The co-mitogenic combination of transforming growth factor β_1 and bombesin protects protein kinase C- δ from late-phase down-regulation, despite synergy in diacylglycerol accumulation

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Bombesin induces the down-regulation of protein kinase C- δ (PKC- δ) and PKC- ϵ in Swiss 3T3 cells. Simultaneous addition of transforming growth factor β_1 (TGF β_1) selectively blocks PKC- δ down-regulation at mid-S-phase, whereas PKC- ϵ levels continue to decline. Northern blot analysis shows that PKC- ϵ levels could be controlled in part at the level of transcription; PKC- δ mRNA levels remained constant at these later times. Bombesin induces a sustained elevation of some species of diacylglycerol (DAG), consistent with the observed loss of PKC- δ and PKC- ϵ . Interestingly, the combination of bombesin and TGF- β_1 produces

an even greater DAG response. Flow cytometric analysis demonstrates that bombesin induces only 15% of the cells to enter the cell cycle, in contrast to the combination of TGF β_1 plus bombesin which induces 75–80% of the cells to progress through the cycle. The protection of PKC- δ from down-regulation under conditions of sustained DAG elevation correlates with the mitogenic response and implies that the down-regulation process itself is regulated. Consistent with this, it is demonstrated that bombesin plus TGF β_1 protects PKC- δ from phorbol ester-induced down-regulation.

INTRODUCTION

Protein kinase C (PKC) represents a multigene family which comprises at least 12 isotypes and can be divided into four classes, namely the conventional PKCs (α , β_1 , β_2 and γ), the novel PKCs (δ , ϵ , η and θ), the atypical PKCs (ζ , ι and λ), and mPKC μ /PKD. Each of these groups is activated differentially by cofactors and in response to stimuli [1]. These kinases have been implicated in a number of cellular responses [1,2], including the control of cell division.

The mammalian cell cycle has been well defined and studied in fibroblasts (see [3]). In particular, Swiss 3T3 cells have been used as a model system to study the early events during the G1 stage of the cell cycle [4]. Four major PKC isotypes in fibroblasts have been identified as PKC- α , - δ , - ϵ and - ζ , and it has been shown that they are differentially regulated by various agonists [5–9].

Stimulation of Swiss 3T3 cells with bombesin induces a number of early events in the G1-phase of the cell cycle, resulting in the initiation of DNA synthesis and cell division [10]. With respect to PKC, prolonged (> 10 h) exposure to bombesin induces selective membrane association and down-regulation of PKC- δ and PKC- ϵ in Swiss 3T3 cells [9]. These effects are mimicked by exogenously added diacylglycerols (DAGs) such as OAG and DiC8, supporting previous studies indicating that DAG is one of the major activators of the PKC family of enzymes (see [11]).

It has been reported that bombesin can induce acute biphasic production of DAG in Swiss 3T3 cells [12–14]. In addition to the possible *de novo* synthesis of DAG, the activation of phospholipases that hydrolyse either phosphatidylinositol 4,5-bisphosphate or phosphatidylcholine is implicated in the generation of this second messenger. However, the pattern of

endogenous DAG production in relation to PKC expression is not established. In particular, conditions that modify bombesin-induced DAG production and mitogenicity have not been analysed with respect to PKC, and it remains to be established whether sustained DAG accumulation necessitates PKC loss.

Here we have employed the combination of bombesin and transforming growth factor β_1 (TGF β_1) as potent co-mitogens in Swiss 3T3 cells to demonstrate that the loss of specific PKC isotypes does not parallel DAG accumulation. This is shown not to be a function of compartmentalization but to reflect a regulation of the process of PKC down-regulation itself. This process appears to be under cell cycle control.

MATERIALS AND METHODS

Materials

Bombesin (porcine) was from Calbiochem Corp. TGF β_1 (porcine) was from British Biotechnologies. Antisera to PKC isotypes α , δ , ϵ and ζ were as described previously [15–18]. Further antisera to PKC- δ were provided by Dr. S. Jaken (W. Alton Jones Cell Science Center, Lake Placid, NY, U.S.A.). The horseradish peroxidase-conjugated donkey anti-(rabbit IgG)s and the enhanced chemiluminescence detection reagents were from Amersham. Growth factors were diluted and stored according to the manufacturer's instructions.

