Isoform specificity of activators and inhibitors of protein kinase C γ and δ

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Abstract Expression of certain mammalian protein kinase C (PKC) isoforms inhibits the proliferation of *Schizosaccharomyces pombe* (Goode et al., Mol. Biol. Cell 5 (1994) 907–920). We have taken advantage of this fact to determine the in vivo isoform preference of a number of PKC inhibitors, using a microtitre plate assay which allows rapid screening. This in vivo model has revealed previously unreported preferences; calphostin C is a more efficient inhibitor of the novel PKCδ than chelerythrine chloride whereas the efficiencies are reversed for inhibition of the classical PKCγ. We have also shown that the anti-leukaemic agent bryostatin 1 inhibits or activates in vivo in an isoform-specific manner.

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Key words: Protein kinase C; Isoform; Inhibitor; Activator; *Schizosaccharomyces pombe*

1. Introduction

Protein kinase C (PKC) is a major transducer of signals in response to the generation of diacylglycerol (reviewed in [1,2]). As the receptor for the tumour-promoting phorbol esters [3] it has excited much interest as a potential target for tumour therapy and both activators and inhibitors of PKC have entered the clinical trial stage [4,5]. In order to investigate the role of PKC in signal transduction, extensive use is made of pharmacological agents as both activators and inhibitors of PKC activity. If a response can be elicited by treatment of cells with an activator such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) then PKC activation is assumed to be sufficient for signal transduction. If a response is blocked by an inhibitor of PKC then PKC activity is necessary, though not necessarily sufficient, for the response. In addition, prolonged treatment of cells with TPA leads to a loss of PKC in a process known as down-regulation in which activated PKC is degraded. Such cells are rendered deficient in PKC signalling.

Although PKC was originally described as a single activity, cloning has revealed the existence of a complex family of enzymes with at least ten genes encoded in the human genome and evidence for alterative splice forms to further increase the potential number of proteins involved (reviewed in [1,2]). These isoforms can be subdivided on the basis of sequence homology and biochemical properties into three families: conventional, novel and atypical PKCs. Where investigated, cells appear to express a range of isoforms in varying concentrations (e.g. [6,7]). The expression of multiple isoforms is supportive of isoform-specific functions. There is increasing evidence, mainly from overexpression studies, that different isoforms do carry out unique functions in the cell [8–10]. Immunohistochemical analysis has also revealed distinct isoform-specific compartmentalisation consistent with this theory [11,12]. Thus a full understanding of a signalling pathway must include definition of which isoform of PKC is involved and knowledge of the isoform specificity of activators and inhibitors of PKC is vital in the interpretation of experiments involving their use. The development of compounds which modulate the activity of an individual isoform will allow manipulation of a single response with minimal effect on other PKC-dependent pathways.

The isoform specificity of a small number of PKC inhibitors has been determined using purified PKCs [13,14]. The purification of individual PKC isoforms from tissue or cell sources is not trivial as most express a range of isoforms with similar chromatographic properties. PKCs expressed in bacteria have no activity [15,16] and so cannot be used for such analysis, so the most efficient method of isolating a single isoform for biochemical studies is expression using a baculovirus expression system with subsequent purification [17]. This is labour-intensive and the endogenous PKCs of Sf9 insect cells have not been characterised so there is a risk of contamination of expressed protein with an insect PKC. Furthermore, the in vitro behaviour of PKCs may not fully represent their behaviour in the whole cell where there may be compartmentalisation effects as well as metabolism of compounds and competition with other cellular components. Thus an in vivo assay to determine the effect of modulators of PKC activity will provide a more accurate assessment of the consequences for the whole cell.

We have devised a rapid in vivo assay for identifying PKC activators and inhibitors which allows determination of their isoform specificity. Expression of individual isoforms of mammalian PKCs in the fission yeast *Schizosaccharomyces pombe* causes a TPA-dependent growth inhibition which depends on the activity of the expressed PKC [18]. We report here that this effect can be reversed by the addition of known inhibitors of PKC activity. We have devised a microtitre assay which can be used as a rapid screening system to determine if pharmacological agents act as inhibitors of individual PKC isoforms. This system has been used to determine the relative efficacy on individual PKC isoforms of some inhibitors which are currently widely in use. The relative efficiency of growth restoration varies between isoforms suggesting that treatment of intact cells with these inhibitors will not have an equal effect on inhibiting all isoforms of PKC present.

