Comparison of diglyceride production from choline-containing phosphoglycerides in human neutrophils stimulated with *N*-formylmethionyl-leucylphenylalanine, ionophore A23187 or phorbol 12-myristate 13-acetate

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The turnover of choline-containing phosphoglycerides (PC) in response to agonist stimulation is well documented in human neutrophils. We have now compared the enzymic pathways of *N*-formylmethionyl-leucylphenylalanine (fMLP)-, A23187- and phorbol-12-myristate 13-acetate (PMA)-induced diglyceride (DG) and phosphatidic acid (PA) generation in these cells. In order to distinguish between phospholipase C- and D-mediated PC breakdown, human neutrophils were radiolabelled with 1-*O*-[³H]alkyl-2-acyl-glycero-3-phosphocholine and stimulated in the presence of ethanol or propranolol. The addition of 0.5% ethanol to the incubation mixture resulted in the production of phosphatidylethanol, indicative of phospholipase D activation, in response to all three stimuli. Concomitant with phosphatidylethanol formation was a partial block of PA production. The production of DG was also partially blocked by addition of ethanol. Propranolol (200 μM) was also used to assess the contributions of phospholipases C and D toward DG generation. Inhibition of PA phosphohydrolase by propranolol resulted in the complete abolition of DG generation when neutrophils were stimulated with fMLP. In contrast, propranolol only partially inhibited DG generation in response to A23187 and PMA. These results suggested that DG production in response to fMLP stimulation is mediated via the activation of phospholipase D, whereas A23187- or PMA-induced DG generation may involve more than one pathway. However, examination of the water-soluble choline metabolites produced indicated that phospholipase D was responsible for the production of PA and DG in response to all three stimuli.

INTRODUCTION

The stimulated metabolism of choline-containing phosphoglycerides (PC) plays an important role in a number of cell types, including human polymorphonuclear neutrophils (PMN). It is now well established that the alkyl-linked subclass of PC serves as the precursor of platelet-activating factor [1–3] and a donor of arachidonic acid, which may be further metabolized to 5-hydroxyicosatetraenoic acid and leukotriene B₄ [4–7]. More recently, however, the role of PC as a precursor for 1-acyl-2-acyl-glycerols (DG, 'diglycerides') and phosphatidic acid (PA) has come to light. The turnover of PC in response to agonist stimulation has been reported in a number of cell types [8–15], including human PMN and HL-60 granulocytes [6,16–22]. Recent studies in our laboratory [16,17] and those of others [18–22] have shown that both DG and PA are released from PC in response to the stimulation of human neutrophils with a variety of agents. These findings have generated interest in elucidating the enzymic pathways responsible for the production of these lipid products, since they have been shown to modulate neutrophil function [23,24] and protein kinase C activity [24–28].

The generation of PA and DG can be accounted for by either a phospholipase C- or a phospholipase D-mediated pathway. Studies of human PMN and HL-60 granulocytes double-labelled with 1-*O*-[³H]alkyl-2-acyl-*sn*-glycero-3-[³²P]phosphocholine have

led investigators to conclude that a phospholipase D pathway results in production of PA in these cells [16–22]. Since PA production precedes that of DG, DG formation has been attributed to a phospholipase D-mediated process. In many studies, however, the actual production of DG was not reported, and a contribution of phospholipase C toward DG production could not be excluded.

In the present studies, the enzymic mechanism responsible for the generation of DG from PC was investigated in intact human PMN stimulated with *N*-formylmethionyl-leucylphenylalanine (fMLP), ionophore A23187 or phorbol 12-myristate 13-acetate (PMA). Various agents were used to block or inhibit DG generation via phospholipase D, in order to determine the contribution by phospholipase C toward this product. The results of these approaches suggest that phospholipase D and not phospholipase C is activated in response to fMLP, A23187 and PMA. However, some differences in the responses of PMN to these agents are observed, which warrant further investigation.

