

Distinct phospholipase C-regulated signalling pathways in Swiss 3T3 fibroblasts induce the rapid generation of the same polyunsaturated diacylglycerols

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Abstract Prostaglandin $F_{2\alpha}$, platelet-derived growth factor (PDGF) and calcium ionophore A23187 stimulated the rapid (within 25 s) generation of polyunsaturated 1,2-diacylglycerol (DAG) species, in particular 18:0/20:3n-9, 18:0/20:4n-6 and 18:0/20:5n-3, in Swiss 3T3 fibroblasts. This was followed by a second sustained phase characterised by saturated, monounsaturated and diunsaturated DAG species derived, at least partially, from a phospholipase D/phosphatidate phosphohydrolase-linked pathway. This could be directly activated by phorbol ester. Assay of rat brain protein kinase C (PKC) in lipid vesicles showed that first phase, polyunsaturated-enriched DAG isolated from Swiss 3T3 cells was a more potent activator of kinase activity compared to that achieved with DAG from control or 5 min stimulated cells. Thus activation of distinct members of the phospholipase C family leads to the rapid and almost identical generation of polyunsaturated DAG species which are capable of preferentially activating protein kinase C (PKC).

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Key words: Cell signalling; Diacylglycerol; Phospholipase C; Protein kinase C; Swiss 3T3

1. Introduction

The mechanism and role of lipid messenger generation during signal transduction is still incompletely understood. However, one of the earliest events following stimulation is the phospholipase C (PLC)-catalysed generation of inositol 1,4,5-trisphosphate (IP_3) and the diradylglycerol (DRG) 1,2-diacylglycerol (DAG), the physiological activator of protein kinase C (PKC) [1–5]. The other DRGs, 1-alkyl,2-acylglycerol and 1-alkenyl,2-acylglycerol, are poor PKC activators and indeed may act physiologically to downregulate this kinase [6].

At least three different PLCs appear able to generate the rapid first phase in Swiss 3T3 cells: phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$)- PLC_β which is coupled to receptors through the heterotrimeric G-protein G_q/G_{11} (e.g. bombesin, vasopressin [7,8,20]), $PtdInsP_2$ - PLC_γ which is activated via

receptor tyrosine kinases (e.g. platelet-derived growth factor (PDGF) [9,20]) and a PKC-independent, probably polyunsaturated $PtdCho$ -specific PLC (epidermal growth factor (EGF) [10,11]). Despite reports of an interaction with the G_{hi} GTP-binding protein, it is unclear if $PtdInsP_2$ - PLC_δ can couple to a receptor via a G-protein, however it may be activated by changes in IP_3 and Ca^{2+} concentration [20].

PKC has a multiplicity of roles including the attenuation of the initial PLC reaction (preventing sustained $PtdInsP_2$ hydrolysis) and activation of a second phase of DAG production via a sequential phospholipase D (PLD)/phosphatidate phosphohydrolase (PAP) pathway and possibly another PLC pathway [12–14]. The first phase of stimulated DAG generation is normally maximal within 30 s, whilst the second phase rises shortly thereafter, often remaining elevated for 30 min or more.

In 3T3 fibroblasts PLD is activated by many agents (e.g. bombesin [7,8], EGF [11], PDGF [9], $PGF_{2\alpha}$ [7], vasopressin [7], calcium ionophore, A23187 [8], tetradecanoyl phorbol acetate (TPA) [8]) but only some produce a PAP-catalysed DAG accumulation (e.g. TPA, bombesin [8,11]). Thus, acute DAG production is not necessarily the consequence of PLD activation, suggesting that $PtdOH$ has a signalling role in its own right [14,15]. If so, PAP activity would be a mechanism to attenuate the $PtdOH$ signal and enable re-synthesis of the parent phospholipid. The observation that only DAG derived from phosphatidylcholine ($PtdCho$) via a PLD/PAP-linked pathway was required to induce cytidyltransferase translocation and subsequent choline incorporation to reform $PtdCho$ in neutrophils [16], together with our recent work on porcine aortic endothelial cells where we presented evidence that PLD/PAP-generated DAG (saturated, monounsaturated and diunsaturated species) is a poor activator of PKC *in vivo* [17], supports this proposal. This implies that PKC activation *in vivo* requires a different set of DAG species, probably the polyunsaturated forms.