This assay can also be used to identify and evaluate the

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Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

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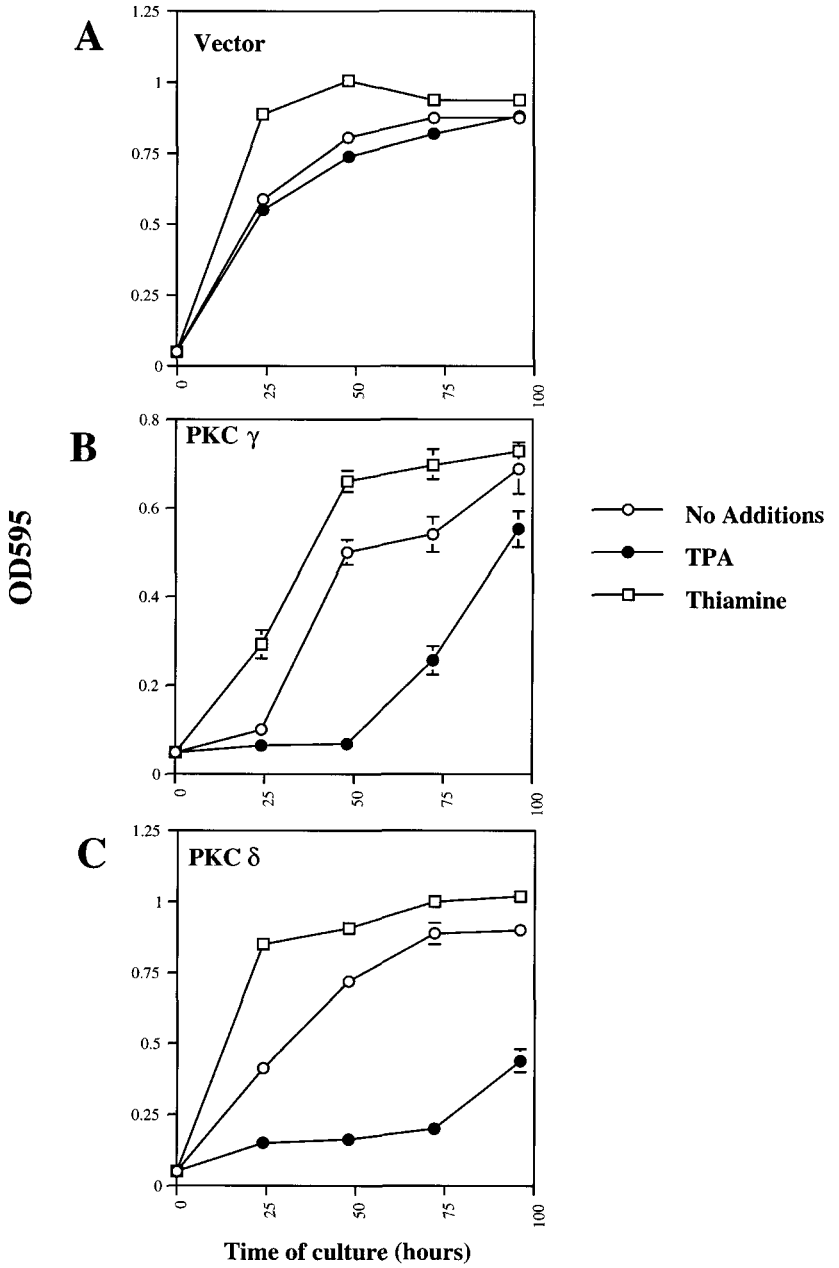


Fig. 1. Growth phenotype and effect of TPA on *S. pombe* strains expressing PKC. *S. pombe* strains expressing either the vector control (A), PKCγ (B) or PKCδ (C) were precultured in minimal selective medium without thiamine to allow induction of PKC expression until an OD_{595} of 0.4–0.6 was reached. The strains were then diluted to an OD_{595} of 0.03 (5×10^5 cells/ml) in minimal selective media with no additions, 1 μM thiamine or 200 ng/ml TPA as indicated. The cell density was determined after the indicated intervals of culture at 30°C. Results are the mean of triplicate samples ± standard deviation and are from one experiment, representative of 10. The control, PKCγ and PKCδ transformants reached saturation densities (OD) of 0.87, 0.69 and 0.89 respectively.

