Ro31-8220 inhibits protein kinase C to block the phorbol ester-stimulated release of choline- and ethanolamine-metabolites from C6 glioma cells: p70 S6 kinase and MAPKAP kinase-1β do not function downstream of PKC in activating PLD

Angela Morreale, Barbara Mallon[1], Gary Beale, John Watson, Martin Rumsby*

Department of Biology, University of York, York YO1 5YW, UK

Received 17 August 1997; revised version received 22 September 1997

Abstract The use of bisindolylmaleimide derivatives of staurosporine as selective inhibitors of protein kinase C (PKC) is in doubt following the report by Alessi [FEBS Lett. 402 (1997) 121–123] that Ro31-8220 and GF109203X are potent in vitro inhibitors of p70 S6 kinase and mitogen-activated protein kinase-activated protein kinase-1β, as well as of PKC. Here we show that the phorbol ester-stimulated release of choline- and ethanolamine-metabolites from C6 glioma cells due to phospholipid hydrolysis by phospholipase D (PLD) is not inhibited by rapamycin or PD98059, specific inhibitors respectively of p70 S6 kinase and MAPKK (MEK) and thus of MAPKAP kinase-1β but is still completely blocked by Ro31-8220. We conclude therefore that $p70^{S6k}$ and MAPKAP kinase-1β as well as MAPK are not involved in signalling pathways downstream of PKC that regulate phorbol ester-stimulated phospholipid turnover and that the inhibitory action of Ro31-8220 occurs by blocking PKC which regulates at least one pathway to PLD activation. The PI-3 kinase inhibitor, wortmannin, inhibits the phorbol ester-stimulated release of ethanolamine- but not choline-metabolites from C6 cells suggesting that different PLD isoforms regulate the turnover of PtdEth and PtdCho in C6 cells. Both PLD isoforms are activated via PKC but the PtdEth-PLD is also regulated via a wortmannin-sensitive pathway.

1. Introduction

The microbial metabolite, staurosporine, was used in the past as a specific inhibitor of protein kinase C (PKC). However, due to its competitive inhibition of the ATP-binding site, it is now known to be a potent but rather unselective inhibitor of several serine/threonine and tyrosine kinases [1]. Various bisindolylmaleimide derivatives of staurosporine show an improved selectivity for inhibition of protein kinase C (PKC) compared with some other kinases while retaining the potency of staurosporine [2,3]. Ro31-8220, for example, has an IC_{50} of 23 nM for inhibition of rat brain PKC in vitro compared with 1.5 μM and 17 μM for PKA and Ca_{2+}/calmodulin-dependent protein kinase respectively [3]. Ro31-8220 does not show significant PKC subspecies specificity [3] but has been widely used to investigate the involvement of PKC in cell signalling pathways (e.g. [4,5,9]).

Conclusions about PKC based on the use of bisindolylmaleimides are now in doubt following the recent report from Alessi [6] that Ro31-8220 and GF109203X are potent inhibitors in vitro of p70 S6 kinase ($p70^{S6k}$) and MAP kinase-activated protein (MAPKAP) kinase-1β, in addition to PKC. This finding makes it necessary, when using Ro31-8220 and similar compounds to establish a role for PKC in signalling pathways, to eliminate an involvement of $p70^{S6k}$ and MAPKAP kinase-1β. We [7,8] have used Ro31-8220 to define a role for PKC in the phorbol ester-stimulated turnover of phosphatidylcholine (PtdCho) and Eth-containing phospholipids (PtdEth) in C6 glioma cells by phospholipase D (PLD), as have others in different cell lines (e.g. [4,9]). Ro31-8220 completely blocks the phorbol ester-stimulated release of choline-lipid headgroup metabolites to the extracellular medium [7,8], a finding taken previously to indicate an involvement of PKC but which from Alessi's report [6] may also indicate a role for $p70^{S6k}$ and/or MAPKAP kinase-1β. Here we show that phorbol ester-stimulated Cho- and Eth- metabolite release from C6 cells is not affected by rapamycin which inhibits $p70^{S6k}$ or by PD98059 which inhibits MAPKK and thus prevents activation of MAPKAP kinase-1β [6] while Ro31-8220 is still inhibitory. The results show that neither $p70^{S6k}$ nor MAPKAP kinase-1β function downstream of PKC to activate PLD and confirm the previous view that phorbol ester-stimulated turnover of PtdCho and PtdEtn is mediated, at least partially, through PKC [7,8].