Cell culture and extraction

Swiss mouse 3T3 cells were grown in culture as previously described [7,9]. Cells were judged quiescent after 8 days and were

Abbreviations used: DAG, diacylglycerol; PKC, protein kinase C; TGF, transforming growth factor; PMA, phorbol 12-myristate 13-acetate.

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stimulated with growth factors for different times as indicated in the text or Figure legends. The cells were extracted in 2 × Laemmli sample buffer [19] as previously described [9], and these total lysates were analysed by Western blotting. Where indicated, cell fractionation was carried out as previously described [9].

Immunoblot analysis

Samples containing 50 µg of protein were subjected to SDS/PAGE [19]. The proteins were transferred to Immobilon (Millipore) and probed with PKC isotype specific antisera as previously described [9,17]. For quantification, duplicate lanes in each experiment were scanned with an LKB Ultrascan and the amount of PKC in the treated cells was calculated as a percentage of that in untreated quiescent cells.

Flow cytometric analysis

Flow cytometry was carried out as described previously [9,20].

Northern blot analysis

Two 15 cm plates of cells were employed for each time point following stimulation with bombesin, TGFβ₁ or a combination of the two. Total RNA was isolated according to Chomczynski and Sacchi [21]. Total RNA (30 µg) was loaded on to a denaturing formaldehyde gel and transferred to Hybond N⁺ (Amersham) according to the manufacturer's instructions. cDNAs encoding mouse PKC-ε [22], rat PKC-δ [17] and bovine PKC-α [23] and mouse 18 S RNA were labelled by random priming and used to probe the membranes employing standard conditions [24]. Briefly, the blots were hybridized in 5 × Denhardt's, 0.5 % SDS, 50 % formamide, 2 × SSC at 42 °C for 16 h. The membranes were then washed once in 2 × SSC/0.1 % SDS at 42 °C for 15 min and then twice in 0.1 × SSC/0.1 % SDS at 65 °C for 15 min each. To determine the loading, the radioactivity associated with the 18 S RNA was quantified with an Ambis Scan. The amount of PKC mRNA was quantified by scanning densitometry of autoradiographs (LKB Ultrascan). The mRNA sizes detected for PKC-δ and PKC-ε were 3.4 kb and 7.1 kb respectively. Two mRNA species were observed for PKC-α (3.5 kb and 8.1 kb) (see Figure 3).

Diradylglycerol species analysis

Fibroblasts (two 15 cm plates per sample; 3.5 × 10⁷ cells) were treated as above and incubations arrested by aspiration of the medium followed by addition of 2 ml of ice-cold methanol to each plate. The cells were scraped from the dishes and transferred into a glass tube. A further 1 ml of methanol was used to wash each plate and this was added to the tube. The lipids were extracted and derivatized (with 1 µg of 1,2-12:0/12:0-DAG as internal standard) as previously described [14,25]. Briefly, the lipid extract was resuspended in dry pyridine (500 µl) containing 25 mg of 3,5-dinitrobenzoyl chloride and heated at 60 °C for 15 min. The reaction was stopped by addition of 2 ml of methanol/water (4:1, v/v) followed by 2 ml of water. This was then passed on to a C₁₈ Sep-Pak mini-column (Waters–Millipore), washed with 2 × 10 ml of methanol/water (4:1, v/v) and the dinitrobenzoylated diradylglycerols eluted with 10 ml of redistilled diethyl ether. After drying under nitrogen and resuspension in 20 µl of cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.), the diradyl classes (1,2-DAG, 1,3-DAG, 1-

alkyl-2-acylglycerol and 1-alkenyl-2-acylglycerol) were separated on a Kromasil column (5 µm; 2.1 mm × 250 mm; Hichrom Ltd., Reading, U.K.) using a linear solvent gradient of 90 % hexane/cyclohexane/diethyl ether/ethanol (49:49:2:0.1, by vol.) plus 10 % cyclohexane/diethyl ether/ethanol (85:15:0.1, by vol.), changing to 100 % of the second solvent in 30 min at 0.5 ml/min with detection at 250 nm. Each dinitrobenzoylated diradylglycerol class was collected, dried under nitrogen, resuspended in 20 µl of acetonitrile/propan-2-ol (1:1, v/v) and separated into molecular species on a Spherisorb S50DS2 column (5 µm; 4.6 mm × 250 mm) using a linear gradient of 100 % acetonitrile/propan-2-ol (9:1, v/v) changing to 100 % acetonitrile/propan-2-ol (1:1, v/v) in 45 min at 1 ml/min with UV detection at 250 nm. Identification was by reference to authentic standards and previously obtained relative retention time data [25].

