Diversity of roles of protein kinase Cα in the proliferation of Swiss 3T3 cells

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We examined the role of protein kinase Cα (PKCα) in the stimulation of DNA synthesis of Swiss 3T3 cells induced by bombesin, platelet-derived growth factor (PDGF) and phorbol 12-myristate 13-acetate (PMA). We found that cells in which this kinase had been down-regulated showed a partially abrogated mitogenic response to bombesin. The response to PDGF was unaltered; however, the response to PMA was completely suppressed. The mitogenic effect of maximal doses of bombesin and PMA combined was greater than that of either agent alone, suggesting that bombesin does not fully activate the PKC pathway. Accordingly, bombesin-induced PKCα translocation from cytosol to membranes was partial, while that observed with PMA was essentially complete. Moreover, exposure to Ro-31-8220, a PKC inhibitor, had signficantly greater effects on the response to PMA than on that to bombesin. Our findings point out different roles that PKCα may play in diversely activated cells: while, in the case of PMA, stimulation of this kinase may be necessary and sufficient to induce proliferation, it appears to be necessary only for a full response to bombesin, and redundant among the mechanisms triggered by PDGF.

INTRODUCTION

Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), have mitogenic effects on a number of cell types. These agents are thought to cause this effect through the stimulation of various protein kinase C (PKC) isoforms [1,2]. The discovery that many growth factors induce phosphoinositide (PI) breakdown producing diacylglycerol (DG), a physiological activator of PKC, led to the suggestion that this kinase is directly involved in the mitogenic response to such growth factors [3,4]. One example of a mitogen that enhances PI turnover is platelet-derived growth factor (PDGF). This agent stimulates the tyrosine kinase activity of its receptor which, in turn, phosphorylates PI-phospholipase C-γ (PI-PLC-γ) and activates PI breakdown to produce DG and inositol 1,4,5-trisphosphate [5,6]. The resulting stimulation of PKC activity is hypothesized to participate in the proliferative response to PDGF [6]. However, this view has recently been challenged. For instance, it has been found that PI breakdown can be abolished by a transient exposure to genistein, a tyrosine kinase inhibitor, without affecting the mitogenic response to PDGF in CH310T1/2 mouse fibroblasts [7]. Likewise, overexpression of PI-PLC-γ does not enhance the effects of the growth factor in NIH 3T3 cells, which led to the suggestion that PI-PLC-γ-mediated breakdown of PIs is not relevant to the mitogenic response [8]. By extension, these findings raise doubts about the role that PKC plays in this type of cellular response.

Bombesin represents another type of growth factor. In contrast with the PDGF receptor, the bombesin receptor does not possess tyrosine kinase activity [6,9]. Interaction of bombesin with its receptor leads to PI breakdown [10] and this action is due to the activation of the β isoform of PI-PLC, involving a guanine-nucleotide-binding protein of the G_q family [11–14]. Bombesin also stimulates phosphatidylcholine breakdown through the activation of phospholipase D [15] and phospholipase A_2 [16]. The latter releases free arachidonate, a fatty acid with mitogenic activity [17]. In addition, bombesin stimulates Ca^{2+} influx, and the hormone-elicited enhancement of DNA synthesis depends on the extracellular Ca^{2+} concentration [18]. Bombesin also causes a small increase in cellular cyclic AMP content [19] and such an increase may constitute another mitogenic signal [20].

Over a decade ago, it was proposed that bombesin stimulated mitogenesis through the activation of the PKC pathway [21]. In support of this view, it was reported that down-regulation of this kinase by exposure to phorbol dibutyrate almost completely abolished the response to bombesin [22]. This report is in conflict with the observation that PKC-independent mitogenic mechanisms are activated by this growth factor. In this respect, especially important is the recent discovery of a novel pathway involving tyrosine phosphorylation of specific proteins, unrelated to those phosphorylated by tyrosine kinase growth factor receptors [23–25].

We undertook this study to examine these issues in Swiss 3T3 cells. We used a protocol for phorbol ester-induced PKC down-regulation different from those previously employed in this kind of study. Using this protocol, we examined the mitogenic responses to bombesin, PDGF and PMA. We also compared the effects of a new PKC inhibitor, Ro-31-8220, on the responses to PMA and bombesin. Our results are compatible with the notion that PKCα activation is needed for a full mitogenic response to bombesin, but that additional PKC-independent mechanisms are activated by this growth factor. In contrast, PKCα activation is not necessary in order for PDGF to elicit a full mitogenic response.

EXPERIMENTAL

Cells and cultures

Swiss 3T3 fibroblasts (A.T.C.C., Rockville, MD, U.S.A.) were cultured in Dulbecco's modified essential medium (DMEM, GIBCO BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum and 2 mM glutamine (both from Sigma Chemical Co., St. Louis, MO, U.S.A.) at 37 °C in a 5% CO_2 humidified atmosphere. Stock cultures were not allowed to reach confluency.