To address this issue we investigated the effect of four dissimilar agents upon DAG generation: the growth factor PDGF which acts through a tyrosine kinase, the eicosanoid $PGF_{2\alpha}$ which acts through a G-protein-coupled receptor, TPA which directly activates PKC and the calcium ionophore A23187 which induces elevated intracellular $[Ca^{2+}]$. The results show that a common cellular response to a variety of stimuli is the rapid and transient generation of an almost identical set of polyunsaturated DAG species even though the PLC enzyme involved may be different. This is followed by the more sustained production of mostly saturated and monounsaturated DAG species coming, at least partially, via a $PtdCho$ -PLD/PAP-linked pathway. In a lipid vesicle assay, the polyunsaturated-linked species are stronger activators of

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Abbreviations: DRG, diradylglycerol; DAG, diacylglycerol; $PtdOH$, phosphatidic acid; $PtdCho$, phosphatidylcholine; $PtdEtn$, phosphatidylethanolamine; $PtdIns$, phosphatidylinositol; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate; $PtdBut$, phosphatidylbutanol; $PtdSer$, phosphatidylserine; IP_3 , inositol 1,4,5-trisphosphate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PLC, phospholipase C; PLD, phospholipase D; PAP, phosphatidate phosphohydrolase; PKC, protein kinase C; DNB, 3,5-dinitrobenzoyl (derivative); HBS, Hanks' buffered saline

PKC than those species found in control cells or generated during the second phase of acute DAG production.

2. Materials and methods

All solvents were of high-performance liquid chromatography (HPLC) or analytical grade, purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland, UK. Human recombinant $\beta\beta$ -PDGF was from Gibco-BRL. Calcium ionophore A23187, $\text{PGF}_{2\alpha}$, TPA, histone and all other chemicals were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK. Swiss 3T3 mouse fibroblasts were cultured in Dulbecco's Modified Eagle's Medium containing 10% newborn calf serum until approximately 95% confluent and then in medium containing 2% serum for a further 24 h to quiesce the cells.

Fibroblasts were stimulated in triplicate as previously described [10,18]. Optimum, maximally effective concentrations were determined in earlier work (PDGF, 30 ng/ml [9]; $\text{PGF}_{2\alpha}$, 1 μM [7]; A23187, 5 μM ; TPA, 100 nM [8]). Where required, 30 mM butan-1-ol (to block DRG generation via the PLD/PAP pathway) or 10 μM Ro-31.8220 (to block the kinase activity of PKC) was added to the HBG 5 min prior to stimulation. Incubations were stopped by aspiration of the medium followed by addition of 2 ml ice-cold methanol together with internal standard (1 μg 1,2-12:0/12:0 DAG). Lipids were extracted, derivatised with 3,5-dinitrobenzoylchloride, separated by HPLC and identified as in earlier work [10,18]. DAG for the PKC assay was quantified using the DAG kinase mass assay [21].

PKC assays were performed in glass tubes (50 μl /sample) using a mixed lipid vesicle system (sonicated until fully dispersed) containing 450 μM egg PtdCho, 50 μM bovine brain phosphatidylserine (PtdSer), 0.5 μM DAG (or 0.5 μM TPA for positive control), 200 $\mu\text{g}/\text{ml}$ histone type IIIIS (Sigma) in kinase buffer (50 mM HEPES, pH 7.5, 75 mM KCl, 20 mM β -glycerophosphate, 10 mM MgCl_2 , 1 mM sodium orthovanadate, 0.1 mM CaCl_2) and rat brain mixed PKCs (Calbiochem). Reactions were started by addition of 0.5 μCi [γ - ^{32}P]-ATP at a final concentration of 20 μM . Following incubation for 2 h at 35°C the reaction was stopped with 50 μl boiling 2 \times gel loading buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromophenol), boiled for a further 5 min and separated on 17.5% SDS-polyacrylamide gels. The phosphorylated histone band was detected and quantified using a phosphorimager.