MATERIALS AND METHODS

Materials

1-*O*-[9,10-³H₂]Hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine (56 Ci/mmol) was synthesized as described previously [29] and purified by reverse-phase h.p.l.c. when radiolabelled decom-

Abbreviations used: DG, 1-acyl-2-acyl-glycerol ('diglyceride'); fMLP, *N*-formylmethionyl-leucylphenylalanine; GPC, glycerol-3-phosphocholine; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, choline-containing phosphoglycerides; PEt, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil.

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Fig. 1. Effect of ethanol on fMLP-mediated stimulation of PC hydrolysis

Human PMN labelled with 1- O -[^3H]alkyl-2-acyl-GPC were treated with (●, ■) or without (○) fMLP (1 μM) in the presence (■) or absence (●, ○) of 0.5% ethanol for the indicated time. DG (a), PA (b) and PEt (c) were quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of three experiments.

position products accumulated. Lipid standards, except phosphatidylethanol (PEt), used for t.l.c. were purchased from Serdary Research Laboratories (London, Ont., Canada). PEt was synthesized as follows: 50 mg of PA was dissolved in 2.5 ml of pyridine, and 70 mg of tri-isopropylbenzenesulphonyl chloride and 15 μl of ethanol were added. The reaction mixture was incubated for 2 h at 70 $^{\circ}\text{C}$, followed by stirring at room temperature for 3 h. The pyridine was dried under N_2 , the residue dissolved in water and the mixture extracted by the method of Bligh & Dyer [30]. PEt was isolated from the organic phase by chromatography on silica gel 60 plates developed in the organic phase of ethyl acetate/iso-octane/acetic acid/water (11:5:2:10, by vol.) (R_f = 0.33).

PMA, cytochalasin B and BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A23187 was from Calbiochem (La Jolla, CA, U.S.A.). Silica gel 60 plates were obtained from E. Merck (Darmstadt, Germany), and silica gel G plates were from Analtech (Newark, DE, U.S.A.). Plasmagel was purchased from Cellular Products (Buffalo, NY, U.S.A.), and Isolymph was from Gallard-Schlesinger Chemical Manufacturing Corp. (Carle Place, NY, U.S.A.). All solvents were of h.p.l.c. grade from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Isolation of human PMN

Human PMN were isolated as described previously [31]. Heparinized venous blood was collected from healthy donors,

and the erythrocytes were removed by sedimentation at 1 g for approx. 45 min with Plasmagel. Leucocytes were pelleted from the Plasmagel by centrifugation (225 g for 10 min), resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS; Sigma) layered over Isolymph, and centrifuged (400 g for 30 min). The neutrophil pellet was washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, and the residual erythrocytes were removed by hypo-osmotic lysis. The cells were collected by centrifugation (225 g for 10 min), suspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, and counted on a Coulter counter.

Radiolabelling of cellular lipids

PMN ($3.5 \times 10^7/\text{ml}$) were radiolabelled by incubating with 0.5 μCi of 1- O -[9'- ^3H]hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine/ml as described previously [32]. The mixture was incubated at 37 $^{\circ}\text{C}$ with gentle shaking for 30 min, after which the cells were washed with ice-cold PBS. Preliminary studies demonstrated that > 95% of the radiolabel had been taken up by the PMN at this time. Furthermore, > 90% of the cell-associated radioactivity was 1- O -[^3H]alkyl-2-acyl-GPC.

Incubation conditions and extraction of lipids

Radiolabelled PMN were suspended in PBS at $3.5 \times 10^7/\text{ml}$ and prewarmed for 5 min at 37 $^{\circ}\text{C}$ before addition of the stimulating agents. Where appropriate, samples were incubated with propranolol (200 μM) for 5 min before addition of the stimulating agents. Samples were stimulated with fMLP (1 μM , with preincubation with 5 μg of cytochalasin B/ml), A23187 (5 μM) or PMA (0.1 μM). All three agents were added to the incubation mixtures in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide did not exceed 0.1%. At the indicated times (see the Figures), the reaction was terminated by withdrawing 0.5 ml of the sample into 1.5 ml of chloroform/methanol (1:2, v/v) (methanol contained 2% acetic acid). The lipids were then extracted by the method of Bligh & Dyer [30].