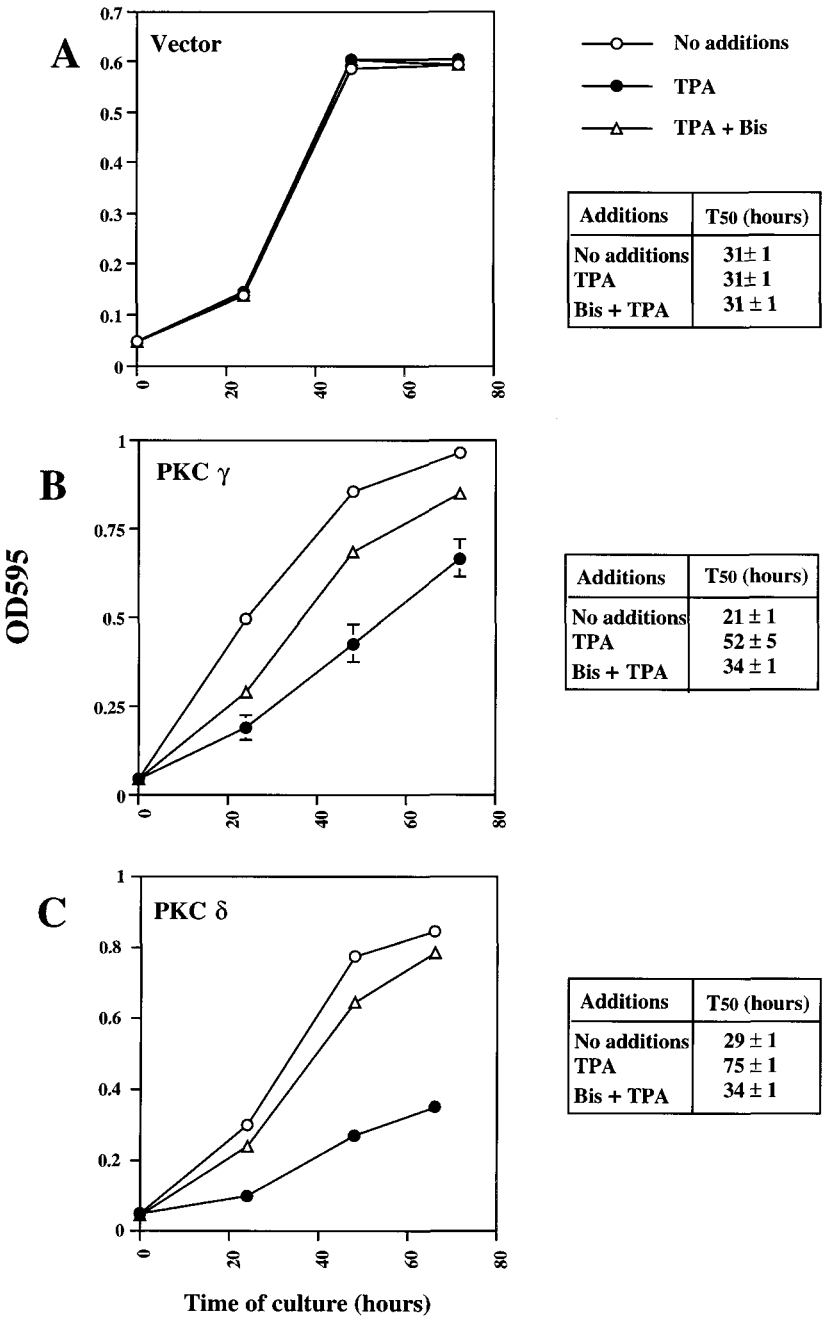
isoform specificity of PKC activators. PKC activators also have potential as anticancer agents as, for example, they cause the terminal differentiation of a number of haematopoietic cell types. Highly specific agents which activate only the required isoforms would be of interest for use in such cases. We have examined the effects of the mixed PKC agonist/antagonist bryostatin 1 on two isoforms of PKC and report opposing effects on two isoforms.

2. Materials and methods

All PKC inhibitors were purchased from Calbiochem; TPA and amino acids were purchased from Sigma. The PKC activator bryostatin was a kind donation from G. Radda, Oxford. The monoclonal 36G9 PKCγ antibody [19] was obtained from Peter Parker (ICRF, London). All other antibodies used and the enhanced chemiluminescence (ECL) detection system were obtained from Amersham International.

2.1. Growth of S. pombe

Mammalian PKCs were expressed in *S. pombe* strain *h-, ade6-704, leu1-32, ura4-Δ18*, under the control of the thiamine-repressible promoter in the pREP series of vectors [18]. Cells were transfected with either empty vector or the PKC expression vector [18]. The resulting transformants were tested for the ability of TPA to inhibit growth in the absence of thiamine by streaking on fully supplemented Edin-



