Bombesin, Platelet-derived Growth Factor, and Diacylglycerol Induce Selective Membrane Association and Down-regulation of Protein Kinase C Isotypes in Swiss 3T3 Cells*

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Swiss 3T3 cells contain protein kinase C (PKC) isotypes α, β, δ, ε and ζ (Olivier, A. R., and Parker, P. J. (1992) J. Cell. Physiol. 152, 240–244). Acute stimulation of quiescent cells with the neuropeptide bombesin decreases the mobility of PKC-δ and PKC-ε on SDS-polyacrylamide gels. These slower migrating forms of PKC-δ and PKC-ε rapidly (within 1 s) and selectively are found associated with the Triton X-100-soluble membrane fraction. No change in the mobility or distribution of PKC-α or PKC-ζ is detected. Long-term treatment of cells with bombesin induces selective membrane association and down-regulation of PKC-δ and PKC-ε (decreasing 70 and 65%, respectively). No change in the long-term distribution of PKC-α and PKC-ζ was detected. Bombesin did, however, increase PKC-α protein levels by 60% compared to control cells. PKC-ζ levels remained unchanged. Both the shift in mobility and down-regulation of PKC-δ and PKC-ε were only induced by mitogenic doses of bombesin. The potent mitogen platelet-derived growth factor induced similar effects on the PKC isotypes δ and ε. PKC-α and PKC-ζ levels were unaffected. Repeated doses of the synthetic diglyceride 1-oleoyl-2-acetyl-sn-glycerol induced PKC-δ and PKC-ε down-regulation and stimulated the cells to divide. Again PKC-α and PKC-ζ levels were unaffected. These results show a correlation between the membrane association and down-regulation of PKC-δ and PKC-ε and the entry of cells into S phase.

Protein kinase C (PKC) consists of a family of related polypeptides that are dependent on phospholipids for activity and their activation has been implicated in the regulation of a number of cellular responses (for review, see Refs. 1 and 2). This enzyme family can be divided into three groups: (i) α, β1, β2, and γ which are Ca2+- and phorbol ester/diacylglycerol (DAG)-dependent; (ii) ε, δ, η, and θ which are phorbol ester/DAG-dependent but do not require Ca2+; and (iii) ζ and λ, the former of which is not dependent on Ca2+ and phorbol ester/DAG (see Refs. 1 and 3, and references therein); PKC-ζ can be activated by phosphorylinoisitol 3,4,5-trisphosphate (4). The conservation of PKC isotypes between species suggests that they play specialized roles in cellular regulation, however, there is only limited information on their selective operation in vivo (1, 2). The events leading to cell proliferation have been extensively studied using Swiss 3T3 cells as a model system (5). Recently it was reported that fibroblasts express PKC-δ, -ε, and -ζ in addition to PKC-α (6–8). These enzymes are differentially down-regulated in response to phorbol esters, implying different regulation/compartimentalization for each isotype (6, 7). While phorbol esters provide pharmacological evidence of distinct operation of these PKC isotypes, their role/behave in response to physiological agonists with defined mitogenic properties has not been investigated previously in fibroblasts. In view of the complex, multiphasic nature of DAG production in response to various cellular agonists (e.g. Refs. 9–11) it has been pertinent to determine directly how such agonists impinge upon specific PKC isotypes.

Bombesin binds to a G-protein-coupled receptor (12), inducing a number of early responses including phospholipid hydrolysis (5, 13–15) which results in the acute changes in second messengers such as Ca2+ and DAG that could activate PKC (13, 14). Acting through its tyrosine kinase receptor, platelet-derived growth factor (PDGF) can also induce a similar series of events to generate second messengers (16), including those involved in PKC activation. As with other mitogens, stimulation needs to be sustained for a prolonged period in order to commit cells to division. The prolonged signals that are required to establish commitment are poorly understood.

With the use of isotype-specific antisera it is shown here that both bombesin and PDGF can induce marked changes in PKC isotype expression and distribution after both acute and prolonged exposure to the mitogens. In addition, the repeated addition of DAG can mimic these prolonged effects. The results are consistent with a role for PKC activation and consequent down-regulation in G1 progression, perhaps contributing to the sustained mitogen responses necessary for commitment to S phase.