2. Materials and methods

2.1. Materials

C6 glioma cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, England). Cell culture plastic was from Corning (Bibby-Sterilin, Stone, England). Trypsin/EDTA (10×) and DMEM were from Life Technologies (Paisley, Scotland). FBS and staurosporine were from Sigma (Poole, Dorset, England). Ro31-8220 was a gift from Dr. G. Lawton, Roche Research Centre, Welwyn, England. Rapamycin and wortmannin were from LC Laboratories (Alexis Corp., Nottingham, England). PD98059 was a gift from Professor P. Cohen (University of Dundee). [^{3}H]choline and [^{3}H]ethanolamine or [^{14}C]ethanolamine were from Amersham (Little Chalfont, Bucks., England). 12-0-tetradecanoylphorbol 13-acetate

*Corresponding author. Fax: +44 (1904) 432860.
E-mail: mgr1@york.ac.uk

[1]Present address: Dept. of Neurosciences, Cleveland Clinic Foundation, 9500 Euclid Av., Cleveland, OH 44195, USA.

Abbreviations: Cho, choline; DMEM, Dulbecco's modified Eagle's medium; Eth, ethanolamine; FBS, foetal bovine serum; MAPKK (MEK), mitogen0-activated protein kinase kinase; MAPKAP kinase-1β, mitogen-activated protein kinase-activated protein kinase-1β; PEth, phosphoethanolamine; PLD, phospholipase D; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-tris phosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PI-3 kinase, phosphatidylinositol-3 kinase; PKC, protein kinase C; $p70^{S6k}$, p70 S6 kinase; TBS, Tris-buffered saline