Abbreviations used: DMEM, Dulbecco's modified essential medium; PKC, protein kinase C; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; PI, phosphoinositide; DG, diacylglycerol; PLC, phospholipase C; [^{3}H]TdR, [*methyl*-^{3}H]thymidine.

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Drugs

Bombesin and PMA were from Sigma. PDGF was from GIBCO. PMA was prepared in a 2 mM stock solution in DMSO (Sigma). Compound Ro-31-8220 was donated by Dr. Geoffrey Lawton, Roche Products Limited (London, U.K.). Stock solutions were freshly prepared in DMSO prior to use. The final DMSO concentration was below 0.5% and was kept constant in all samples of each experiment.

Measurement of proliferation rates

Confluent cultures plated in 24-well plates were transferred to DMEM containing 2 mM glutamine without fetal calf serum (serum-free medium) for 24 h prior to the experiments. Exposure to the different agents was then carried out for 18 to 20 h after which time 1 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (40–60 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.) was added to each well and the cells were further incubated for 1 h. The pulses were terminated by trichloroacetic acid precipitation, which was carried out directly on the dishes, and the precipitates were dissolved in 0.5 M NaOH and radioactivity was counted by liquid scintillation counting after neutralizing the samples with concentrated HCl. All assays were carried out in triplicate and the values obtained for each condition routinely differed by less than 10%.

Assessment of PKCα down-regulation and translocation by Western blotting

To verify PKCα down-regulation, cultures of Swiss 3T3 fibroblasts, grown to confluency in 60 mm × 15 mm Petri dishes, were transferred to serum-free medium and exposed to 1 μM PMA, 30 nM bombesin or vehicle for 12 to 72 h, after which time PKCα was detected in cell homogenates as described by Ganesan et al. [26]. Briefly, the cells were washed three times with PBS and 0.2 ml of cold homogenization buffer, consisting of 20 mM Tris/HCl, 0.5 mM EGTA, 1 mM PMSF, 0.1% 2-mercaptoethanol, 50 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μM pepstatin, 0.5 unit/ml α_2-macroglobulin and 0.25 mM phosphoramidon, was added to each plate. The cell lysates were scraped off the plates with a rubber policeman and transferred to Eppendorf vials. The samples were immediately placed on ice and homogenized with the aid of a Branson sonifier (20 W, 3 × 1 min), after which they were concentrated by vortex evaporation and dissolved in Laemmli's sample buffer. This was followed by SDS/PAGE using 10% gels and the separated proteins were electroblotted on to Millipore Immobilon-P membranes. The blots were probed with anti-PKCα antibodies raised in rabbits using specific peptide sequences coupled to keyhole limpet haemocyanin and purified by immunoaffinity using the same immobilized peptides as affinity medium. The reactive bands were visualized by incubation with ^{125}I-Protein A and autoradiography [26].

A similar procedure was carried out in the studies of translocation of PKCα from the cytosol to the particulate fraction. In this case, after sonication, the samples were centrifuged in a Beckman Airfuge [172.5 kPa (25 p.s.i.) for 10 min], to separate the particulate fraction containing the membranes and insoluble components (pellet) from the cytosolic fraction (supernatant). The pellets were resuspended in 200 μl of homogenization buffer and all fractions were concentrated by vortex evaporation and processed for immunoblotting as described above.

Densitometric quantification of PKC bands was carried out on scans of the autoradiograms, obtained with a Microtek ScanMaker IISP, using the NIH Image program devised by Wayne Rasband (National Institutes of Health, U.S.A.), version 1.55, in a Performa 475 Macintosh computer.

Statistical analysis

Statistical analysis was performed using Student's *t* test for comparison of paired data and the Student–Newman–Keuls test for comparison of multiple means.

RESULTS AND DISCUSSION

Down-regulation of PKCα upon prolonged exposure to phorbol esters has been observed in a number of cell types [2]. Our results confirm this observation. After 48 h of exposure to 1 μM PMA, a striking decrease in the levels of this protein could be observed by immunoblotting with specific antibodies directed against PKCα, reported to be the only Ca^{2+}-dependent isoform expressed in these cells [27] (Figure 1). We used this experimental condition to test the mitogenic response to stimulation with 30 nM bombesin, 10 ng/ml PDGF or an additional 1 μM PMA. We observed that PKCα-depleted cells still responded to bombesin (Table 1).