T.l.c.

Radiolabelled products in the organic phase were separated with the following solvent systems. Neutral lipids were separated by chromatography on commercially prepared silica gel G plates in hexane/ethyl ether/acetic acid (40:60:1, by vol.). PA and PEt were separated from each other and other lipid components by chromatography on silica gel 60 plates developed in the organic phase of ethyl acetate/iso-octane/acetic acid/water (11:5:2:10, by vol.). Phospholipids and neutral lipids were identified by their migration with authentic standards.

Measurement of water-soluble choline metabolites

Water-soluble hydrolysis products of the phospholipase C and/or D reactions were quantified as follows. PMN were isolated and stimulated with fMLP, A23187 or PMA as described above. At the indicated times 0.5 ml samples were removed and placed in Eppendorf tubes for rapid centrifugation (10 s) to pellet the cells. The supernatant was aspirated and the cell pellet was resuspended in 0.5 ml of deionized water and extracted by the method of Bligh & Dyer [30], except that the methanol contained 2% acetic acid. Samples (850 μl) of the aqueous phase (total volume 2 ml) were placed in clean glass tubes and dried in a 60 $^{\circ}\text{C}$ oven. After differential-ion-pair extraction, the masses of cell-derived choline and phosphocholine were separately determined by radioenzymic phosphorylation *in vitro* by using exogenous choline kinase and [^{32}P]ATP as described in detail by Murray *et al.* [33].

Statistical analysis

Owing to donor-to-donor variability representative experiments are present in the figures. However, the data from replicate

experiments were pooled and compared by a paired Student's *t* test in order to determine statistical significance. These values are present in the text. These statistical analyses were performed by using Statworks statistical software for the Macintosh computer.

RESULTS

The stimulation of human PMN with a variety of agents has been shown to stimulate the hydrolysis of PC to yield PA and DG [6,16–22]. Consistent with these earlier studies, fMLP, A23187 and PMA were found to stimulate the hydrolysis of 1-*O*-[³H]alkyl-2-acyl-GPC from prelabelled cells to yield radiolabelled alkyl-PA and alkyl-DG (Figs. 1–3). (We [16] and others [22,34,35] have shown that both the alkyl- and acyl-linked species of PC are hydrolysed to yield PA and DG in PMN stimulated with a variety of agents. In addition, similar results are obtained when PMN are radiolabelled with an alkyl- or acyl-linked precursor [35]. Therefore, in these and all subsequent experiments PA and DG were measured as [³H]alkyl-PA and [³H]alkyl-DG, both of which are likely to reflect mass changes.) With all stimuli investigated, the formation of radiolabelled DG lagged behind that of PA. Such kinetics suggest a precursor/product relationship in which PC is first acted on by a phospholipase D to yield PA, which is then degraded by PA phosphohydrolase to yield DG. As such, these two enzymes provide an indirect means of

Fig. 2. Effect of ethanol on A23187-mediated stimulation of PC hydrolysis

Prelabelled human PMN were stimulated with (●, ■) or without (○) A23187 (5 μ M) for the indicated time in the presence (■) or absence (●, ○) of 0.5% ethanol. DG (a), PA (b) and PET (c) were quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of five experiments.

Fig. 3. Effect of ethanol on PMA-mediated stimulation of PC hydrolysis

Prelabelled human PMN were stimulated with (●, ■) or without (○) PMA (0.1 μ M) for the indicated time in the presence (■) or absence (●, ○) of 0.5% ethanol. DG (a), PA (b) and PET (c) were quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of three experiments.

DG generation from PC, rather than the direct pathway catalysed by phospholipase C.