0014-5793/97/$17.00
PII S0014-5793(97)01252-0

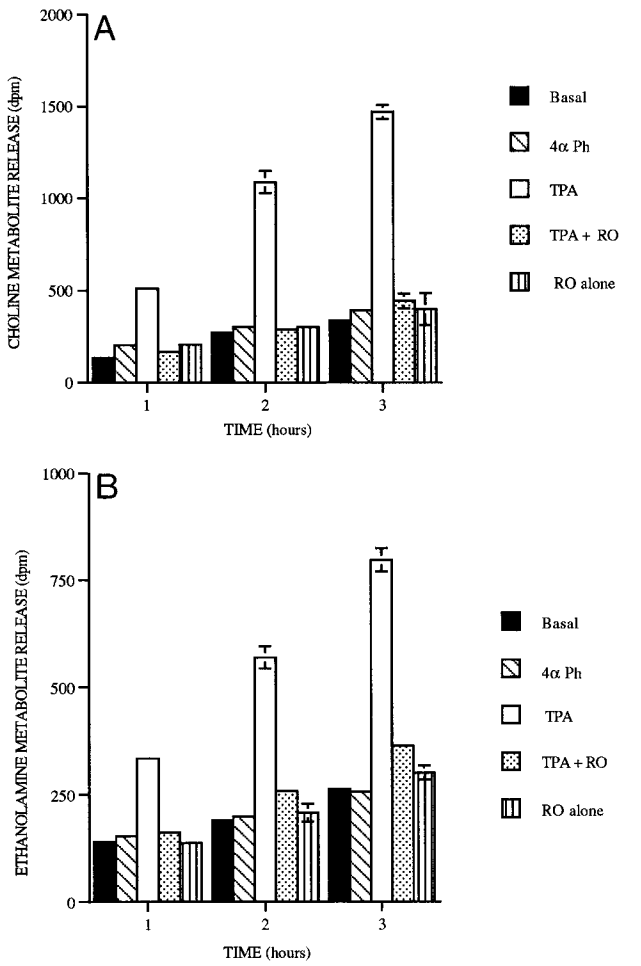

Fig. 1. Action of the phorbol ester TPA and the bisindolylmaleimide Ro31-8220 on: A, choline- and, B, ethanolamine-metabolite release to the extracellular medium from C6 glioma cells. Cells were labelled to equilibrium as described and treated for the time periods shown with TPA or 4α-phorbol (100 nM) or Ro31-8220 (1 μM). Choline- and ethanolamine-metabolite release to the extracellular medium was assayed as described. Results are means from at least three separate wells of cells with radioactivity counted in duplicate and are ± S.E.M.

(TPA, PMA) and 4α-phorbol were from the Alexis Corporation (UK) Ltd. (Bingham, Notts., England).

2.2. Incubation of C6 cells

C6 glioma cells were grown in DMEM supplemented with 10% FBS in 25 cm^2 flasks. Medium was changed every third day. Cells were passaged when just confluent by rinsing with Tris-buffered saline followed by releasing with 1×trypsin/EDTA for 2 min. The trypsin was inactivated by addition of fresh DMEM/10% FBS and cells were passaged at a ratio of 1:3 or 1:4 into 12- or 24-well plates for experiments. When cells were about 70–80% confluent medium was removed and fresh DMEM/10% FBS containing 0.5 μCi radiolabelled choline or ethanolamine was added back to each well for 24 h since about 15–20 h are needed for equilibration of choline and ethanolamine pools in C6 cells [10].

2.3. Stimulation of cells and release of lipid headgroup metabolites

Labelling medium was removed from wells and cells were rinsed twice with DMEM/10% FBS at 37°C. Fresh DMEM/10% FBS (500 μl/well in a 24-well plate) containing suitable concentrations of TPA or 4α-phorbol with Ro31-8220, rapamycin or PD98059 as appropriate were then added and 30 μl aliquots of medium were immediately withdrawn into Eppendorf tubes on ice at time zero and at hourly intervals thereafter. These aliquots of supernatant were centrifuged at 13000 rpm to pellet any cell fragments and duplicate aliquots of the supernatant were removed for scintillation counting to follow the phorbol ester-stimulated release of radiolabelled lipid headgroup metabolites [8,10]. Results are expressed as the mean dpm/aliquot of supernatant and are ± the S.E.M. calculated from triplicate or quadruplicate wells treated identically. Individual experiments were repeated at least twice to ensure similar trends.

3. Results

3.1. Phorbol ester-stimulated release of lipid headgroup metabolites

We have shown previously [7,8,10] that TPA-treatment of Cho- and Eth-labelled C6 cells stimulates the release of phospholipid headgroup metabolites to the extracellular medium and that the bulk of the radioactivity is radiolabelled Cho or Eth indicative of PLD activation. Such findings are confirmed in Fig. 1A where TPA at 100 nM markedly stimulates the release of Cho-metabolites from C6 cells: this release increases with time in an almost linear manner up to at least 3 h. Treatment of C6 cells with the inactive 4α-phorbol had no such stimulatory effect, values for metabolite release being similar to basal samples treated with DMSO alone. The stimulatory effect of TPA on Cho-metabolite release was almost completely inhibited by treatment of cells with 1 μM Ro31-