MATERIALS AND METHODS

Bombesin (porcine), insulin (human), and platelet-derived growth factor, and (human recombinant) β-chain homodimer were from Calbiochem Corp. Antisera to the carboxyl termini of PKC-α, PKC-β, PKC-ε, and PKC-ζ were as previously described (17–20). The ECL Western blotting detection reagents and the horseradish peroxidase-conjugated donkey anti-rabbit IgGs were from Amersham, United Kingdom. Synthetic diglycerides, 1,2-diacyl-sn-glycerol (DiC2), and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were from Sigma. Growth factors were diluted according to the manufacturer’s procedures and the DiC2 and OAG dissolved in Me2SO.

Cell Culture and Extraction—Swiss 3T3 cells were maintained as previously described (6). Cells were judged quiescent after 8 days, a time at which 95–97% of the cells were in G0–G1, as judged by flow cytometry (see Fig. 5) and immunofluorescence (data not shown). Cells
The immunoblots were visualized using the ECL detection reagents and loaded per lane and analyzed by 8% (panel heated 25°C) SDS-PAGE and Western blotting. The same membrane was stripped and reprobed with the different isotype-specific antisera. The immunoblots were visualized using the ECL detection reagents and autoradiography as described under “Materials and Methods.”

were stimulated with growth factors or lipids for increasing times as indicated in the text or figure legends. For total cell lysates, the cells were placed on ice and washed twice with phosphate-buffered saline (PBS). Twofold concentrated Laemmli (21) sample buffer (150 μl/6-cm plate, 300 μl/10-cm plate) was added to each plate. The cell lysate was scraped to the side of the plate and transferred to a tube which was placed on ice and washed two times with phosphate-buffered saline (PBS). %ce concentrated Laemmli (panel 25°C) SDS-PAGE and Western blotting. The same membrane was stripped and reprobed with the different isotype-specific antisera. The immunoblots were visualized using the ECL detection reagents and autoradiography as described under “Materials and Methods.”

RESULTS

Effect of Acute Bombesin Treatment on PKC Isotypes—Purification of recombinant PKC-δ (19, 24) or endogenous PKC-δ and PKC-ε from tissue (25–27) have shown that these enzymes exist as phosphorylated entities displaying heterogeneity on SDS-PAGE. The neuropeptide bombesin has been shown to induce a number of early events in Swiss 3T3 cells, including increased phosphorylation of proteins (5, 28). To determine whether this agonist has any effect on the mobility of these PKCs on SDS-PAGE, Swiss 3T3 cells were stimulated for 15 min with increasing doses of bombesin. This acute treatment of the quiescent cells is found to induce a size shift of both PKC-δ and PKC-ε as seen by analysis on SDS-PAGE and subsequent Western blotting (Fig. 1). The extent of this shift is dependent on the bombesin concentration, maximum shift is achieved by mitogen concentrations (see below and Ref. 29) of the neuropeptide (Fig. 1, lanes 5–7). PKC-ε appears as a very distinct doublet when analyzed on 7.5% SDS-PAGE with a lower bisacrylamide concentration (see Fig. 1). To determine whether this slower migrating form is a result of phosphorylation of this enzyme, extracts from untreated and bombesintreated extracts were subjected to Fast Flow S chromatography to remove the endogenous serine threonine phosphatases (30). The eluates were then incubated with protein phosphatase 2A either in the presence or absence of the phosphatase inhibitor microcystin. The results in Fig. 2 show that treatment of both quiescent and stimulated cell preparations caused an increase in the mobility of the respective proteins and microcystin blocked this effect. Dephosphorylation of PKC-ε in quiescent cells resulted in an increase in the mobility of the protein (denoted a in Fig. 2), indicating that PKC-ε has a basal level of phosphorylation (denoted b in Fig. 2) in quiescent cells (Fig. 2, compare lane 3 with I, 4, and 5). Treatment of the stimulated cell eluates with phosphatase caused the dephosphorylation of...
both of the bands resulting only in the lower molecular weight forms (a and b). These results therefore indicate that PKC-ε is a phosphoprotein and stimulation with bombesin increases this phosphorylation. This is most likely also the case for PKC-δ (25, 26), however, unlike PKC-ε, it was not possible to separate PKC-δ from endogenous protein phosphatases by cation exchange chromatography (not shown). PKC-α is also known to be phosphorylated in response to growth factors (31). A shift in mobility is, however, not detected (Fig. 1). PKC-ζ does not appear to change migration under any condition.

