.05% BSA). These results did in fact support the idea that serum maintains either a higher state of phosphorylation or higher level of expression of cPLA2 and that long-term exposure to PMA reduces this effect. This reduction in activity matches the decrease in BK- or ionophore-induced AA release seen in cells with downregulated PKC (Fig. 2).

Examination of the levels of cPLA2 after long-term downregulation of PKC by phorbol ester as measured by Western blotting. To determine whether long-term incubation of MDCK-D1 cells with PMA causes a reduction in the expression level of cPLA2, Western blots were performed on cell lysates by means of a rabbit polyclonal antibody directed against the 85-kDa form of phospholipase A2. As shown in Fig. 4, downregulation with 100 nM PMA did not alter cPLA2 expression in MDCK-D1 cells. Furthermore, cells that were serum starved failed to display any significant differences in cPLA2 levels compared with those in serum-treated cells. These results demonstrate unequivocally that the decrease of basal cPLA2 activity due to downregulation of PKC is not due to a reduction in the expression level of this phospholipase.

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Determination of level of phosphoserine phosphorylation of cPLA2 after long-term phorbol ester pretreatment. A likely explanation for the observed decrease of cPLA2 activity brought about by long-term PMA expo-
Ser228 (34). Indeed, it was demonstrated in vitro that MAPK can carry out the phosphorylation of Ser505, an event that increases the enzyme's ability to hydrolyze substrate (23). One could then hypothesize that basal PKC activity, being enhanced through signaling induced by the presence of serum, might serve to maintain a variable portion of cPLA2 in a phosphorylated state through its positive effects on the MAPK cascade (39). Alternatively, MAPK could be activated by PKC-independent signaling. To determine whether long-term downregulation of PKC levels could reduce the basal phosphorylation state of cPLA2, Western blotting of cPLA2 immunoprecipitates was performed with a rabbit polyclonal antibody directed specifically toward phosphoserine residues. As depicted in Fig. 5, downregulation of PKC levels (by 100 nM PMA) markedly reduced the phosphoserine content of cPLA2.

Effect of inhibiting MAPK kinase on BK-stimulated AA release. Experiments based on BIS inhibition did not support a direct role of PKC in mediating the onset responses to BK stimulation. Notwithstanding, it appeared that conditions which affected the phosphorylation of cPLA2 in a PKC-dependent manner affected responses to BK and ionophore. The question then arose as to whether steady-state phosphorylation levels of cPLA2 were sufficiently high to account for BK responses under the usual conditions tested and whether the nonapeptide acted solely through its generation of a Ca2+ signal without causing increased phospholipase phosphorylation. At this stage, it was decided to test whether the MAPK cascade might be implicated in mediating onset effects of BK. To this end, a specific MAPK kinase inhibitor, PD-98059, was employed. Indeed, this agent very significantly reduced the ability of BK to elicit AA release from MDCK-D1 cells (by 64 ± 3%, P < 0.01). It should be noted that, based on the average results of four trials, this agent did not significantly change the basal level of AA release, indicating a targeting of the BK-coupled signaling. Also, adding significance to this result are the facts that in vitro PL2 activity was not altered in the presence of PD-98059, and trypan blue exclusion revealed no detectable cytotoxic effects on the cells.

DISCUSSION

We have previously showed that much of the propensity of BK to induce a release of AA from MDCK-D1 cells is dependent on activation of phosphatidylcholine (PC)-specific PLC, which produces the bioactive lipid diacylglycerol (DAG) (18). Accordingly, inhibition of PC-PLC by D609 resulted in substantial loss of BK-stimulated AA release. The question arose as to how DAG served in the signaling process. In agreement with the conclusions from other studies, we (18) found that this lipid can enhance in vitro PLA2 activity when sonicated into exogenous PC vesicles (19). Thus the enhancement appeared to result, at least in part, from favorable effects on the lyotropic mesomorphism of the substrate. Interestingly, BK stimulation also increased PKC activity within the 1st min, an effect that could be abolished with D609 (18). Thus BK-enhanced release of AA appeared to be coincidental with stimulation of PKC after production of DAG by PC-PLC. Indeed, PKC by activation of the MAPK cascade (24) would be expected to cause an increase in the phosphorylation and activity of cPLA2 (23, 27); however, from other evidence at hand, the role of this protein kinase in mediating BK responses appeared uncertain (35, 36).