3. Results and discussion

Quiescent Swiss 3T3 mouse fibroblasts contain approximately 4 nmol DRG/ 10^7 cells and have a basal DAG molecular species profile most closely resembling that of total cellular PtdCho (see [18]). Stimulation with A23187, PDGF, $\text{PGF}_{2\alpha}$ or TPA caused time-dependent changes in total DAG mass (Table 1). PDGF, $\text{PGF}_{2\alpha}$ and A23187 all stimulated an elevation at 25 s which returned to basal levels within 5 min for A23187 and $\text{PGF}_{2\alpha}$ and within 30 min for PDGF. Only with the phorbol ester was total DAG mass still significantly elevated at 30 min. This was probably due to PKC activation causing sustained stimulation of downstream phospholipases hence leading to a prolonged DAG signal. Using the DAG kinase-linked assay we previously showed that

PDGF stimulated a biphasic elevation in total DRG mass [9] whereas TPA only stimulated a monophasic response [8]. However, this methodology was unable to distinguish between the DRG classes. HPLC separation of these classes demonstrated no changes in 1-alkyl,2-acylglycerol or 1-alkenyl,2-acylglycerol even though they represent approximately 30% of the total basal DRG (30% alkyl,acylglycerol, <1% alkenyl,acylglycerol). This is similar to stimulation with bombesin [18] and EGF [10], neither of which had any acute effect on these classes even though the most likely substrates for second-phase DRG generation, PtdCho and PtdEtn, contain significant amounts of alkyl,acyl and alkenyl,acyl forms [18]. Thus diacyl phospholipids are selectively hydrolysed to generate DAG in response to receptor stimulation. This may be due to compartmentalisation where the relevant phospholipase only has access to diacyl species or, alternatively, the enzyme(s) may be specific for lipids containing an ester bond (with two oxygen atoms) at the *sn*-1 position. This is not specific to Swiss 3T3 cells since a similar response was obtained in TPA-stimulated Madin-Darby canine kidney cells where diacyl- but not alkyl,acyl glycerol species were elevated [19].

PDGF, $\text{PGF}_{2\alpha}$ and A23187 stimulated the greatest changes in DAG profiles (relative to the basal profile) at the 25-s time point. Most of the increased mass was due to an elevation of polyunsaturated species, in particular 18:1n-9/20:3n-9, 18:0/20:3n-9, 18:0/20:4n-6 and 18:0/20:5n-3 (Fig. 1). $\text{PGF}_{2\alpha}$ stimulates a receptor/G-protein/PtdInsP₂-PLC β coupled mechanism in these cells [7] whilst PDGF activates PtdInsP₂-PLC γ via a receptor tyrosine kinase. Since elevated cytosolic [Ca^{2+}] can activate phosphoinositide-PLC [2,20], in particular PLC δ , the A23187 may initially activate this enzyme. Therefore the results in Fig. 1 could suggest that PLC δ , like PLC β and PLC γ , generates primarily polyunsaturated DAGs. Agonists which, through the generation of IP₃ by either PLC β or PLC γ , stimulate an elevation of cytosolic [Ca^{2+}] could also activate PLC δ , giving a combined effect which might enhance the polyunsaturated DAG changes. The small, rapid and transient elevation of total DAG often seen in control samples, probably resulting from the small, unavoidable environmental fluctuations induced during the experimental protocol (e.g. temperature, shear forces, mixing effects), may be caused by transient cytosolic Ca^{2+} changes activating this PLC δ .

The rapid initial phase of DAG generation is followed by a second, more sustained phase. Close examination of the molecular species profiles indicated that for PDGF, $\text{PGF}_{2\alpha}$ and A23187 another source is utilised to produce this second phase of DAG elevation. This profile is characterised predominantly by saturated, monounsaturated and diunsaturated species (18:1n-9/18:2n-6+16:1/18:1n-9, 16:0/16:1+14:0/18:1n-9,

Table 1
Changes in total 1,2-diacylglycerol mass following stimulation of 3T3 fibroblasts with PDGF, $\text{PGF}_{2\alpha}$, calcium ionophore (A23187) or phorbol ester (TPA)

	% of basal DAG \pm S.D.			
	PDGF	$\text{PGF}_{2\alpha}$	A23187	TPA
Control	100 \pm 7	100 \pm 6	100 \pm 6	100 \pm 12
25 s	182 \pm 32*	167 \pm 26*	125 \pm 19*	ND
5 min	142 \pm 26*	100 \pm 20	98 \pm 14	140 \pm 7*
30 min	117 \pm 23	104 \pm 24	112 \pm 25	148 \pm 24*
<i>n</i>	4	3	3	3

Basal DAG = 3 nmol/ 10^7 cells. ND = not determined. *Significantly elevated above controls ($P < 0.05$).