RESULTS

TGFβ₁ modifies bombesin-induced changes in PKC-δ

Because of the documented effect of TGFβ₁ on late-phase DAG, this co-mitogen was chosen in order to study the relationship between DAG accumulation (see below) and PKC expression [26]. Initial experiments revealed that TGFβ₁ modified bombesin-induced down-regulation of PKC-δ in a time-dependent fashion (Figure 1, left panels). In response to the combination of bombesin + TGFβ₁ there was no net loss of PKC-δ after 30 h, contrary to the effect of bombesin alone. Interestingly, at 18 h the initial down-regulation of PKC-δ induced by bombesin was not affected by TGFβ₁. Contrary to these effects on PKC-δ, the down-regulation of PKC-ε was indistinguishable in cells treated with bombesin or bombesin + TGFβ₁ (Figure 1). The levels of PKC-α were reduced in cells co-treated with TGFβ₁ (see below). Expression of PKC-ζ was not grossly affected by the presence of TGFβ₁.

The apparent biphasic behaviour of PKC-δ in cells treated with bombesin + TGFβ₁ led to the examination of a more detailed time course of PKC expression following treatment of Swiss 3T3 cells with bombesin or bombesin + TGFβ₁ (Figure 1, right panels). Consistent with the PKC-δ blots described above, PKC-δ was shown to behave in a biphasic fashion when TGFβ₁ was added as a co-mitogen with bombesin. The initial phase of bombesin-induced down-regulation of PKC-δ was quite unaffected by TGFβ₁. However, by 24 h the level of PKC-δ in bombesin + TGFβ₁-treated cells was twice that in cells treated with bombesin alone, and by 39 h it was 149 % of the starting concentration compared with 12 % for bombesin alone. The point of bifurcation for PKC-δ expression under these two conditions coincides with the time at which the cells start to enter the G2–M-phase of the cell cycle (see below). TGFβ₁ alone had a modest effect on PKC-δ, causing a gradual increase to 220 % (range 180–260 %) of initial levels by 39 h (results not shown).

It was also observed that PKC-ζ expression remained essentially unaltered by either bombesin or bombesin + TGFβ₁ (Figure 1, right panels); this was also true for TGFβ₁ alone (results not shown). PKC-ε was down-regulated in response to bombesin, an effect that was modestly increased when TGFβ₁ was also present. TGFβ₁ alone had no significant effect upon PKC-ε protein expression (results not shown). The increased expression of PKC-α observed following bombesin treatment was significantly reduced by exposure to TGFβ₁. This pattern of PKC-α expression was similar to that seen for TGFβ₁ alone, with PKC-α levels being 230 % (range 204–255 %) of control levels at 39 h (compare with Figure 1, panel α).

Figure 1 PKC levels in response to bombesin and TGF β_1

Left-hand panels: Swiss 3T3 cells were untreated (0), or treated with 50 ng/ml bombesin (BOMB) or 50 ng/ml bombesin plus 5 ng/ml TGF β_1 (B + T) for the indicated times. The cells were harvested as described in the Materials and methods section. Samples of 70 μ g of total protein were analysed by SDS/PAGE and Western blotting. Numbers on the left indicate molecular mass (kDa). The same membrane was stripped and reprobed with the different isotype-specific antisera. This is one of five similar experiments. Right-hand panels: cells were treated with bombesin either alone ($\square$) or in combination with TGF β_1 ($\bullet$) for the indicated times. The cells were harvested and all lysates subjected to SDS/PAGE and Western blot analysis. The intensities of the PKC-containing bands (PKC- ζ , PKC- α , PKC- ϵ and PKC- δ) were quantified by densitometry as described in the Materials and methods section. This is one of two similar experiments.