The activation of PKC is frequently measured through its ability to associate stably with membranes upon acute treatment with phorbol esters or growth factors that induce DAG production (1, 2). To determine which of the identified PKC isotypes can translocate in response to bombesin, fractionated cell lysates were subjected to 10% SDS-PAGE and the PKC content analyzed by Western blotting (Fig. 3). In addition to translocation, bombesin induces maximal size shift of PKC-δ and PKC-ε within 1 s of stimulation and it is this form which is associated with the membrane (Fig. 3, and date not shown). The shift in mobility of PKC-ε is not as well resolved on this gel system compared to the 7.5% SDS-PAGE low bisacrylamide used in Figs. 1, 2, 4, 5, and 7. At later times, between 3 and 45 min, a portion of the PKC-δ and PKC-ε population redistributes to the cytosol again, this is clearly shown by the increase in cytosolic protein at 45 min. This suggests a less stable association of the enzymes with the membrane at this time. The distribution of PKC-α and PKC-ζ appears to be unchanged under these conditions. These results, therefore, show that bombesin induces an acute shift in apparent size of PKC-δ and PKC-ε and that this slower migrating form is predominantly associated with the membrane.

**Effect of Prolonged Treatment of Mitogens on PKC Isotypes**—A well documented characteristic of phorbol esters is to induce an increased rate of degradation of PKC when administered for prolonged periods (32). Until recently investigators have been unable to show that down-regulation occurs in vivo upon physiological stimulation. It has only been since additional members of the PKC family were identified that it was found that PKC-ε down-regulates in response to prolonged TRH treatment in GH3 cells (33–35). To determine whether bombesin has any effect on the stability of the PKC isotypes, δ, ε, and ζ, cells were treated for up to 30 h with increasing doses of the neuropeptide. Fig. 4 shows that mitogenic concentrations of bombesin (12.5–50 ng/ml) lead to partial down-regulation of PKC-δ and PKC-ε (Fig. 4, lanes 5–7), resulting in a 79 and 65% decrease in the PKC-δ and PKC-ε levels, respectively, at 30 h post-stimulation (Table I). PKC-α levels, however, appear to be induced upon bombesin treatment increasing 60% above control levels at this time (Fig. 4 and Table I). The down-regulation of PKC-δ and PKC-ε, and induction of PKC-α, are time dependent as shown in Fig. 5B, where cells were treated with bombesin for up to 44 h. Under no conditions did PKC-ζ levels change. By comparison, a non-mitogenic dose of bombesin had no reproducible effect on the expression levels of the PKC proteins throughout the time course tested (Fig. 5A).

If activation is reflected by the stable association of PKC isotypes with the membrane fraction, the results would suggest that only the two Ca²⁺-independent isotypes (δ and ε) are activated by acute bombesin treatment. However, it was important to determine whether bombesin could also induce membrane association of these different enzymes at later times during progression through the cell cycle. Cell extracts were fractionated after prolonged treatment with bombesin and the PKC content in each fraction determined by Western blotting after electrophoresis on 10% SDS-PAGE. Fig. 6 shows that even at later times, between 7 and 36 h post-stimulation, the major proportion of PKC-δ and PKC-ε is associated with the detergent-soluble membrane fraction. Also, as with the acute treatments, the distribution of PKC-α and PKC-ζ does not appear to change. The decrease in the PKC-ζ levels seen at 36 h is not reproducible. In each case there is a net change in the amount of PKC-δ, -ε, and -α at the later times, consistent with the data in Figs. 4 and 5 and Table I. The results imply that it is the membrane-associated populations of PKC-δ and -ε which are down-regulated.