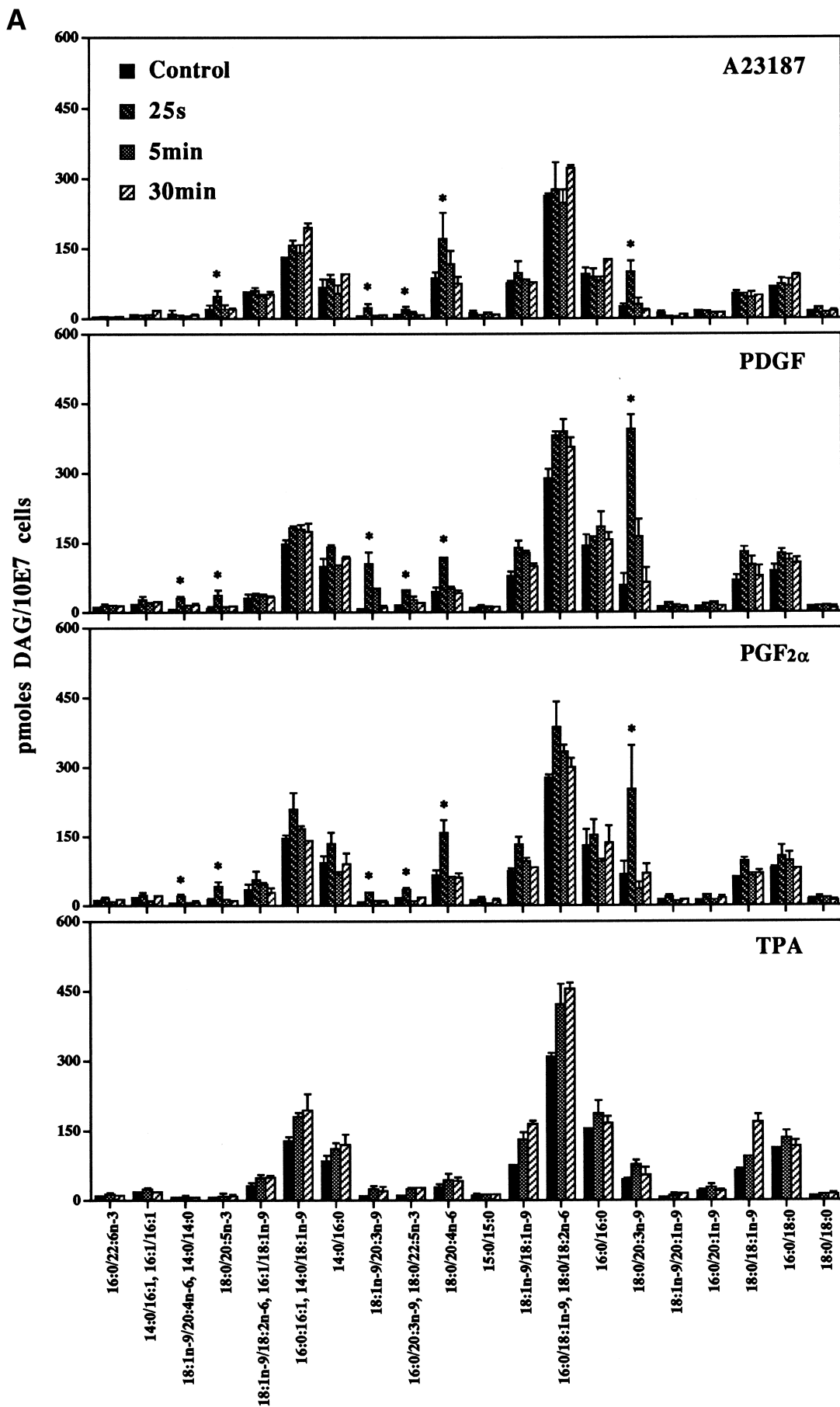


Fig. 1.