Additions	T50 (hours)
No additions	31± 1
TPA	31± 1
Bis + TPA	31 ± 1

Additions	T50 (hours)
No additions	21 ± 1
TPA	52 ± 5
Bis + TPA	34 ± 1

Additions	T50 (hours)
No additions	29 ± 1
TPA	75 ± 1
Bis + TPA	34 ± 1

Fig. 2. Restoration of TPA-induced growth inhibition by bisindolylmaleimide. Cell growth was assayed as described in the legend to Fig. 1, with no additions, 200 ng/ml TPA or the combination of 200 ng/ml TPA with 3 μM bisindolylmaleimide (Bis). The T_{50} value (time taken to reach 50% of saturation density) for each condition is expressed in the associated tables for vector control (A), PKCγ (B) or PKCδ (C). Results are the mean of triplicate samples ± standard deviation from one experiment, representative of 10. The control, PKCγ and PKCδ transformants reached saturation densities (OD) of 0.59, 0.92 and 0.84 respectively.

burgh minimal medium (EMM) plates [20] containing 100 ng/ml TPA. Single colonies were picked and grown in culture overnight in the presence of thiamine. Cells were then washed three times to remove the thiamine and cultured overnight in the absence of thiamine. Cells (OD_{595} 0.4–0.6) were diluted into 150 μl (final volume) in triplicate in a 96-well microtitre plate (flat bottom wells) to an OD_{595} of 0.03 in EMM (supplemented with 2% w/v glucose, adenine (150 mg/l), uracil (150 mg/l) or leucine (150 mg/l)), and containing thiamine (1 μM), TPA (200 ng/ml) or inhibitors as indicated. For those inhibitors dissolved in DMSO control cells with the appropriate volume of DMSO confirmed that the solvent had no effect on cell growth. The cells were incubated with slow shaking at 30°C in a sealed box containing moist tissues. The outer wells of the plate were not used as these sometimes dried out on prolonged incubation at 30°C. The growth was monitored by determining the OD_{595} at various time intervals using a plate reader (Molecular Devices, Raleigh Court, Crawley).

The effect of each inhibitor was assessed by comparing the time taken for the cells incubated in the presence of TPA with or without inhibitors to reach 50% of the final saturation density of cells incubated without additives (T_{50}). Final saturation density used to calculate the T_{50} value for each experiment is indicated in the figure legends. The relationship between the T_{50} value and the growth profile of *S. pombe* strains is outlined in Fig. 2. T_{50} values were obtained for each experiment by visual inspection of the data. The densities of each

Table 1
The effects of Gö 6976 and rottlerin on growth of *S. pombe* PKC transformants

Culture conditions	T_{50} (h)		
	PKCγ	PKCδ	Vector
No additions	30 ± 1	29 ± 1	31 ± 1
TPA (200 ng/ml)	60 ± 5	75 ± 1	30 ± 1
TPA+rottlerin (15 μM)	59 ± 6	63 ± 3	31 ± 1
TPA+Gö 6976 (10 μM)	43 ± 1	72 ± 8	32 ± 1

S. pombe PKC transformants were incubated without additions, in the presence of TPA or in the presence of TPA and either rottlerin or Gö 6976 at the indicated concentrations. OD_{595} readings were taken at 12 h intervals and the T_{50} value (h) for each condition was determined. The results shown are the means of triplicate samples from a single experiment, representative of four. All the values in this table were obtained in a single experiment.

individual culture well were plotted independently and three T_{50} values determined for each condition. These were then averaged to determine the mean T_{50} with a standard deviation.

2.2. Western blot analysis

Denatured protein extracts were made from equivalent numbers of cells as described [20]. Protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotted as described elsewhere [21]. The PKCγ 36G9 monoclonal antibody was followed by a sheep anti-mouse horse radish peroxidase-conjugated antibody and the ECL detection system.

3. Results

S. pombe cells expressing either a conventional isoform of PKC (PKCγ) or a novel isoform (PKCδ) under a thiamine-repressible promoter were examined for their ability to show TPA-dependent growth inhibition when grown in microtitre plates. Treatment of cells expressing either isoform with TPA led to growth inhibition compared with the cells not expressing PKC (cultured in the presence of thiamine) ([18] and Fig. 1). The strains expressing PKC show some growth inhibition in the absence of TPA, presumably due to background activation of the expressed PKC. Within an experiment the saturation density for a particular cell strain was always identical, irrespective of the presence of thiamine, TPA or inhibitor – only the time taken to reach this density varied. Differences in the saturation density are seen between experiments due to slight differences in growth conditions. The time at which the TPA-dependent inhibition was lost varied between isoforms, presumably dependent on the rate of down-regulation of the PKC from the *S. pombe* cells. As TPA causes down-regulation and loss of the PKC from the cell, the growth returned gradually to normal rates ([18] and Fig. 1).