To investigate further the contribution of these two pathways towards DG generation, we took advantage of the fact that, in the presence of ethanol, phospholipase D catalyses a transphosphatidyl reaction which results in the formation of PET [36,37]. Thus this reaction serves as a tool for detecting phospholipase D activity and for blocking phospholipase D-mediated PA formation, and hence DG production. In these experiments, ethanol was added to the incubation mixtures at a concentration of 0.5%, and its effect on DG production was monitored. Billah *et al.* [22] have reported that, in human PMN, PET is derived exclusively through the action of phospholipase D. Furthermore, the PET which is formed is metabolically stable and does not contribute significantly to DG production. A23187, fMLP and PMA effectively stimulated PET production when ethanol was included (bottom panels of Figs. 1–3), indicating that all three agents were able to stimulate phospholipase D activity. Concomitant with the production of PET was a decrease in PA production (middle panels of Figs. 1–3). PA production was blocked to the greatest extent at the early time points. However, the time course of this response varied slightly among the three stimuli. Treatment with ethanol resulted in a statistically significant ($P < 0.01$) block of PA generation during the first 1 min of stimulation with A23187 (Fig. 2, middle panel). This was not observed in response to fMLP and PMA.

Fig. 4. Effect of propranolol on fMLP-stimulated PA and DG production

Human PMN labelled with 1- O -[^3H]alkyl-2-acyl-GPC were preincubated with (■) or without (●, ○) 200 μM -propranolol for 5 min before stimulation with fMLP (1 μM) (■, ●) or vehicle for the indicated times. DG and PA were quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of three experiments.

Fig. 5. Effect of propranolol on A23187-stimulated PA and DG production

Prelabelled human PMN were preincubated with (■) or without (●, ○) 200 μM propranolol for 5 min before stimulation with A23187 (5 μM) (■, ●) or vehicle for the indicated lengths of time. DG and PA were quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of three experiments.

Fig. 6. Effect of propranolol on PMA-stimulated PA and DG production

Prelabelled human PMN were preincubated with (■) or without (●, ○) 200 μM -propranolol for 5 min before stimulation with PMA (0.1 μM) (■, ●) or vehicle for the indicated lengths of time. DG and PA were quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of three experiments.

Of particular interest was the effect of ethanol on the production of DG. Again, the time course of ethanol's effect was stimulus-dependent. In PMN stimulated with fMLP (Fig. 1), DG continued to accumulate throughout the incubation period in the absence of ethanol. However, when ethanol was included in the incubation mixture, DG production was completely blocked at 30 s. DG levels then increased until 2 min and then reached a plateau, such that DG production was significantly ($P < 0.001$) inhibited after 5 min of incubation. In contrast, when PMN were stimulated with A23187 or PMA, the blockage of DG formation by ethanol was not evident until after 10–15 min of incubation. (Figs. 2 and 3, top panels). This finding was particularly interesting since, in cells stimulated with A23187, ethanol completely blocked PA production at 1 min, but no significant effect on DG production was observed. In contrast, ethanol significantly blocked the production of DG after 15 min in samples stimulated with PMA. The finding that ethanol only partially blocks fMLP- and PMA-stimulated PA and DG formation and had little effect on A23187-induced DG production prompted further investigation, since PC-specific phospholipase C activation was suggested.

To distinguish between phospholipase C- and D-mediated DG production, an inhibitor of PA phosphohydrolase was employed. Propranolol is a cationic amphiphilic drug which is a potent inhibitor of PA phosphohydrolase [38]. As such, propranolol effectively inhibits the conversion of PA into DG. In this manner, propranolol is useful in determining the contributions of phospholipases C and D toward DG production. In these experiments PMN were preincubated with 200 μM -propranolol for 5 min before the addition of fMLP, A23187 or PMA. When PMN were stimulated with fMLP in the presence of propranolol, the production of DG was completely abolished, with a concomitant increase in the accumulation of PA (Fig. 4). These

Fig. 7. Effect of propranolol on PC hydrolysis in stimulated human PMNs

Human PMN labelled with 1- O - ^3H alkyl-2-acyl-GPC were preincubated with (■) or without (●, ○) 200 μM -propranolol for 5 min before stimulation (■, ●) with 1 μM fMLP (a), 5 μM -A23187 (b) or 0.1 μM -PMA (c) (○, unstimulated). At the indicated times, samples were removed and the PC fraction was quantified as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of three experiments.

results support a role for phospholipase D in the production of PA and DG in response to fMLP.