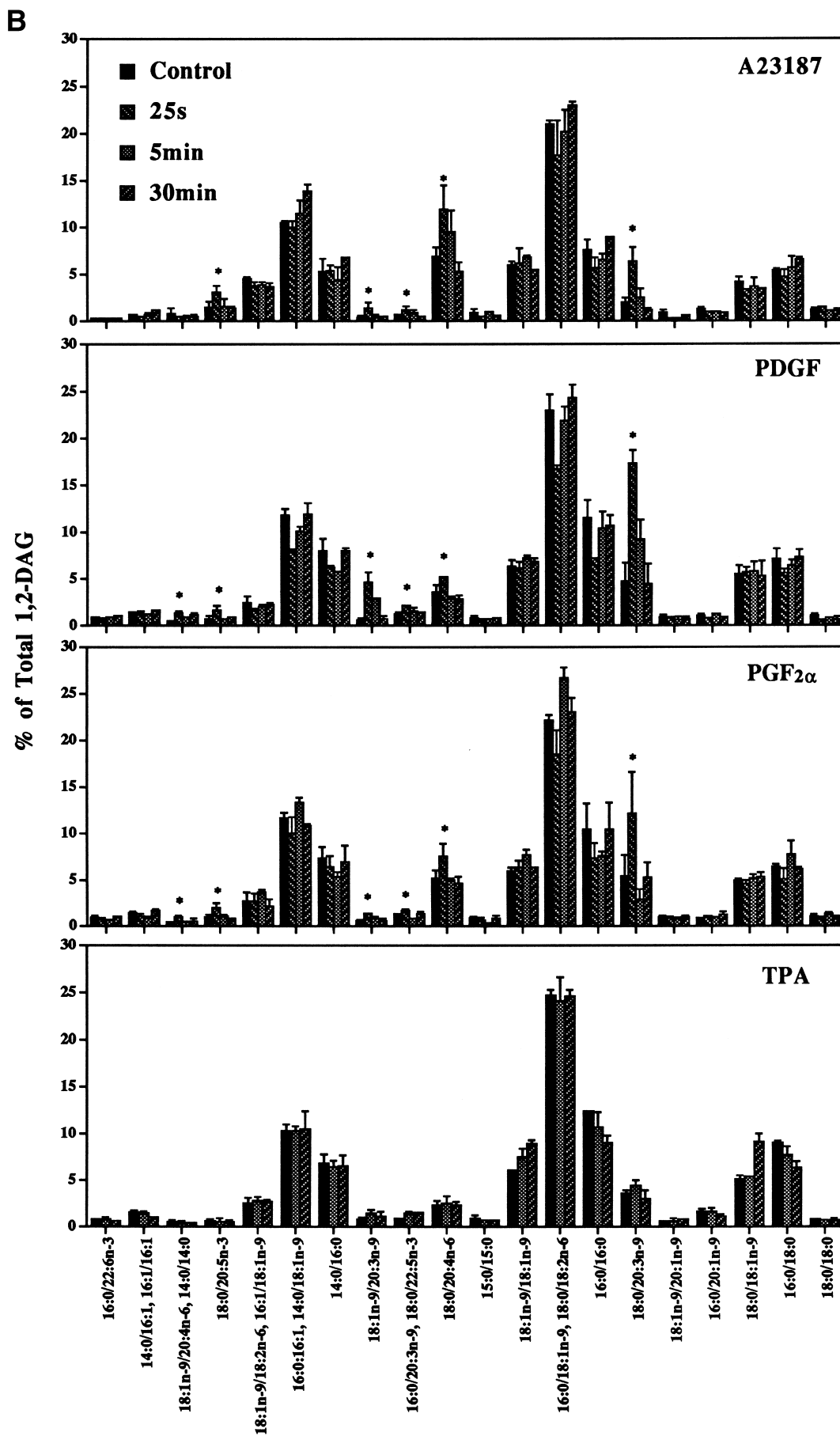


Fig. 1 (Continued).

Fig. 1. Changes in DAG species profiles following stimulation with calcium ionophore (A23187), PDGF, $\text{PGF}_{2\alpha}$ or phorbol ester (TPA). Data expressed as (A) absolute mass of each species ($\text{pmol}/10^7$ cells) \pm S.D. ($n=3-4$) or (B) as a percentage of total cellular DAG mass \pm S.D. ($n=3-4$). Over 30 DAG species were identified; however, the minor species (each representing $<0.5\%$ of total DAG) have been omitted from the figure. ●, species significantly elevated at 25 s; $P<0.05$ using Student's paired t -test.