3.1. The effects of PKC inhibitors

The inhibition of growth induced by TPA in both PKCγ and PKCδ transformants could be reversed by the inclusion of known inhibitors of PKC. Fig. 2 illustrates the growth curves for the PKCδ- and PKCγ-expressing cells cultured in the presence of TPA and the PKC inhibitor bisindolylmaleimide (GF109203X). A reversal of growth inhibition is apparent while growth of control cells transfected with vector alone was not significantly altered. At high concentrations the inhibitors did have effects on control cells alone so for all inhibitors the concentrations were chosen to be below this threshold level. We have used the time taken to reach 50% of the T_{50}, of cells alone, as a measure of the initial growth rate in strains under various conditions as this includes differences in lag time as well as in doubling time and we have found it to give an accurate reflection of the efficacy of the inhibitors (Fig. 2 and data not shown). The absolute values of T_{50} varied between experiments, presumably due to slight differences in the initial plating density. However all the trends reported here were reproducible. The ability of PKC inhibitors to reverse the growth defect in these strains, therefore, provides a rapid screening method for testing new compounds for their ability to inhibit individual isoforms of PKC. In PKCγ expressing cells treated with TPA the inclusion of bisindolylmaleimide (3 μM) reduced the T_{50} to 34 ± 1 h from 53 ± 5 h. In PKCδ transformants bisindolylmaleimide induced a 41 h decrease in T_{50}.

The ability of this assay to distinguish isoform-specific effects was confirmed by comparing the effect of the ATP analogue Gö 6976 on the growth of cells expressing PKCδ and PKCγ. This compound is reportedly specific for the conventional PKCs with IC_{50} of 2–6 nM [13]. This was reflected in the reversal of growth inhibition at a given concentration of Gö 6976 (10 μM) having no effect on the growth time for PKCδ expressing cells while inducing a 17 h decrease in T_{50} in PKCγ-expressing cells (Table 1). Conversely rottlerin has been reported to be specific for PKCδ [23] and this specificity

Table 2
The effects of calphostin, chelerythrine and D-*erythro*sphingosine on the growth of *S. pombe* PKC transformants

Culture conditions	T_{50} (h)		
	PKCγ	PKCδ	Vector
No additions	36 ± 1	35 ± 1	37 ± 1
TPA (200 ng/ml)	76 ± 2	115 ± 1	37 ± 1
TPA+calphostin (125 nM)	64 ± 1	39 ± 3	36 ± 1
TPA+chelerythrine (26 μM)	56 ± 1	75 ± 7	36 ± 1
TPA+D-*erythro*sphingosine (1 μM)	55 ± 2	36 ± 3	35 ± 1

S. pombe PKC transformants were incubated without additions, in the presence of TPA or in the presence of TPA and either calphostin, chelerythrine or D-*erythro*sphingosine at the indicated concentrations. OD_{595} readings were taken at 12 h intervals and the T_{50} value (h) for each condition was determined. The results shown are the means of triplicate samples from a single experiment, representative of four. All the values in this table were obtained in a single experiment.

Table 3
A comparison of the in vitro IC_{50} values of PKC inhibitors with the in vivo percentage inhibition of PKC-induced growth effect

Inhibitor	IC_{50}			% Reversal	
	Mix	γ	δ	γ	δ
Rottlerin	NA	40 μM	3–6 μM	3	57
Gö 6976	NA	2–6 nM	*	37	7
Calphostin	50 nM	NA	NA	30	95
Chelerythrine	660 nM	NA	NA	50	50
D-*Erythro*sphingosine	600 nM	NA	NA	53	99
Bis	10 nM	20 nM	NA	58	45

The table outlines a comparison between the published data on in vitro IC_{50} values [13,22,23] for each inhibitor examined and the results obtained with the in vivo assay described in this paper. The IC_{50} values presented are for either a mixture of PKC isoforms (Mix), or individual isoforms where data are available. NA indicates IC_{50} values not available, * indicates not detectable, Bis is bisindolylmaleimide. The in vivo assay results are presented as % reversal of the growth defect and are calculated as follows:

$$\frac{T_{50}(\mathrm{TPA}) - T_{50}(\mathrm{TPA} + \mathrm{inhibitor})}{T_{50}(\mathrm{TPA}) - T_{50}(\mathrm{no\ additions})} \times 100.$$

The % reversal values were calculated from the data in Tables 1 and 2 and Fig. 2, and hence the concentration of inhibitors examined and the final saturation densities are as outlined in the relevant legends.

was readily apparent using our assay system. Rottlerin induced a 12 h decrease of T_{50} in cells expressing PKCδ whereas an insignificant difference in growth rate was observed in control cells and those expressing PKCγ.