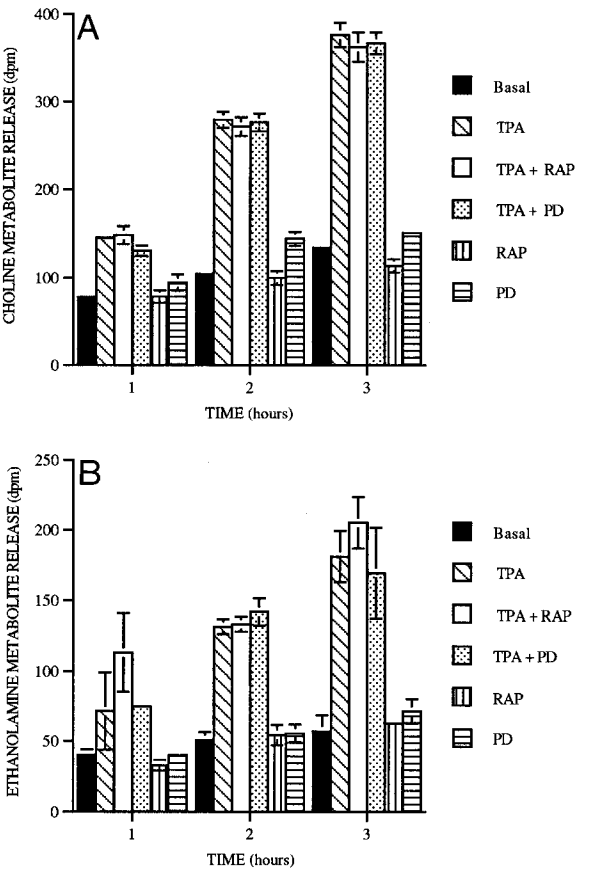

Fig. 2. Action of rapamycin and PD98059 on the TPA-stimulated release of: A, choline- and B, ethanolamine-metabolites to the extracellular medium from C6 glioma cells. Cells were treated as in the legend to Fig. 1. TPA was at 100 nM, rapamycin was at 100 nM and PD98059 was at 50 μM. Results are means from at least three separate wells of cells with radioactivity counted in duplicate and are ± S.E.M.

8220 (Fig. 1A) added at the same time as TPA. Ro31-8220 also effectively inhibited Cho-metabolite release at a 100 nM concentration. When added to labelled C6 cells in the absence of TPA 1 μM Ro31-8220 had no effect on Cho-metabolite release.

C6 glioma cells also show a well-defined TPA-stimulated release of Eth-lipid headgroup metabolites (Fig. 1B) which is not observed with 4α-phorbol. This TPA-stimulated release of Eth-headgroup metabolites is also blocked by 1 μM Ro31-8220. In the absence of TPA 1 μM Ro31-8220 had no effect on Eth-metabolite release from C6 cells over a 3 h period.

3.2. *Effects of rapamycin and PD98059*

The results in Fig. 2A demonstrate clearly that the immunosuppressant, rapamycin, at a concentration of 100 nM had no inhibitory effect on the TPA-stimulated release of Cho-metabolites to the extracellular medium. Higher concentrations of rapamycin (1 μM) were also without effect. As shown in Fig. 2B rapamycin also had no effect on the TPA-stimulated release of Eth-metabolites to the extracellular medium. Rapamycin alone at 100 nM caused no change in the basal release of Cho- or Eth-lipid headgroup metabolites to the medium.

The TPA-stimulated release of Cho- or Eth-lipid headgroup metabolites from C6 cells was also not inhibited by the MAPKK inhibitor PD98059, which blocks activation of MAPKAP kinase-1β. PD98059 was used at a concentration of 50 μM as reported [11]. At this concentration, in the absence of TPA, PD98059 alone had no effect on Cho- or Eth-metabolite release.