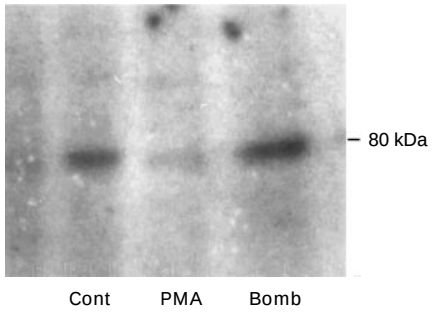

Figure 1 Down-regulation of PKC as shown by immunoblotting

Confluent cultures of Swiss 3T3 cells were exposed to vehicle (Cont), 1 μM PMA (PMA) or 30 nM bombesin (Bomb) in serum-free medium for 48 h. After these periods, the cells were homogenized and the presence of PKCα was detected by immunoblotting using specific antibodies, incubation with ^{125}I-Protein A and autoradiography, as described in the Experimental section. Densitometry of the PKC bands yielded the following values: Cont, 100%; PMA, 17.8%; Bomb, 128.9%.

Table 1 Effect of PKC down-regulation on the mitogenic responses to bombesin and PDGF

Cultures of Swiss 3T3 cells were first incubated with vehicle (DMSO) or, to induce down-regulation of PKC, with 1 μM PMA for 48 h in serum-free medium. Then, vehicle, 30 nM bombesin, 10 ng/ml PDGF or 1 μM PMA were added and, after 18 h, mitogenicity was assayed by a 1 h pulse of [^{3}H]TdR, as described in the Experimental section. The data represent the radioactivity incorporated into trichloroacetic acid-precipitable material and correspond to the means ± S.E.M. of three independent determinations. Column *P* shows the statistical significances of the differences between the responses of PKC-down-regulated and non-down-regulated cells to each agent. N.S., not significant. The statistical significance of the differences between the corresponding control cultures (vehicle) and cultures treated with each agent are indicated by the superindices [a] ($P < 0.001$) and [b] (not significant).

Treatment	Non-down-regulated PKC (c.p.m./well)	Down-regulated PKC (c.p.m./well)	*P*
Vehicle	6707 ± 772	10261 ± 1231	< 0.02
Bombesin (30 nM)	163880 ± 2976[a]	90448 ± 2611[a]	< 0.001
PDGF (10 ng/ml)	320020 ± 14055[a]	309740 ± 23283[a]	N.S.
PMA (1 μM)	225660 ± 14769[a]	11737 ± 3192[b]	< 0.001

Table 2 Partially additive mitogenic effects of bombesin and PMA

Cultures incubated for 24 h in serum-free medium were exposed to vehicle (DMSO) or 1 μM PMA, either in the absence [bombesin (0 nM), control] or in the presence of 30 nM or 100 nM bombesin. Mitogenicity was assayed 18 h later as in Table 1. The data show radioactivity incorporated into trichloroacetic acid-precipitable material and represent the means ± S.E.M. of three independent determinations. Similar results were obtained in two additional experiments. Column *P* shows the statistical significances of the differences between the responses to each dose of bombesin in vehicle- versus PMA-treated cells. The statistical significance of the differences between the corresponding control cultures (Bombesin 0 nM) and cultures treated with 30 or 100 nM bombesin are indicated by the superindices [a] ($P < 0.001$), [c] ($P < 0.05$) and [d] ($P < 0.1$).

	Radioactivity (c.p.m./well)		
	+ Vehicle	+ 1 μM PMA	*P*
Bombesin (0 nM)	37209 ± 4204	209302 ± 19674	< 0.001
Bombesin (30 nM)	139535 ± 14372[a]	260465 ± 25265[d]	< 0.005
Bombesin (100 nM)	144186 ± 14274[a]	293023 ± 30494[c]	< 0.005

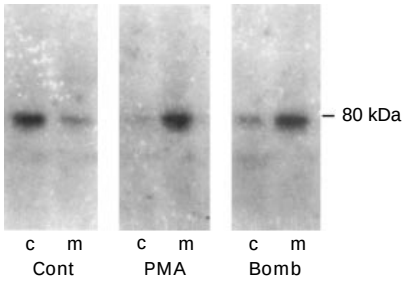

Figure 2 Translocation of PKC by exposure to PMA and bombesin

Cultures in serum-free medium were exposed for 10 min to vehicle (Cont), 1 μM PMA (PMA) or 30 nM bombesin (Bomb) at 37 °C, after which they were homogenized. The particulate material (m), containing cell membranes, was separated from the cytosol (c) by centrifugation and PKCα was detected in each fraction as in Figure 1. Densitometric quantification of the PKC distribution between cytosol and membrane resulted in the following values. Cont: c, 81.1%/m, 18.9%; PMA: c, 5.5%/m, 94.5%; Bomb: c, 26.2%/m, 73.8%.