Having determined that the assay system was capable of distinguishing the isoform specificity of PKC inhibitors, the relative ability of some widely used PKC inhibitors to restore growth of cells expressing individual PKC isoforms was determined (Table 2). One classical isoform (PKCγ) and one novel isoform (PKCδ) were investigated as representatives of the subfamilies of PKC. The PKC inhibitors tested included one targeting the ATP binding site (chelerythrine chloride) and others that target regulatory sequences (calphostin C, D-*erythro*sphingosine) [22]. The isoform specificity of these inhibitors has not been previously reported. The results indicated that significant differences occur. PKCγ was relatively insensitive to calphostin C (125 nM) whereas chelerythrine chloride (26 μM) inhibited PKCγ to a greater extent. In contrast cells expressing PKCδ showed reversed sensitivity with significant inhibition by this same concentration of calphostin C, whereas the cells were relatively refractory to 26 μM chelerythrine chloride. D-*Erythro*sphingosine had an effect similar to chelerythrine chloride in PKCγ transformants but was much more effective than chelerythrine in PKCδ expressing cells. Thus previously unreported isoform preferences of these PKC inhibitors in the intact cell have been revealed. Table 3 outlines a comparison of the results obtained in this study with the reported IC_{50} values for each inhibitor. This table further indicates that reported in vitro isoform specificities for the PKC inhibitors examined have been confirmed using our in vivo system.

3.2. The effects of a PKC activator

In addition to examining the effects of PKC inhibitors, this assay system can also be used to look at the isoform specificity of PKC activators. We have chosen to look at the effect of bryostatin as this compound will activate PKCs in vitro but has significantly different effects to phorbol esters when added to whole cells. Bryostatins are natural products that have antileukaemic activity [24] and are in use in clinical trials although the mechanism of action is not well established. We compared the effects of bryostatin 1 with TPA on the growth of the PKCγ and PKCδ transformants (Fig. 3). The results suggest that bryostatin 1 activates PKCδ, as indicated by a decrease in growth rate compared to PKCδ transformants incubated in the absence of PKC activators (Fig. 3C). A similar effect was anticipated for cells expressing PKCγ but in fact the growth rate of PKCγ transformants was increased by the presence of bryostatin (Fig. 3B); it appears to antagonise PKCγ activity. Several explanations are possible. Rapid loss of immunoreactive PKC in response to bryostatin treatment has been reported in a variety of cell lines and used to explain differences between cellular responses to bryostatin and phorbol esters [25–27]. Thus it is possible that bryostatin induced extremely rapid down-regulation of PKCγ in *S. pombe* leading to a rapid relief of PKCγ-induced growth inhibition and a subsequent return to normal growth rates. However, in our system PKCγ expression levels were comparable to untreated cells after culture with bryostatin for 24 h, whereas this isoform was almost completely down-regulated in TPA treated cells (Fig. 4A). Stripping and re-probing the same Western blot with an antibody against a protein whose expression levels are not expected to change was used to control for the levels of protein loading (Fig. 4B). These results suggest that bryostatin does not activate PKCγ but may, in fact, act as an inhibitor of activity, reversing the small degree of growth inhibition seen in the absence of TPA treatment. Thus in our in vivo experimental system bryostatin exhibits a distinct isoform specificity, activating PKCδ while leading to inhibition of PKCγ.

4. Discussion

We have developed a rapid assay for determining the ability of reagents to inhibit individual isoforms of PKC. This method is applicable to screening unknown compounds firstly for their ability to inhibit PKC and secondly to demonstrate specific inhibition of individual isoforms. The assay depends on the TPA-dependent growth inhibition exhibited by *S. pombe* cells expressing individual isoforms of PKC. As this growth inhibition depends on PKC activity [18] we have shown that the inclusion of inhibitors of PKC can reverse this growth defect. Reversal of growth inhibition is also apparent in the absence of TPA but the inclusion of TPA, by increasing the growth inhibition, greatly increases the sensitivity of the assay and allows small differences in inhibitor efficiency to be deter-