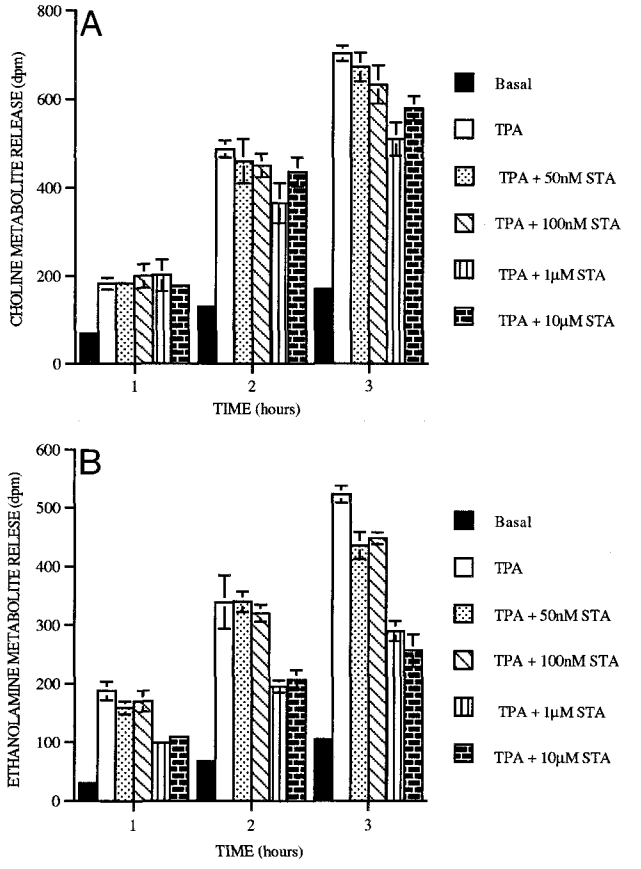

Fig. 3. Action of staurosporine (STA) at different concentrations on the TPA-stimulated release of: A, choline- and, B, ethanolamine-metabolites to the extracellular medium from C6 cells. Cells were treated as in the legend to Fig. 1. Results are means from at least three separate wells of cells with radioactivity counted in duplicate and are ±S.E.M.

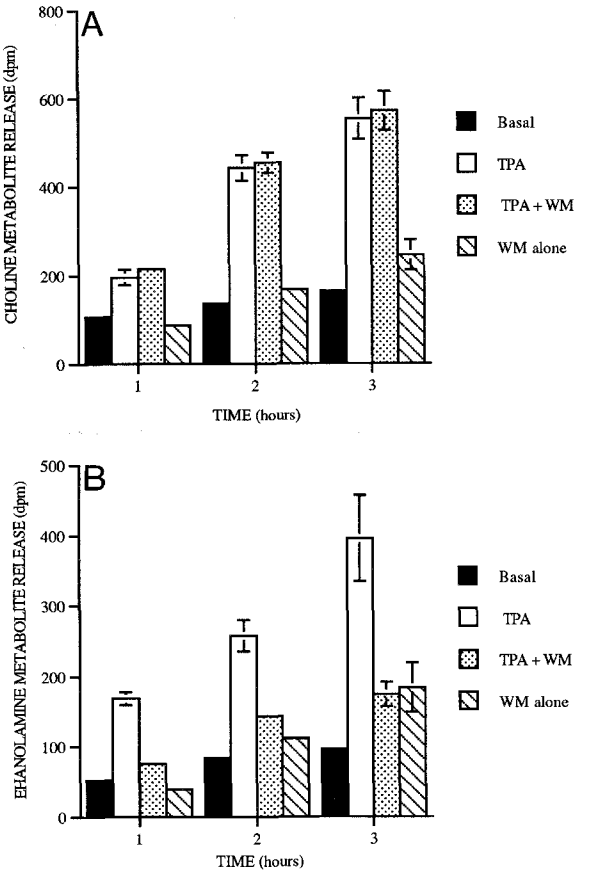

Fig. 4. Action of wortmannin (WM, 5 μM) on the TPA-stimulated release of: A, choline- and B, ethanolamine-metabolites to the extracellular medium from C6 cells. Cells were treated as in the legend to Fig. 1. Results are means from at least three separate wells of cells with radioactivity counted in duplicate and are ±S.E.M.