Although the [^{3}H]TdR incorporation was smaller in PMA-treated than in vehicle-treated cultures upon exposure to bombesin, this growth factor induced an almost 9-fold stimulation of mitogenesis in PKCα-depleted cells, as compared with the corresponding controls (PKC-down-regulated cells exposed to vehicle instead of bombesin). Our results contrast with previously reported ones, in which the proliferative response was almost completely abrogated in cells with down-regulated PKC [18,28]. However, our experiments follow a different protocol. First, we used PMA as the phorbol ester, instead of the more polar and easily washed out phorbol dibutyrate used in other studies. Secondly, we did not wash the cultures prior to the addition of bombesin. In the previous studies, extensive washing preceded exposure to the mitogen; thus, recovery of PKC during the period of exposure could potentially occur and becomes a complicating factor of unknown consequences. In our studies, the phorbol ester was present during the whole period examined and thus the kinase is more likely to remain down-regulated throughout the entire experiment.

PKCα down-regulation, according to our protocol, had no significant effects on mitogenesis stimulated by 10 ng/ml PDGF (Table 1), agreeing with a previous report [29]. Parallel cultures showed an almost completely suppressed response to 1 μM PMA (Table 1).

If Swiss 3T3 cells were exposed to bombesin and PMA at maximal doses, the mitogenic response observed was higher than that obtained with either agent alone (Table 2). These partially additive effects on the stimulation of DNA synthesis suggest that bombesin and PMA act, in part, by different mechanisms. This notion was further supported by experiments in which the PKC inhibitor Ro-31-8220 was used. When the cells were exposed to 1 μM PMA or 30 nM bombesin in the presence of Ro-31-8220 the inhibitory effect was much more potent on PMA-induced than on bombesin-induced mitogenesis ($P < 0.001$): the relative mitogenic responses in the presence of Ro-31-8220 were 8.16 ± 3.2% for PMA and 25.9 ± 0.52% for bombesin (mean ± S.E.M., $n = 3$), with respect to control cells exposed to PMA or bombesin in the absence of inhibitor. A possible explanation for these results is the following. In the case of bombesin, the inhibitor blocks the pathway involving PKC, but those pathways that operate independently from this kinase remain unaffected, yielding only a partial inhibition. In the case of PMA, on the other hand, the strong inhibition of mitogenesis is consistent with the notion that only a PKC-dependent mechanism is activated by the phorbol ester.

Bombesin and PMA at maximal mitogenic doses induce PKCα translocation to different extents. We have compared the activation of PKCα in bombesin- and PMA-treated cells by immunoblotting of cytosol and sedimentable cell fractions with antibodies specifically raised against this isotype. We found that 30 nM bombesin caused partial translocation of PKCα to a sedimentable fraction, whereas, with PMA, translocation was essentially complete (Figure 2). These findings suggest that PKCα is maximally stimulated by 1 μM PMA but only partially stimulated by 30 nM bombesin, a dose of bombesin that causes a maximal mitogenic response. Olivier and Parker [30] recently reported that bombesin does not enhance translocation of PKCα. However, the homogenization buffer used by these authors has a relatively high concentration of Ca^{2+} chelators, which could result in the loss of membrane-associated PKC during the preparation of the cell fractions and account for the difference from our results.

We found an additional important difference between the effects of PMA and bombesin on cellular PKCα. While prolonged exposure to PMA elicited down-regulation of the kinase, no such effect was observed upon a similarly extended exposure to bombesin (Figure 1). Since PKC-stimulated mitogenesis requires at least 18 h to become apparent, it could be argued that the late down-regulation of the enzyme, rather than the early stimulatory effects of PMA, is responsible for cell proliferation. In other words, PKC activity could be a suppressor of cell replication. In this case, the phorbol ester mechanism of action could involve the elimination of suppressive effects through the down-regulation of the kinase. A similar mechanism could be involved in growth factor action since many of these agents also induce sustained stimulation of PKC. Our results indicate that this is not the case for bombesin, although it is possible that the effects of other growth factors could be mediated through such a mechanism [30]. Indeed, Isales and co-workers have recently presented evidence that suggests a negative regulatory role for PKC in mitogenesis [31].

In conclusion, the results presented here indicate that bombesin-induced mitogenesis requires PKCα activation for a maximum response but that mechanisms independent of PKC may also be involved in the response to this growth factor. This is in agreement with mounting evidence from other laboratories [19,23–25,32]. In the case of PDGF, mitogenesis does not require

PKCα activation at all for full expression, despite the fact that activation of the PKC pathway is a normal event induced by this growth factor [20]. The data indicate that PKCα activation by PDGF is redundant with respect to the enhancement of DNA synthesis; what other role it may be playing remains to be elucidated. In the case of stimulation by PMA, however, PKCα activation is sufficient and also necessary, since PKC-down-regulated cells fail to respond to this agent. Thus, in different situations, PKCα appears to play necessary, sufficient or redundant roles depending on the agonist that activates the kinase. Numerous signalling pathways operate simultaneously in activated cells [33–35]. Our results emphasize that the role of this kinase cannot be considered isolated from the complex set of cellular signals concomitantly activated in each condition.

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