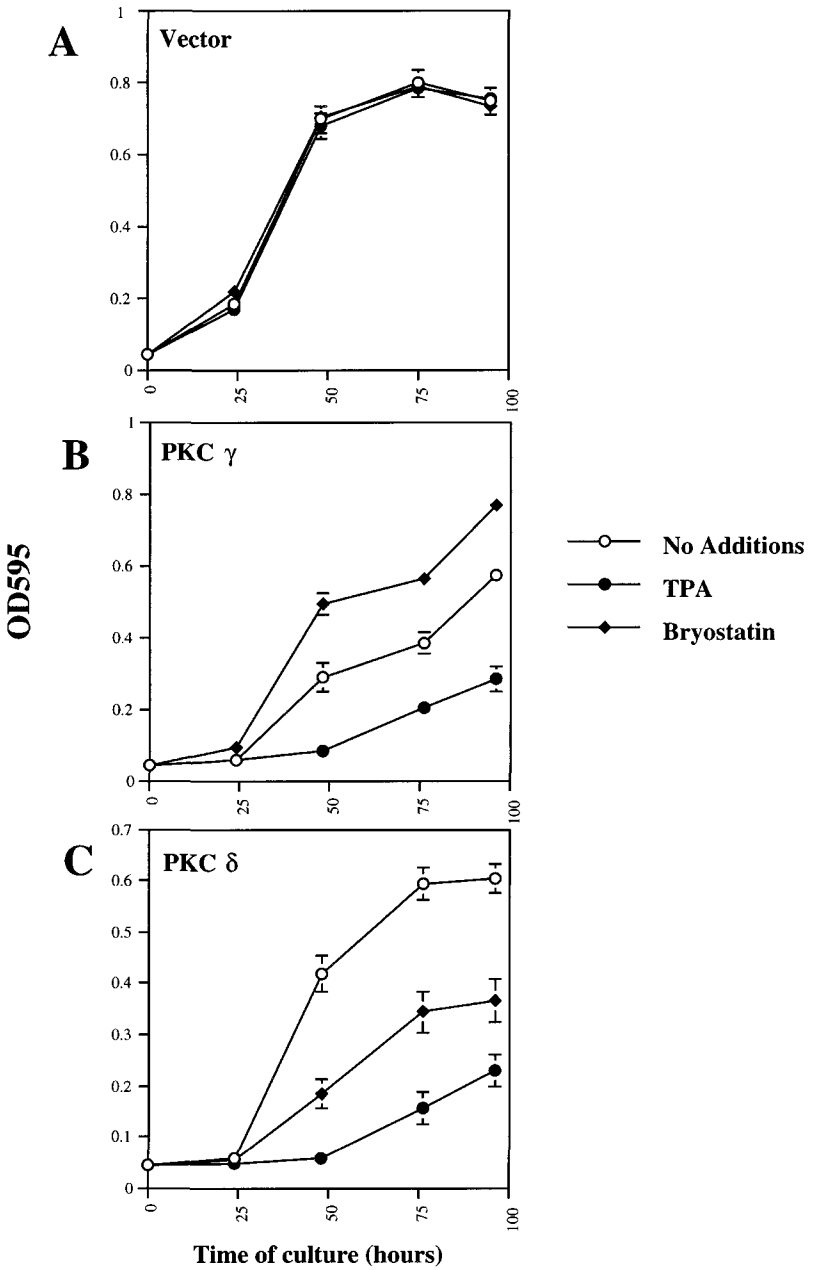


Fig. 3. The isoform-specific effects of the PKC activator bryostatin 1. Cell growth was assayed as described in the legend to Fig. 1, in the presence of no additions, 200 ng/ml TPA or 200 ng/ml bryostatin as indicated. The cell density was determined after the indicated intervals of culture at 30°C. Results are the mean of triplicate samples ± standard deviation, from one experiment, representative of 3. The control, PKCγ and PKCδ transformants reached saturation densities (OD) of 0.75, 0.57 and 0.60 respectively.

mined (unpublished observations). TPA has no effect on control cell growth (Figs. 1–3) and so is included only to activate all the expressed mammalian PKC. It is not clear whether it is the activation of the PKC itself or its subsequent downregulation which is causing the growth defect. However, as all are dependent on PKC activity [18] this does not effect the validity of the assay and as both events will be occurring in response to PKC activators in mammalian cells, this in vivo assay is a better model for the effect of inhibitors in the intact cell than an in vitro determination of IC_{50}. Both isoforms tested here do downregulate on prolonged TPA treatment (Fig. 4 and [18]) with similar efficiency. We have used the time taken to reach 50% of the T_{50} as a convenient measure of the growth rate of cells under varying conditions. Comparison of the T_{50} values allows the efficacy of inhibitors to be assessed on a single PKC isoform in an intact cell. This may be significantly different from the effect in an in vitro kinase assay due to the influence of other cellular components. The use of a microtitre plate assay system allows rapid screening of a large number of compounds at varying concentrations.