In contrast with the effect of propranolol on fMLP-stimulated PMN, this drug only partially inhibited DG production in response to A23187 (Fig. 5) and PMA (Fig. 6). Although propranolol augmented PA production in response to A23187 and PMA at the later time points ($P < 0.05$), its effect was not as dramatic as that seen with fMLP stimulation. Of interest was the observation that propranolol inhibited PA production during the first 5 min of stimulation with A23187 ($P = 0.008$) (Fig. 5). This finding led us to investigate the effect of propranolol on PC degradation. Propranolol had no effect on the breakdown of PC stimulated by the chemotactic peptide fMLP (Fig. 7a). However, it did inhibit the breakdown of PC in response to PMA ($P < 0.05$) and A23187, although the latter was not statistically significant (Figs. 7b and 7c). Thus in human PMN propranolol not only inhibits PA phosphohydrolase, but may also inhibit phospholipid breakdown by some unknown mechanism. The inhibition of PC breakdown by propranolol may account in part of its effect on DG generation in response to A23187 and PMA.

The inability of propranolol (200 μM) to block completely A23187- and PMA-induced DG formation at a dose where fMLP-stimulated DG production was completely blocked led to further investigations of possible phospholipase C activity. To this end, the question of enzymic pathway was addressed by

Fig. 8. Production of water-soluble metabolites in response to stimulation of human PMN with fMLP, A23187 or PMA

Human PMN were stimulated (●) with fMLP (1 μM) (a), A23187 (5 μM) (b) or PMA (0.1 μM) (c) (○, unstimulated), and at the indicated times samples were removed and cellular choline was determined as described in the Materials and methods section. Each point represents the mean of duplicate determinations from a representative of five experiments.

measuring the mass of cellular choline and phosphocholine generated in human PMN after stimulation with fMLP, A23187 or PMA. In these experiments activation of phospholipase C would lead to an increase in cellular phosphocholine, whereas phospholipase D activation would result in an increase in cellular choline concentrations.

When PMNs were stimulated with fMLP, A23187 or PMA (Fig. 8), dramatic increases in cellular choline levels were observed. Stimulation with fMLP resulted in an increase in cellular choline from approx. 450 pmol/ 10^7 cells to 1500 pmol/ 10^7 cells within 30 s. Similar elevations of cellular choline were observed 5 min after stimulation with A23187 or PMA. The levels of choline remained elevated for the remainder of the incubation period with all three stimuli.

In contrast with the increased cellular choline levels observed after stimulation of PMN, no changes in the levels of phosphocholine were observed (results not shown). The inability of all three agents to produce increases in cellular phosphocholine levels suggest that phospholipase C is not activated in response to fMLP, A23187 or PMA.

DISCUSSION

Various studies suggest that PC-derived DGs may play an important role in neutrophil function. Diacyl- and alkylacyl-glycerols have been shown to prime phospholipase A_2 activation

[23] and the rate and/or duration of the respiratory burst [24]. Furthermore, diacylglycerols may stimulate protein kinase C [25–27], whereas alkylacylglycerols have been reported to inhibit protein kinase C [24,28]. In spite of this, the enzymic pathways underlying DG production have not been thoroughly investigated. The hydrolysis of choline-containing phosphoglycerides has been attributed to phospholipase C or D in a variety of cell types. Daniel *et al.* [12] proposed a phospholipase C-mediated hydrolysis of PC in MDCK cells, based on the production of radiolabelled DG and phosphocholine. However, phospholipase D activity was not ruled out. On the other hand, PC breakdown has been attributed to phospholipase D action in rats hepatocytes, endothelial cells and REF 52 cells [39–41]. In human PMN and HL-60 granulocytes, a phospholipase D-mediated pathway of PC hydrolysis leads to PA generation [16–22].