Alterations in PKC expression do not correlate with changes in DAG

Use of TGF β_1 as a co-mitogen reflected its documented ability to suppress late-phase diacylglycerol accumulation [26]. However, contrary to this previous study, we found that TGF β_1 greatly enhanced the extent of bombesin-induced diacylglycerol accumulation. Analysis of this lipid found it to consist almost entirely of DAG, with very little alkylacylglycerol or alkenylacylglycerol being detected. No significant changes in the total mass or species profiles of these latter two classes were observed following stimulation with bombesin (in the presence or absence of TGF β_1) for 7, 15 or 30 h (results not shown). In contrast, the total DAG mass increased markedly following bombesin stimulation in the presence of TGF β_1 but not with bombesin alone (Table 1). DAG analysis revealed over 30 different molecular species, although many were relatively minor components (results not shown; but see previously [14]). Bombesin alone appeared to elicit some mass changes (relative to the 0 h time point) in specific DAG species, particularly in the 16:0/18:1, n -9+18:0/18:2, n -6, 16:0/16:0 and 16:0/18:0 fractions (which increased) and the 18:0/20:4, n -6 and 18:0/20:3, n -9 fractions (which decreased) (Figure 2a). However, these changes are largely artificial since any manipulation of cells, particularly the unavoidable temperature shock at the start of experiments caused by medium changes, will generate a rapid, transient elevation of polyunsaturated DAG species (T. R. Pettitt and M. J. O. Wakelam, unpublished work) that is similar but of smaller magnitude to that seen with acute agonist stimulation (e.g. [14]). As a result of this, the 0 h time point will

Table 1 Changes in total DAG mass in response to bombesin in the presence and absence of TGF β_1

Values (means $\pm$ S.D.; $n = 3$) were determined from the total peak area of the individual molecular species relative to a 1,2-12:0/12:0-DAG internal standard.

Incubation time (h)	DAG mass (pmol/ 10^7 cells)	
	Bombesin	Bombesin + TGF β_1
Control	1220 $\pm$ 91	1220 $\pm$ 91
7	1254 $\pm$ 155	2081 $\pm$ 173
15	1224 $\pm$ 10	1736 $\pm$ 100
30	1371 $\pm$ 104	2322 $\pm$ 52

be slightly enriched in these species at the expense of more saturated species; thus when the cells have returned to homeostasis they will show an apparent reduction in polyunsaturated species and an elevation in more saturated species. Comparison of the DAG profiles obtained following bombesin stimulation showed that, whereas inclusion of TGF β_1 increased the amounts of most molecular species (Figure 2a, lower panel), it caused a significantly greater percentage elevation in the proportions of several polyunsaturated species, in particular 16:0/20:4, n -6, 20:3/20:3, 18:0/20:5, n -3, 18:0/20:4, n -6 and 18:0/20:3, n -9, which were elevated at all time points (Figure 2b). TGF β_1 caused an approximate doubling of the relative contribution to the total DAG mass made by these five polyunsaturated species at each

Figure 2 Effects of bombesin in the presence and absence of TGFβ₁ on 1,2-DAG accumulation

(a) Changes in the mass of DAG molecular species (pmol/10⁷ cells) following stimulation with bombesin alone (upper panel) or bombesin + TGFβ₁ (lower panel) for 0, 7, 15 and 30 h. (b) Effect of TGFβ₁ on the relative proportions of DAG species in bombesin-stimulated Swiss 3T3 fibroblasts, expressed as a percentage of the total DAG mass for each condition. Over 30 species were detected, but the minor forms (each representing less than 1% of total DAG) have been omitted. Values are means ± S.D. (*n* = 3). * Polyunsaturated species whose relative contribution was significantly elevated (*P* < 0.05) at all three time points in bombesin-stimulated cells treated with TGFβ₁.

time point, from 13.8, 10.9 and 14.4% at 7, 15 and 30 h respectively with bombesin alone to 29.4, 21.2 and 22.1% with bombesin + TGFβ₁.

The ability of TGFβ₁ to cause further elevation of DAG over and above that induced by bombesin alone implies that under these conditions the presence of DAG must be insufficient to induce PKC-δ down-regulation. This contrasts with the exposure of quiescent cells to DAG alone [9]. Furthermore, in quiescent cells both PKC-δ and PKC-ε behave in a similar fashion.

Bombesin + TGFβ₁-induced changes in the level of PKC-ε, but not that of PKC-δ, correlate with changes in mRNA levels

The discrepant behaviour of PKC-ε and PKC-δ under conditions of elevated DAG is contrary to the previously documented behaviour of these isotypes on exposure of Swiss 3T3 cells to exogenous DAG or phorbol 12-myristate 13-acetate (PMA). In order to investigate the basis for this altered behaviour, mRNA levels were monitored for PKC-δ and PKC-ε as well as for PKC-α (PKC-ζ could not be detected even on prolonged exposure of

Northern blots). PKC-δ mRNA displayed a characteristic size of 3.4 kb (Figure 3, top panels). Quantification of the mRNA levels by normalization to the content of 18 S RNA (Figure 3, bottom panels) indicates that there was a transient increase in PKC-δ mRNA that had returned to basal levels by 24 h. A similar pattern was observed for the action of TGFβ₁ alone (results not shown). The sustained (bombesin alone) or transient (bombesin + TGFβ₁) loss of PKC-δ protein (see below) clearly does not correlate with any changes in mRNA levels.