3.3. *Ro31-8220 and staurosporine differ in their effects*

Staurosporine, the parent compound from which Ro31-8220 is derived, was less effective in inhibiting TPA-stimulated Cho-metabolite release from C6 cells than Ro31-8220 (Fig. 3A). At 1 μM staurosporine induced a slight inhibition of Cho-metabolite release but this was not so obvious at a concentration of 10 μM. Curiously, staurosporine was more effective at inhibiting TPA-stimulated Eth-metabolite release from C6 cells: concentrations of 1 μM and 10 μM produced about a 50% inhibition (Fig. 3B).

3.4. *Effect of the PI-3 kinase inhibitor, wortmannin*

Wortmannin, at a concentration of 5 μM, had no inhibitory effect on the TPA-stimulated release of Cho-metabolites from C6 cells (Fig. 4A) and had no influence used alone on basal Cho-metabolite release values. On the other hand, the same concentration of wortmannin almost completely inhibited TPA-stimulated Eth-metabolite release (Fig. 4B). In the absence of TPA 5 μM wortmannin alone had no effect on basal Eth-metabolite release values (Fig. 4B).

4. Discussion

PKC, the only kinase activated by phorbol esters such as TPA, is a major regulator of PLD [12–15,27] which catalyses the stimulated turnover of PtdCho and PtdEth by both PKC-dependent and PKC-independent routes [4,12,16,17,27]. It is not yet resolved whether PKC activates PLD directly or via other signalling molecules such as Rho and ARF [27]. In most cells activation of PLD via PKC results in the stimulated turnover of PtdCho but in C6 glioma cells and type 1 astrocytes in primary culture a stimulated turnover of ethanolamine-containing phosphoglycerides, including both diacyl and plasmalogenic forms, also occurs [8,10,12,18]. Release of Cho- and Eth-metabolites to the extracellular medium is a direct way to follow PKC-stimulated turnover of complex lipids via PLD and the bulk of the radioactivity released is Cho or Eth indicative of PLD activation [8,10]. Radiolabelled Eth released from cells in such experiments does not arise by the action of an extracellular phosphatase on PEth [18]. To observe radiolabelled lipid headgroup metabolite release most efficiently cold Cho or Eth has to be in the extracellular medium [8,10,18] because released lipid headgroups are channelled back into cells for biosynthesis [19].

Here we show again that TPA, but not inactive 4α-phorbol, clearly stimulates the turnover of PtdCho and PtdEth in C6 glioma cells as revealed by the almost linear increase in Cho- and Eth-metabolites in the external medium over basal levels up to 3 h. The steady basal release of headgroup metabolites presumably represents the normal turnover of phospholipid headgroups and release of choline- and ethanolamine-metabolites for re-use [19]. The TPA-stimulated turnover of complex lipids is completely blocked by 100 nM Ro31-8220, a concentration which is well below the 2.5–5 μM level at which this inhibitor causes activation of the stress-activated protein kinase JNK1 [20]. Until recently such findings with Ro31-8220 would have indicated an involvement of PKC in PLD activation. However, the recent report of Alessi [6] that Ro31-8220 is a good inhibitor of $p70^{S6k}$ and MAPKAP kinase-1β in vitro, in addition to its inhibition of PKC, means that such conclusions cannot be drawn until the involvement of these other kinases has been examined. Alessi states [6] that rapamycin is a specific inhibitor of $p70^{S6k}$ while PD98059 specifically blocks MAPKAP kinase-1β activity by inhibiting activation of MAPKK-1 [11]. These two inhibitors can therefore be used to define the role of $p70^{S6k}$ and MAPKAP kinase-1β in signalling pathways. Our results (Fig. 2) show that neither rapamycin nor PD98059 inhibit the TPA-stimulated release of Cho- and Eth-metabolites. This shows that $p70^{S6k}$ and MAPKAP kinase-1β are not involved in signalling pathways downstream of PKC to PLD. PD98059 acts by preventing activation of MAPKK-1 by Raf so our findings suggest that MAPK also does not function between PKC and PLD. Rapamycin and PD98059 alone had no effect on basal Cho- and Eth-lipid head group release from C6 cells indicating that these substances do not directly stimulate PLD, PC-PLC or other signalling molecules regulating pathways to PLD and PC-PLC. The concentrations of rapamycin used (100 nM, 1 μM) are greatly in excess by 20 and 200 times respectively of the 5 nM concentration reported to completely inhibit $p70^{S6k}$ from several different cell types [28,29]. PD98059 was used at 50 μM as reported for intact cells by Alessi et al. [11] to inhibit MAPKK-1. We know from related work that 100 nM rapamycin fully inhibits synthesis of PKC-ε in C6 cells (Beale, G., unpublished results) and that 50 μM PD98059 inhibits PKC-ε synthesis in 3T6 fibroblasts (Watson, J., unpublished results).