Although studies utilizing ethanol strongly support a role for phospholipase D in fMLP-stimulated PA production, the contribution of phospholipase D towards DG production is not clear, nor is its role in PMA- or A23187-stimulated cells. Therefore, we investigated the enzymic pathways underlying PC-derived DG production in human PMN stimulated with fMLP, A23187 or PMA. Ethanol and propranolol were added to the incubation mixtures in an attempt to delineate between phospholipase C- and D-mediated DG production. The ability of PMN to generate PET when stimulated with fMLP, PMA and A23187 was indicative of an activatable phospholipase D which was responsive to all three stimuli. However, ethanol's usefulness in distinguishing between the enzymic pathways of DG generation was limited by its inability to block completely phospholipase D-mediated PA production. Thus, although ethanol did block PA and DG production to some extent with all three stimuli, it was not possible to determine whether the remaining DG was due to phospholipase C activity or simply to the remaining phospholipase D activity.

In contrast with ethanol, propranolol at first appeared to be more useful in distinguishing between the apparent contributions of phospholipase C and D forward DG generation. Propranolol's ability to completely inhibit DG production in response to fMLP indicated that phospholipase D was probably the only enzyme responsible for DG formation with this stimulus. Adding strength to this conclusion was the finding that fMLP, although dramatically increasing intracellular choline levels, had no effect on the levels of phosphocholine.

Of additional interest in samples stimulated with fMLP in the presence of propranolol were the findings that (1) the inhibition of DG production was accompanied by a change in PA levels which was equal in magnitude but opposite in direction to that of DG, and (2) that propranolol had no effect on PC breakdown. These findings suggest that propranolol was exerting its effect only on PA phosphohydrolase. Such findings were not observed in response to A23187 and PMA. In PMN stimulated with these agents, propranolol only partially blocked DG formation, and the extent to which propranolol inhibited DG production was greater than that to which PA levels were augmented. Further investigation revealed that propranolol inhibited the breakdown of PC, probably accounting for its inhibition of DG production. This finding was somewhat surprising, since this effect was not observed with fMLP stimulation.

In order to rule out phospholipase C activation, we examined the water-soluble products of PC hydrolysis, namely choline and phosphocholine. These two molecules are direct products of phospholipase C and D activity respectively. Consistent with the activation of phospholipase D was an increase in cellular choline levels when PMNs were stimulated with A23187 or PMA. However, we were unable to demonstrate an increase in cellular phosphocholine levels in response to these agents at the times

investigated. Therefore, a PC-hydrolysing phospholipase C is not likely to play a role in the DG production stimulated by fMLP, A23187 or PMA. Consistent with these conclusions are findings in human PMN labelled with phosphonate-containing analogues of PC. Such phosphonate-containing lipids cannot be hydrolysed by phospholipase D; any DG produced from the phosphonate-containing precursor would be the result of phospholipase C-mediated cleavage. Human PMN labelled with phosphonate-containing PC stimulated with various agents did not hydrolyse this substrate, suggesting that phospholipase C was not activated (A. Nixon, J. Strum, J. Schmitt, A. Emilsson, L. Daniel & R. Wykle, personal communication).

In conclusion, the predominant mechanism responsible for DG production from choline-containing PCs in stimulated PMN appears to be the activation of phospholipase D with the subsequent breakdown of PA by PA phosphohydrolase.