**Effect of PDGF on PKC Isotypes**—To determine whether PDGF, a major serum growth factor for these cells, would elicit
similar effects on the PKC isotypes, cells were treated for increasing times with mitogenic concentrations of PDGF and the PKC content at each time was evaluated by Western blotting. Fig. 7 shows that PDGF also induces a size shift of PKC-δ and PKC-ε at early times, 15 min and 1 h. Both of these PKC isotypes are also partially down-regulated at later times, where the protein levels decreased by approximately 60% after 19 h for both PKC-δ and PKC-ε (Table I). PDGF, however, does not induce PKC-α expression. The slight increase at 24 and 36 h after treatment was not reproducible. PKC-ζ sometimes appears as a doublet on 8% SDS-PAGE but no change is ever seen in stimulated compared to control cells. These effects of PDGF, like those of bombesin, are dose-dependent and correlate with mitogenicity (data not shown). PDGF also induces membrane association of PKC-δ and PKC-ε, but not of PKC-α and PKC-ζ under the extraction and fractionation conditions described for bombesin (data not shown).

Taken together, the results show that bombesin and PDGF can elicit both acute and prolonged effects on PKC-δ and -ε; bombesin also induces PKC-α expression. Neither mitogen had reproducible effects upon PKC-ζ.

Effect of Exogenously Added Diacylglycerols on PKC Isoforms—It has been reported that bombesin and PDGF induce biphasic production of DAG in Swiss 3T3 cells (9, 10, 13, 14). The physiological activation of PKC is thought to occur through the binding of DAG, and prolonged exposure to the second messenger could cause down-regulation of PKC-δ and PKC-ε in mitogen-treated cells. To test this possibility, cells were treated either with a single dose or repeatedly with OAG or DiC8 (by adding a fresh amount every hour for up to 11 h) either in the absence or presence of insulin (which is comitogenic with DAG in these cells; Ref. 36). The PKC content after each treatment was analyzed by Western blot analysis. As shown in Fig. 8, both PKC-δ and PKC-ε are down-regulated in response to continuous OAG treatment. Compared to control cells, PKC-ζ and PKC-α levels are unaffected by continuous OAG treatment. However, a single dose of OAG did cause a size shift (still detectable at 12 h) with both PKC-δ and PKC-ε (Fig. 8). DiC8, another DAG, did not cause this prolonged shift, but did cause down-regulation of these isotypes after continuous treatment (data not shown). The addition of insulin had no significant effect on the PKC levels. These results, therefore, suggest that continuous exposure of cells to exogenously added DAG can induce activation and down-regulation of at least PKC-δ and PKC-ε and support the notion that agonist-induced sustained DAG production activates and induces down-regulation of these PKC isotypes in vivo.

Induction of DNA Synthesis—In order to assess the relationship between the stimulation of DNA synthesis and the effects induced by bombesin and OAG on the different PKC isotypes, the DNA content of cells was analyzed by flow cytometry. First, cells were treated with increasing bombesin concentrations for 26 h. Fig. 9A (panels 4 and 5) shows that doses between 25 and 100 ng/ml induce the maximal number of cells to enter S phase (for bombesin this is only approximately 15%). These are similar to the doses at which the shift in mobility and down-regulation of PKC-δ and PKC-ε occur (Figs. 1 and 4).

In order to determine whether a single dose or continuous treatment of OAG is sufficient to induce DNA synthesis, cells were treated with diglyceride in the absence or presence of insulin. The results show that only the cells treated continuously with OAG enter S phase (Fig. 9B, panels 9 and 10). Insulin potentiates this effect (Fig. 9B, panel 10). A single dose of OAG either in the absence or presence of insulin is not sufficient to stimulate a significant number of cells to initiate DNA synthesis (Fig. 9B, panels 7 and 8). Again, as with bombesin, the activation and down-regulation of PKC-δ and PKC-ε induced by OAG correlate with the ability of the cells to enter S phase (Fig. 8 and Fig. 9B).