In contrast to PKC-δ, the mRNA levels of PKC-ε showed a gradual decline in cells treated with bombesin + TGFβ₁ (Figure 3, bottom panels). TGFβ₁ treatment alone did not induce any loss of PKC-ε mRNA (results not shown); bombesin alone induced a modest accumulation of PKC-ε over a 38 h time course. This loss of PKC-ε mRNA in response to bombesin + TGFβ₁ could account in part for the continued loss of PKC-ε protein under conditions in which PKC-δ is apparently 'protected' from down-regulation despite the elevated DAG (see below).

PKC-α mRNA was induced in response to bombesin, in

Figure 3 PKC mRNA levels during the cell cycle

Cells were stimulated with bombesin alone or in combination with TGF β_1 for the indicated times. The total mRNA from two 15 cm dishes of cells was extracted and subjected to Northern blot analysis as described in the Materials and methods section. Top panels: the blot was probed sequentially with cDNAs to PKC- δ , PKC- ϵ and 18 S RNA. The mRNA sizes are 3.4 kb (δ) and 7.1 kb (ϵ). Middle panels: a duplicate blot was probed sequentially with a cDNA to PKC- α and 18 S RNA. The sizes of the two α mRNA species are 3.5 and 8.1 kb. Bottom panels: normalized PKC mRNA levels (relative to 18 S RNA) from cells treated with bombesin alone ($\square$) or with bombesin + TGF β_1 ($\bullet$) plotted against time. This represents one of two similar experiments.

parallel with the accumulation of protein. The inclusion of TGF β_1 reduced the accumulation of PKC- α mRNA, again consistent with the reduced accumulation of PKC- α protein (see above). These observations imply that PKC- α levels are largely controlled by transcription under these conditions.

TGF β_1 synergizes with bombesin in inducing cell cycle progression

The pattern of cell cycle progression was analysed, because of the distinction between PKC- δ and PKC- ϵ behaviour on DAG treatment of quiescent cells [9] and the effects described here for bombesin- and bombesin + TGF β_1 -stimulated cells. Preliminary studies employing [3 H]thymidine incorporation revealed that TGF β_1 alone induced a delayed onset of S-phase compared with bombesin (as observed previously; see [27]). The combined effects of these mitogens produced a synergistic increase in [3 H]thymidine incorporation with entry into S-phase at 15–20 h (results not shown).

A more detailed flow cytometric analysis is shown in Figure 4. It is clear that bombesin (Figure 4B) induced an S-phase peak at 24 h, with G2/M peaking at 32 h. TGF β_1 induced an S-phase peak at 32 h with a broad G2/M peak at 30–46 h. Neither of these mitogens alone induced a substantial proportion of the cells to enter the cell cycle (in both cases approx. 15–20 % of the cells progressed through the cell cycle). Thus, for cells treated with bombesin, 80–85 % of the cells remain in G0/G1, and PKC- δ and PKC- ϵ are largely down-regulated; this is consistent with the effects of DAG on quiescent (i.e. non-cycling) cells. It is noteworthy that even under these conditions some 10–20 % of the PKC- δ remained, a level much greater than that observed on down-regulation in quiescent cells [9].

On co-stimulation with bombesin + TGF β_1 , the vast majority of the cells progressed through the cell cycle (> 75 %). At roughly 18 h cells began to enter S-phase, and this peaked at 24 h, with the peak of G2/M cells being at 26 h. It is during this progression through S-phase and entry into G2/M that these cells 'recover' their PKC- δ content (see Figure 1, panel δ). The cycling behaviour of these co-mitogen-treated cells suggests that the lack of further down-regulation of PKC- δ , despite the elevated DAG levels, may reflect cell cycle-dependent regulation of the process of down-regulation.