Ro31-8220, however, still fully inhibits Cho- and Eth-metabolite release from C6 cells confirming our previous results with C6 cells [7,8] and results of others with neutrophils and fibroblasts [4,9]. The 100 nM concentration of Ro31-8220 used is well below the IC_{50} value at which protein kinase A or calcium/calmodulin-dependent protein kinase are inhibited [2] and, as our findings above also exclude an involvement of $p70^{S6k}$ and MAPKAP kinase-1β, this result with Ro31-8220 indicates a role for PKC in phorbol ester-stimulated phospholipid turnover, as previously believed [12,27]. The α and β subspecies of PKC, but not the δ-, ε- or ζ-forms have been found to activate PLD in fibroblast membranes [21] while overexpression studies in fibroblasts have linked PKC-β_1 to PLD activation [22]. A down-regulation approach in C6 cells also reveals a role for PKC-α in activating PLD [8].

The specific PI-3 kinase inhibitor, wortmannin, blocked the TPA-stimulated release of Eth-lipid metabolites but had no effect on the release of Cho-lipid metabolites (Fig. 4). This suggests that different PLD isoforms regulate the turnover of PtdCho and PtdEth in C6 cells. Both forms are regulated partly through PKC as shown by the common stimulatory effect of TPA and the action of Ro31-8220. The PtdEth-hydrolysing PLD isoform, additionally, is regulated through PI-3 kinase. In C6 cells the PtdEth-specific PLC may have a specific requirement for PIP_3 since such very polar lipids are needed for PLD activity [16,23]. Alternatively, there may be an as yet undefined link between PI-3 kinase and the PtdEth-specific PLD perhaps involving tyrosine kinases and/or small GTP-binding proteins such as Ras and Rho [16]. In adipocytes an insulin-activated PC-PLD has been identified downstream of PI-3 kinase [25]. Different isoforms of PLD co-exist in the same cell and have separate cytosolic and membrane localisations [12,24]. The differing effects of staurosporine (Fig. 3) on TPA-stimulated Cho- and Eth-metabolite release also support the view that separate PLD isoforms regulate the TPA-stimulated turnover of PtdCho and PtdEth in C6 cells. However, staurosporine effects are hard to interpret since this compound can directly activate PLD at high concentrations [26]. At 10 μM the slight inhibitory effect of staurosporine on TPA-stimulated Cho-metabolite release was reversed while this was not the case for TPA-stimulated Eth-metabolite release where inhibition increased.

This study confirms that $p70^{S6k}$, MAPK and MAPKAP kinase-1β are not involved downstream of PKC in signalling to PLD and that Ro31-8220 blocks TPA-stimulated PtdCho and PtdEth turnover by inhibiting PKC. Results with wortmannin suggest that TPA-stimulated turnover of PtdCho and PtdEth in C6 glioma cells may be mediated through separate PLD isoforms. Both isoforms are regulated through PKC but the PtdEth-PLD is regulated additionally via PI-3 kinase.

Acknowledgements: This work is supported by grants from the BBSRC and The Wellcome Trust. We thank Rebecca Turner for help with cell culture.

References

[1] Gordge, P.C. and Ryves, W.J. (1994) Cell. Signall. 6, 871–882.