**DISCUSSION**

The data presented here provide evidence that chronic treatment of cells with specific agonists leads to a late phase translocation of particular PKC isotypes (Fig. 6). Associated with this late phase, prolonged activation, is a loss of steady state expression of these PKC isotypes that emulates a typical down-regulation response (Figs. 4–6). The ability of sustained DAG to induce this response is demonstrated directly through the use of membrane permeant DAG species. In the context of this cell proliferation model system, the mitogens bombesin and PDGF elicit the acute and prolonged changes in PKC-δ and PKC-ε in a way that parallels their potency in stimulating cell division. The implication is that activation of these particular PKC iso-

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**Table I**

| Isotype | Expression % control |
|---------|----------------------|
|         | Bombesin | PDGF |
| PKC-δ   | 29 ± 10   | 38 ± 1 |
| PKC-α   | 161 ± 16  | 83 ± 20 |
| PKC-ε   | 34 ± 11   | 43 ± 13 |
| PKC-ζ   | 109 ± 14  | 91 ± 26 |

![Fig. 5. Bombesin time course.](image-url)
types is at least a biphasic process that forms part of the program of events induced by these mitogens leading to cell division.

Surprisingly, under these growth conditions, there is no evidence of an acute translocation of PKC-α even in the context of the very rapid translocation of PKC-δ/ε. This is in contrast to phorbol ester treatment which under identical extraction conditions induces membrane association of PKC-α (Ref. 6, and data not shown). Whether this reflects some alteration in Ca\(^{2+}\) homeostasis is not clear, although in view of the specific Ca\(^{2+}\) dependence of PKC-α this may prove to be the case. Elevation of Ca\(^{2+}\) during extraction induces membrane association of PKC-α (6, 33). During the revision of the manuscript, Ha and Exton (37) reported that PDGF failed to induce membrane association of PKC-α in IIC9 cells, whereas PKC-ε did translocate to the membrane. By contrast the translocation of PKC-α was observed under conditions where Ca\(^{2+}\) was substantially elevated (37, 38).

Coincident with acute translocation, mitogenic concentra-

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**Fig. 6. Long-term bombesin-induced PKC translocation.** Cells were treated for the indicated times with 25 ng/ml bombesin and fractionated as described under "Materials and Methods." The cytosols were normalized for protein and an equal volume for each fraction was analyzed by 10% SDS-PAGE and Western blotting as described in the legend to Fig. 2. This is one of two similar experiments.

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**Fig. 7. PDGF time course.** Cells were either untreated (zero) or treated for the indicated times with 50 ng/ml PDGF. 70 μg of total cell lysate was analyzed by 8% (panel A) or 7.5% low bisacrylamide (panel B) SDS-PAGE and immunoblot analysis as described in the legend to Fig. 1. This represents one of four similar experiments.

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**Fig. 8. Diacylglycerol induced down-regulation.** Cells were either untreated (zero), treated once with 100 μg/ml OAG (acute), or fresh OAG was added every hour (continuous) for 11 h either in the absence (−) or presence (+) of 5 × 10\(^{-9}\) M insulin. The cells were harvested after 12 h and subjected to 10% SDS-PAGE and immunoblot analyses as described in the legend to Fig. 1. Duplicate samples were loaded in the case of the treated cells. This represents one of two similar experiments.

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9 A. R. Olivier, M. McKinnon, and P. J. Parker, unpublished results.
Mitogen-induced PKC Down-regulation

Fig. 9. Induction of DNA synthesis by bombesin and OAG. A, cells were either untreated (panel 1) or treated with 0.25 (panel 2), 2.5 (panel 3), 25 (panel 4), or 100 (panel 5) ng/ml bombesin. B, cells were either untreated (panel 6) or treated once with 100 μg/ml OAG (panels 7 and 8) or continuously with OAG (panels 9 and 10) in the absence (panels 7 and 9) or presence (lanes 8 and 10) of 5 × 10⁻⁶ M insulin.

down-regulation of PKC-δ and PKC-ε, while PKC-α and PKC-ζ are unaffected. Therefore, a good correlation exists between the presumed activation (as judged by translocation) and down-regulation of these PKC isotypes and the ability of the cells to enter S phase.

The results support the notion that distinct phases of DAG (9, 10) produced in response to Swiss 3T3 cell mitogens lead to the selective activation of PKC-δ and PKC-ε. In the prolonged phase of activation during late G₁ (and beyond) there is a steady decline in the level of PKC-δ and PKC-ε expression. Whether the activation of these PKC isotypes or their induced depletion contribute to cell cycle progression, remains to be established.

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