PKC- δ is not down-regulated by PMA in bombesin + TGF β_1 -treated cells

The hypothesis that down-regulation is cell cycle-dependent was tested directly by driving cells into the cycle with the combination of bombesin + TGF β_1 ; then at 20 h (the point at which PKC- δ loss is arrested; see Figure 1) the cells were challenged with 400 nM PMA. It has been shown previously that quiescent cultures of Swiss 3T3 cells treated with this concentration of PMA lose approx. 50 % of their PKC- δ within 1 h [7]. Here, cycling cells were found to be insensitive to the PMA treatment, with no loss of PKC- δ occurring (Figure 5). By contrast, control cells pretreated for an equivalent time (20 h) with bombesin alone displayed typical PMA-induced down-regulation of PKC- δ .

In order to assess the contribution of protein synthesis versus protein degradation with respect to the protective effect that TGF β_1 has on bombesin-induced PKC- δ down-regulation, the effect of the protein synthesis inhibitor anisomycin was investigated. Inhibition of protein synthesis had no effect on the protection afforded by TGF β_1 (results not shown), indicating that the effect was directed to the PMA-induced degradation itself.

DISCUSSION

The evidence presented here demonstrates that there is no direct correlation between the agonist-induced elevation of DAG and

Figure 4 Flow cytometric analysis of cells stimulated with bombesin and TGF β_1

Cells were stimulated with 5 ng/ml TGF β_1 (**A**), 50 ng/ml bombesin (**B**) or both (**C**), and the cells were harvested at the indicated times. The DNA content was determined by fluorescence-activated cell sorting analysis as described previously [9,20]. The percentages of cells in G0–G1 ($\square$), S-phase ($\bullet$) and G2–M (Δ) are plotted against time. This is representative of three similar experiments.

Figure 5 Bombesin + TGF β_1 blocks PMA-induced down-regulation

Swiss 3T3 cells were treated for 20 h with either bombesin alone (lanes 1–3) or bombesin + TGF β_1 (lanes 4–6) as indicated. Cells were either harvested (lanes 1 and 4) or incubated for a further 1 h in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 400 nM PMA (TPA). Extracts were prepared and analysed for PKC- δ by Western blotting (see the Materials and methods section). The two molecular mass markers indicated are 97 kDa (upper) and 67 kDa (lower).

the down-regulation of a particular PKC isotype. Indeed, the study provides evidence for the operation of two independent agonist-induced regulatory mechanisms operating on PKC, one at the level of mRNA expression and the other at the level of the down-regulation process itself.

The complexity of PKC regulation in these cells has come to light through studies on the combined action of the growth factors bombesin and TGF β_1 . Bombesin itself is a known mitogen for Swiss 3T3 cells [28] and had been documented to activate PKC- δ and PKC- ϵ selectively in these cells [9]. Prolonged bombesin treatment induces sustained activation and consequent

down-regulation of both PKC- δ and PKC- ϵ . In contrast to bombesin, TGF β_1 can inhibit cell division in certain cell types, while having delayed mitogenic effects in fibroblasts (reviewed in [27]). TGF β_1 has been reported to block late-phase activation of phosphatidylcholine-specific phospholipase C and hence might modify PKC responses through such a mechanism [26]; however, this is clearly not the case in Swiss 3T3 cells. It is shown here by direct assessment of DAG accumulation that bombesin or bombesin + TGF β_1 leads to an increase in particular species of DAG (the combination producing a much greater response). Total DAG levels increased with bombesin + TGF β_1 treatment but not with bombesin alone. Part of this is a generalized increase in all species, probably derived from phosphatidylcholine hydrolysis (based on species profile similarities; see [14] for phospholipid profiles from Swiss 3T3 fibroblasts). However, there is an additional increase in polyunsaturated molecular species, particularly 18:0/20:4, $n-6$ and 18:0/20:3, $n-9$. These are the same species that are seen to be elevated rapidly, reaching a maximum within 30 s before returning to basal levels by 5 min, when 3T3 fibroblasts are treated with bombesin [14] or epidermal growth factor [25]. The source of these polyunsaturated DAG species is unclear, but they may be derived from the phosphoinositides which are rich in polyunsaturated species or from a minor phosphatidylcholine pool as postulated for early epidermal growth factor-induced DAG changes [25]. The observation that similar polyunsaturated DAG species are both specifically elevated at very early times and rise again at later time points if cells are induced to undergo division strongly suggests a specific role for these moieties in regulating cell cycle events. TGF β_1 also acts synergistically with bombesin in stimulating these cells to enter the cell cycle, this combination of mitogens being as effective as 10% serum in stimulating cell division (75–80% of quiescent cells enter the cycle; results not shown). Thus TGF β_1 is co-mitogenic with bombesin and enhances at least one of the bombesin-induced sustained responses, i.e. DAG accumulation.