[2] Davis, P.D., Hill, C.H., Keech, E., Lawton, G., Nixon, J.S., Sedgwick, A.D., Wadsworth, J., Westmacott, D. and Wilkinson, S.E. (1989) FEBS Lett. 259, 61–63.
[3] Wilkinson, S.E., Parker, P.J. and Nixon, J.S. (1993) Biochem. J. 294, 335–337.
[4] Briscoe, C.P., Martin, A., Cross, M. and Wakelam, M.J.O. (1995) Biochem. J. 306, 115–122.
[5] Standaert, M.L., Avignon, A., Yamada, K., Bandyopadhyay, G. and Farese, R.V. (1996) Biochem. J. 313, 1039–1046.
[6] Alessi, D.R. (1997) FEBS Lett. 402, 121–123.
[7] Mallon, B.S., Thompson, H.J.M., Turner, R.A., Holden, C.M. and Rumsby, M.G. (1993) Biochem. Soc. Trans. 21, 354S.
[8] Mallon, B.S. (1996) D.Phil. thesis, University of York.
[9] Uings, I.J., Thompson, N.T., Randall, R.W., Spacey, G.D., Bonser, R.W., Hudson, A.T. and Garland, L.G. (1992) Biochem. J. 281, 597–600.
[10] McNulty, S., Lloyd, G.S., Rumsby, R.M., Sayner, R.M. and Rumsby, M.G. (1992) Neurosci. Lett. 139, 183–187.
[11] Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995) J. Biol. Chem. 270, 27489–27494.
[12] Kiss, Z. (1996) Chem. Phys. Lipids 80, 81–102.
[13] Exton, J.H. (1994) Biochim. Biophys. Acta 1212, 26–42.
[14] Billah, M.M. (1993) Curr. Opin. Immunol. 5, 114–123.
[15] Morris, A.J., Engebrecht, J. and Frohman, M.A. (1996) Trends Pharmacol. Sci. 17, 182–185.
[16] Houle, M.G. and Bourgoin, S. (1996) Biochem. Cell Biol. 74, 459–467.
[17] Lacal, J.C. (1997) FEBS Lett. 410, 73–77.
[18] Van Iderstine, S.C., Byers, D.M., Ridgway, N.D. and Cook, H.W. (1996) J. Lipid Med. Cell Signal. 15, 175–192.
[19] George, T.P., Morash, S.C., Cook, H.W., Byers, D.M., Palmer, F.B.S.C. and Spence, M.W. (1989) Biochim. Biophys. Acta 1004, 283–291.
[20] Beltman, J., McCormick, F. and Cook, S.J. (1996) J. Biol. Chem. 271, 27018–27024.
[21] Conricode, K.M., Smith, J.L., Burns, D.J. and Exton, J.H. (1994) FEBS Lett. 342, 149–153.
[22] Pai, J.-K., Pachter, J.A., Weinstein, I.B. and Bishop, W.R. (1991) Proc. Natl. Acad. Sci. USA 88, 598–602.
[23] Brown, H.A., Gutowski, S., Kahn, R.A. and Sternweisk, P.C. (1995) J. Biol. Chem. 270, 14935–14943.
[24] Wang, P., Anthes, J.C., Siegel, M.I., Egan, R.W. and Billah, M.M. (1991) J. Biol. Chem. 266, 14877–14880.
[25] Standaert, H.L., Avignon, A., Yamada, K., Bandyopadhyay, B. and Farese, R.V. (1996) Biochem. J. 313, 1039–1046.
[26] Perianin, A., Combadiere, C., Pedruzzi, E., Djerdjouri, B. and Hakim, J. (1993) FEBS Lett. 315, 33–37.
[27] Exton, J.H. (1997) Physiol. Rev. 77, 303–319.
[28] Price, D.J., Grove, J.R., Calvo, V., Avruch, J. and Bierer, B.E. (1992) Science 257, 973–977.
[29] Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J. (1992) Cell 69, 1227–1236.