18:1n-9/18:1n-9, 16:0/18:1n-9+18:0/18:2n-6, 16:0/18:0; Fig. 1) although the kinetics of their generation varied depending on the stimulus. Since this source produced DAG species with a profile very similar to that of the basal DAG (although the mass of individual species is elevated, Fig. 1A, the relative proportions of each species resembles that of the control, Fig. 1B) which in turn most closely resembles the acyl profile of total PtdCho (see [18]), the data suggests this phospholipid as the probable substrate for phospholipase-catalysed generation of the second-phase diacyl species.

PDGF-stimulated second phase DAG generation was detectable at 25 s, where it underlay the elevation of the polyunsaturated species, was maximal at 5 min and still elevated at 30 min. We previously showed that the PDGF-elevated second-phase DAG is probably derived via a PKC-dependent, PtdCho-specific PLD/PAP-linked pathway [9]. We also showed that phosphoinositide hydrolysis was slower than that seen with bombesin, commencing after a lag phase of approximately 15 s, and was detectable for up to 5 min. The slightly raised levels of 18:1n-9/20:3n-9 and 18:0/20:3n-9 detected at 5 min (although less than at 25 s), but not at 30 min would confirm this. In contrast, bombesin-stimulated PtdInsP₂-PLC activity is rapidly desensitised and a putative PtdCho-PLC might be responsible for many of the late DAG changes. With bombesin, PAP-catalysed hydrolysis of PtdOH only starts making a significant contribution after 5 min, although by 30 min all DAG is generated by this pathway [8,11,17,18].

The $\text{PGF}_{2\alpha}$ -stimulated second phase of DAG generation (characterised by more saturated species) was maximal at 25 s (where it overlay the polyunsaturated DAG of the initial phase) declining at 5 min and was undetectable by 30 min (Fig. 1A). Since the first and second phases partially overlap and the shortest time point examined was 25 s, both phases appear to be maximal at this time. Whilst the identity of the phospholipase(s) responsible for this second phase remains uncertain, a PLD/PAP-linked pathway is implicated since $\text{PGF}_{2\alpha}$ stimulated the formation of phosphatidylalcohol in the presence of butanol [7].

A PLD/PAP-linked pathway is probably also activated by the calcium ionophore since A23187 has been shown to stimulate PLD activity in Swiss 3T3 cells [8]. Treatment with EGTA or a PKC inhibitor reduced A23187-induced PLD activity by approximately 50%, however, the effects were not

additive [8], suggesting that both extracellular Ca^{2+} influx and the inhibitor operate through the same signalling component, possibly the more calcium-dependent PKC_{α} .

To examine the role of PKC activation in DAG generation, phorbol ester was used to stimulate the kinase directly. Since our earlier work found little change in DAG levels before 1 min [8] only the 5- and 30-min time points were investigated. TPA caused no significant changes from the basal DAG species profile at 5 min although total mass was elevated (Table 1). However, a small increase in the proportion of 18:1n-9/18:1n-9 and 18:0/18:1n-9 species was evident at 30 min (Fig. 1). Based on the species profile, the detection of elevated levels of choline metabolites [7] and the inability to detect any release of inositol or inositol phosphates, this DAG must arise from PtdCho hydrolysis and probably corresponds to the second phase of DAG generation seen with the other stimuli. The PKC inhibitor Ro-31.8220 completely abolished the DAG mass increases (Table 2), but had no effect on the species profiles (data not shown). Thus TPA directly stimulates PKC causing the activation of the second phase without the preceding polyunsaturated DAG phase. The DAG changes could be completely blocked by butanol, indicating the PLD/PAP-linked pathway as the primary route of DAG generation (Table 2). Analysis of PtdOH from 3T3 fibroblasts challenged with a variety of stimuli, including TPA, detected the production of predominantly saturated, monounsaturated and diunsaturated species, with almost no polyunsaturated structures at any time [17]. This supports our proposals that only the more saturated DAG species are produced via the PLD/PAP-linked pathway.