We have used the assay to determine the relative efficiency of inhibition of a range of widely used PKC inhibitors on representative members of the conventional and novel PKC subfamilies. Although this method cannot be used to determine an IC_{50} as there is no way of judging the concentration of inhibitor present in the cell, it can be used to determine the

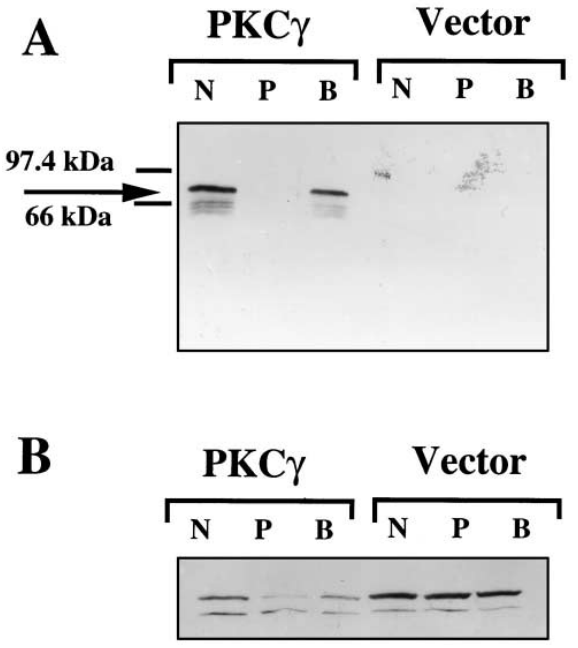


Fig. 4. Expression of PKCγ in cells treated with bryostatin 1 and TPA. A: Denatured extracts collected from vector control and PKCγ-expressing cells cultured for 24 h with no additives (N), 200 ng/ml TPA (P) or 200 ng/ml bryostatin (B) were analysed by Western blot using the 36G9 PKCγ monoclonal antibody. The position of PKCγ is indicated by the arrow. The smaller bands are presumed to be degradation products of full-length PKCγ and have been reported in previous use of this system [18]. B: The Western blot was stripped and reprobed with polyclonal anti-ERK (Santa Cruz K-23) antibody to control for protein loading.

relative efficiency of individual isoform inhibition. The use of an in vivo assay means that the effect of these compounds can be determined in a cell background and is more likely to reflect their true activity in the cell rather than an in vitro system using purified components. The ability of the assay to detect isoform specificity of inhibitors was confirmed by testing the ability of compounds known to show preference for certain isoforms to reverse the growth inhibition of strains expressing individual isoforms. The PKC inhibitor Gö 6976 with specificity for conventional isoforms [13] induced a reversal in growth inhibition only in PKCγ-expressing cells. Conversely rottlerin, which has been reported to be specific for PKCδ [23], induced a reversal of TPA-induced growth inhibition only in cells expressing PKCδ.

The isoform specificity of many widely used PKC inhibitors is not known due to difficulties in obtaining purified preparations of a single isoform. Those which have been reported rely on an in vitro assay rather than quantifying their effects in the cell where many other components may influence the efficiency of PKC inhibition. We have tested some of the commonly used inhibitors in our assay system and are able to detect isoform specificity. For example we found that calphostin C showed a greater efficiency of inhibition of PKCδ while chelerythrine chloride showed a preference for PKCγ. The differences in relative efficiencies of these compounds has wide implications for their use as PKC inhibitors on cells that are expressing a range of PKC isoforms. Treatment with limiting concentrations of inhibitors will preferentially inhibit one isoform and perhaps one pathway rather than all PKC-dependent events. Obviously the effects within a cell will also depend upon the relative concentrations of PKC isoforms, but the specificities demonstrated here will play an important role in developing strategies to modulate cell function using these inhibitors.

Apart from the interest in PKC inhibitors there is a need to establish an assay for PKC isoform-specific activators. Such compounds would be of use experimentally as well as therapeutically. We have also shown that this assay can be used to determine the ability of compounds to activate individual isoforms of PKC. Bryostatin is widely used to modulate PKC function and bryostatin 1 has been used in phase 1 clinical trials [5] but without a full understanding of its mechanism of action. Treatment of cells with bryostatin, however, does not mimic treatment with the other major PKC activators (phorbol esters) and several studies have indicated that bryostatins have mixed activator/inhibitor effects on PKC activity in a variety of cell types [28–30] which may reflect an isoform-specific effect. However, in vitro studies using purified PKCs have not revealed any isoform-specific differences in bryostatin interaction [31,32]. We report here that this compound will activate PKCδ but inhibits PKCγ activity when expressed in *S. pombe* cells. This finding will not only help explain the mechanism of action of bryostatin but may also lead to a clearer understanding of the role of individual PKC isoforms in the process of carcinogenesis.

This novel model has been shown to be valid for the identification of PKC isoform-specific activators and inhibitors. It can be extended to include all the isoforms of PKC which give a growth phenotype in this system and can, therefore, give a great deal of information of general use.

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