Chronic exposure of Swiss 3T3 cells to PMA, DAG or bombesin has been shown previously to lead to the co-ordinate down-regulation of PKC- δ and PKC- ϵ [7,9]. However, in sharp contrast to this, PKC- δ and PKC- ϵ are controlled in an in-

dependent fashion when cells are stimulated with bombesin + TGF β_1 , despite the chronic elevation of DAG. In the case of PKC- δ , there is an initial phase of down-regulation that is complete by approx. 20 h, at which time protein levels re-accumulate to reach a level moderately above that in the unstimulated G0 cells. This re-accumulation occurs at a time when endogenous DAG levels are still grossly elevated. It is evident that PKC- δ is in some way protected from DAG-induced down-regulation.

Unlike PKC- δ , PKC- ϵ becomes down-regulated in response to bombesin or bombesin + TGF β_1 . While this might reflect a lack of 'protection' as alluded to for PKC- δ , it has been established that, in cells stimulated by bombesin + TGF β_1 (but neither alone), PKC- ϵ mRNA disappears with a half-life of approx. 4 h. Thus under these conditions the loss of synthesis may account largely for the loss of steady-state PKC- ϵ protein. No changes in PKC- δ mRNA levels are observed (i.e. increases) that might account for its distinctive behaviour.

The apparent protection of PKC- δ from down-regulation induced by elevated DAG might be due to an inhibition of the process itself, or perhaps to localization of PKC- δ in a DAG-inaccessible compartment. The inability of the protein synthesis inhibitor anisomycin to prevent protection implies an effect that is independent of the synthetic pathway. Based upon studies with PMA, which will penetrate all cellular compartments, it is shown that, during this phase of the bombesin + TGF β_1 response, PKC- δ is resistant to down-regulation. That PKC- δ is actually targeted by PMA under these conditions is suggested by the presence of a slower migrating form on SDS/PAGE, indicative of an altered phosphorylation state (see Figure 5 and [29]). These observations indicate that PKC- δ is not sequestered from PMA and that the protection afforded by TGF β_1 reflects a block in degradation.

It is established, then, that PKC- δ down-regulation, although initially operative, is blocked approx. 20 h into a bombesin + TGF β_1 response, while continuing to operate normally over the same time course of exposure to bombesin alone. A major distinction between these two means of stimulation is that while bombesin only triggers ~ 15 % of the cells to progress through the cell cycle, in combination with TGF β_1 > 75 % of cells enter the cycle. A consistent explanation for the behaviour of PKC- δ is that the down-regulation process is inhibited from mid-S-phase (~ 20 h) onwards. Thus the contribution of the cycling cells to the total PKC- δ content dominates and the total levels are seen to recover. By contrast, for the bombesin-stimulated cells, at most ~ 15 % of cells (those in cycle) will retain PKC- δ on prolonged stimulation; this value in fact corresponds closely with the observed residual PKC- δ (see Figure 1).

Our observations are thus entirely consistent with a cell-cycle-dependence of PKC- δ down-regulation that can be summarized as follows: down-regulation of PKC- δ only operates in the G0/G1-phases of the cell cycle. This is consistent with previous work that has provided evidence for resistance to PKC down-regulation in exponential-phase cells [30]. The degradation process itself has been shown to reflect vesicle traffic/sorting [31], and as such may be controlled in a cell-cycle-dependent manner as documented for Golgi function (reviewed [32]).

With respect to the proliferation of Swiss 3T3 cells, the results indicate that, while PKC- δ and PKC- ϵ are both chronically activated, the sparing of PKC- δ observed in bombesin + TGF β_1 -

treated cells is an effect and not a cause. Thus while more cells enter the cell cycle and progress through S-phase, the sparing of PKC- δ does not occur until beyond the time at which the cells are committed to enter S-phase (approx. 10–12 h). Whether the 're-setting' of PKC- δ levels has an impact upon a further round of division is not established. However, the inability of single mitogens to maintain proliferation of these cells implies that there are longer-term consequences to the action of combined mitogens, and the effects described here for PKC- δ represent one such response.

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