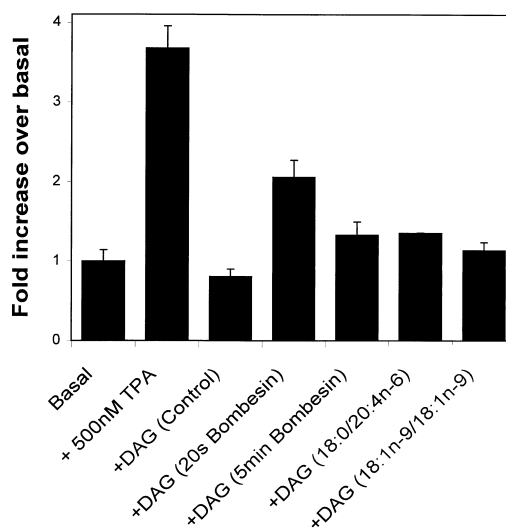


Fig. 2. Activation of rat brain PKC by 500 nM TPA or 500 nM DAG isolated from resting (control), 20-s and 5-min bombesin-stimulated Swiss 3T3 fibroblasts. Lipid vesicles contained 450 μM PtdCho, 50 μM PtdSer, 200 $\mu\text{g}/\text{ml}$ histone type III S and 20 μM [^{32}P]-ATP. Phosphorylated histone was separated on 17.5% SDS-polyacrylamide gels and quantified using a phosphorimager. Values are fold increase in histone phosphorylation \pm S.D. ($n=3$).

Table 2
Inhibition of TPA-stimulated DAG mass changes by butanol and Ro-31.8220

	% of basal DAG \pm S.D.		
	TPA	TPA+butanol	TPA+Ro31.8220
Control	100 \pm 12	89 \pm 16	99 \pm 27
5 min	144 \pm 9	82 \pm 31*	105 \pm 10*
30 min	155 \pm 10	64 \pm 8*	73 \pm 10*
<i>n</i>	6	3	3

Basal DAG = 3 $\text{nmol}/10^7$ cells. *Significantly reduced relative to TPA alone ($P<0.05$).

To investigate PKC activation by the different DAG phases, DAG was isolated from resting (control) Swiss 3T3 fibroblasts and from cells treated for 20 s or 5 min with bombesin (a strong stimulator of biphasic DAG generation [18]). This DAG was then used at a final concentration of 0.5 μ M (0.1 mol%) in a lipid vesicle assay containing PtdCho (90 mol%) and PtdSer (10 mol%), histone and rat brain PKC (a mixture of PKC isoenzymes including α , β , γ , δ , ϵ and ζ). A typical result is shown in Fig. 2 where DAG isolated from cells stimulated for 20 s (and thus enriched in polyunsaturated species [18]) showed an increase in PKC activity as compared to samples containing the DAG from control or 5 min stimulated cells. Experiments using defined, single DAG species confirmed that 18:0/20:4n-6 (the major species generated during the initial, rapid phase) induced a slightly greater PKC activation than 18:1n-9/18:1n-9 (a major species generated during the second phase via the PLD/PAP pathway [17]), a result seen by another group using a slightly different vesicle system [22]. Support for this concept of the importance of the initial polyunsaturated DAG phase comes from work with pancreatic acini where PtdInsP₂-derived DAG (enriched in polyunsaturated species) caused PKC translocation, whereas DAG derived from PtdCho did not [23] and with platelets where thrombin stimulation caused elevation in several DAG species but only elevation of 18:0/20:4n-6 correlated with PKC activation as measured by pleckstrin phosphorylation [24].

In conclusion, acute stimulation of Swiss 3T3 cells induces the rapid generation of polyunsaturated DAG species derived via PtdInsP₂-PLC β (bombesin, PGF_{2 α}), PtdInsP₂-PLC γ (PDGF) or PtdInsP₂-PLC δ (A23187) pathways depending on the stimulatory agent involved. Since the resultant DAG species are effectively identical, the PtdInsP₂-PLC isoenzymes must utilise a common source of PtdInsP₂. This suggests that these DAG species are essential for the activation of an immediate and common downstream target, presumably a specific PKC isoform(s) which often inactivates the initial phase and also stimulates a second phase of DAG generation derived, at least in part, via a PLD/PAP-linked pathway. This DAG, characterised by a more saturated profile, is probably largely inactive as an intracellular messenger. It remains to be confirmed that the PLC-generated polyunsaturated 'early' DAG species are indeed the specific physiological activators of PKC *in vivo*, a function which cannot be effectively achieved by other, more saturated species. However, the re-

sults reported here strongly suggest a structural specificity for the messenger DAG in its capacity as an activator of PKC.

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