The data presented herein represent the first attempt to reconcile differing conclusions drawn from two sets of studies aimed at establishing the role of PKC activation in BK-mediated release of AA in MDCK-D1 cells. The predominant conclusion from studies with chemical inhibitors of PKC has been one that denies a role for this serine-threonine kinase (42). On the other hand, studies involving phorbol ester-mediated downregulation of PKC levels have provided results in support of a role for PKC in the BK-induced liberation of AA in MDCK-D1 (41). We present results demon-

![Fig. 5. Long-term downregulation of PKC levels reduces phosphoserine content of cPLA2.](Image)
strating that the inhibitor of PKC, BIS, although fully capable of blocking the synergistic effect observed with the combination of either PMA and A-23187 or PMA and BK, is unable to alter the release of AA due to BK alone (Fig. 1, A and B). One possibility for this lack of effect with a PKC-specific inhibitor is that a subclass of PKC isozymes may be insensitive to these agents. However, reports indicate that BIS is capable of inhibiting the activities of PKC-α, -β1, -δ, -ε, and -γ with half-maximal inhibitory concentration values of 8.4, 18, 210, 132, and 5,800 nM, respectively (25). The isozymes found in MDCK-D1 cells are entirely comprised in the list just mentioned (4). Another consideration is whether such an inhibitor might nonspecifically inhibit PLA2 activity in these cells. However, we have shown previously that inhibition of PKA by H-89 leads to an enhanced release of AA from MDCK-D1 cells (17) rather than the decreased releases noted presently with BIS. Furthermore, a wide variety of PKC inhibitors, including N-(2-[methylamino]ethyl)-5-isouquinolinesulfonamide-stauroporine (42) and alkylglycerol (40), have proved unsuccessful in blocking BK-mediated AA release but did eliminate the contribution made by phorbol ester treatment. Of the inhibitors tested, only sphingosine, when used at high doses (>10 µM), was able to partially reduce BK-stimulated AA release (32). This lack of effect of PKC inhibitors on agonist-stimulated AA release is not unique to BK and MDCK-D1 cells (5, 6, 14, 28). It should be added, however, that PKC inhibitors have been useful in establishing that agonists such as epinephrine do display in MDCK-D1 cells, a requirement for PKC mediation in their ability to enhance release of AA (42).

Evidence obtained in the present study indicates that long-term treatment of cells with PMA, a process shown to downregulate PKC in MDCK-D1 cells (10), leads to decreased AA release in response to BK stimulation. Such results are often the basis for implicating PKC in signaling phenomena, although the implication can be quite indirect and need not involve this protein kinase in the onset responses to a particular agonist (7, 31). This appears to be the case for BK-stimulated release of AA, which is not affected by PKC inhibitors. Indeed the present results obtained with BIS as inhibitor suggest that although BK can activate PKC, the increase in enzyme activity is insufficient to be functionally apparent or relevant in terms of onset responses leading to AA release. It was observed that the main consequence of PKC downregulation was to lower the activity of cPLA2 by a process which affects its phosphorylation rather than its expression levels (Figs. 3 and 4). It could be concluded from this and the results as a whole, that in MDCK-D1 cells at least, BK need not act by activating PKC but instead simply by initiating a Ca2+ signal. However, stimulation by the nonapeptide relies to some extent on a steady-state pool of phosphorylated cPLA2, the maintenance of which is substantially PKC dependent. The PKC pathway involved would be activated by agonists other than BK, possibly of autocrine origin, or otherwise present in the culture medium, especially when serum is added. This same pathway is also activated by PKC, which accounts for the synergistic increases in AA release observed.

The results presented herein demonstrate that although PKC inhibitors fail to abrogate BK-induced AA release, the MAPK kinase inhibitor, PD-98059, significantly blunts this release. It is thus apparent that BK stimulation of the cells also leads to activation of the MAPK cascade as an onset response and that the latter is involved in the enhancement of AA release. Because MAPK can phosphorylate cPLA2, it is reasonable to assume that some of BK effects are exerted at that level, although this point would require verification. Recently, Xing and Insel (43) demonstrated that epinephrine, signaling through the α1-adrenergic receptor, relies not only on PKC but also on MAPK to phosphorylate and activate cPLA2 and release AA from MDCK-D1 cells. The mechanism for this process is most likely a PKC-mediated activation of RAF-1, which enhances MAPK activity, followed by MAPK recruitment and phosphorylation of cPLA2. However, studies involving agents such as endothelin-1 in both rat astrocytes (16) and Chinese hamster ovary cells transfected with the human ET_A receptor (20) and leukotriene B4 in guinea pig eosinophils (3) have provided evidence for PKC-independent mechanisms of MAPK activation. These signaling pathways seem to rely on the subunits of certain G proteins that increase ras activity through an unknown mechanism. It is possible that BK makes use of such a PKC-independent mechanism to activate the MAPK cascade and phosphorylate cPLA2, a point that we hope to further clarify.

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