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REFERENCES

1. Snyder, F. (1985) *Med. Res. Rev.* **5**, 107–140
2. Wykle, R. L., Olson, S. C. & O'Flaherty, J. T. (1986) *Adv. Inflammation Res.* **11**, 71–81
3. Mueller, H. W., O'Flaherty, J. T. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 6213–6218
4. Walsh, C. E., Waite, B. M., Thomas, M. J. & DeChatelet, L. R. (1981) *J. Biol. Chem.* **256**, 7228–7234
5. Swendsen, C. L., Ellis, J. M., Chilton, F. H., O'Flaherty, J. T. & Wykle, R. L. (1983) *Biochem. Biophys. Res. Commun.* **113**, 72–79
6. Chilton, F. H., Ellis, J. M., Olson, S. C. & Wykle, R. L. (1984) *J. Biol. Chem.* **259**, 12014–12019
7. Albert, D. H. & Snyder, F. (1984) *Biochim. Biophys. Acta* **796**, 92–101
8. Guy, G. R. & Murray, A. W. (1982) *Cancer Res.* **42**, 1980–1985
9. Mufson, R. A. (1985) *Carcinogenesis* **6**, 1693–1698
10. Bocckino, S. R., Blackmore, P. F. & Exton, J. H. (1985) *J. Biol. Chem.* **260**, 14201–14207
11. Besterman, J. M., Duronio, Y. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6785–6789
12. Daniel, L. W., Waite, M. & Wykle, R. L. (1986) *J. Biol. Chem.* **261**, 9128–9132
13. Irving, H. R. & Exton, J. H. (1987) *J. Biol. Chem.* **262**, 3440–3443
14. Glatz, J. A., Muir, J. G. & Murray, A. W. (1987) *Carcinogenesis* **8**, 1943–1945
15. Pessin, M. S. & Raben, D. M. (1989) *J. Biol. Chem.* **264**, 8729–8738
16. Agwu, D. E., McPhail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L. & McCall, C. E. (1989) *J. Biol. Chem.* **264**, 1405–1413
17. Agwu, D. E., McPhail, L. C., Wykle, R. L. & McCall, C. E. (1989) *Biochem. Biophys. Res. Commun.* **159**, 79–86
18. Pai, J.-K., Siegel, M. L., Egan, R. W. & Billah, M. M. (1988) *J. Biol. Chem.* **263**, 12472–12477
19. Pai, J.-K., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) *Biochem. Biophys. Res. Commun.* **150**, 355–364
20. Truett, A. P., III, Snyderman, R. & Murray, J. J. (1989) *Biochem. J.* **260**, 909–913
21. Billah, M. M., Pai, J.-K., Mullman, T. J., Egan, R. W. & Siegel, M. I. (1989) *J. Biol. Chem.* **264**, 9069–9076
22. Billah, M. M., Eckel, S., Mullman, T. J., Egan, R. W. & Siegel, M. I. (1989) *J. Biol. Chem.* **264**, 17069–17077
23. Bauldry, S. A., Wykle, R. L. & Bass, D. A. (1988) *J. Biol. Chem.* **263**, 16787–16795
24. Bass, D. A., McPhail, L. C., Schmitt, J. D., Morris-Natschke, S., McCall, C. E. & Wykle, R. L. (1989) *J. Biol. Chem.* **264**, 19610–19617
25. Berridge, M. J. (1987) *Biochim. Biophys. Acta* **907**, 33–45
26. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–697
27. Nishizuka, Y. (1986) *J. Natl. Cancer Inst.* **76**, 363–370
28. Daniel, L. M., Small, G. W., Schmitt, J. D., Marasco, C. J., Ishaq, K. & Piantadosi, C. (1988) *Biochem. Biophys. Res. Commun.* **151**, 291–297
29. Wyrick, S. D., McManahan, J. S., Wykle, R. L. & O'Flaherty, J. T. (1985) *J. Labelled Compds. Radiopharm.* **22**, 1169–1174

30. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
31. DeChatelet, L. R. & Shirley, P. S. (1982) *Infect. Immun.* **35**, 206–212
32. Chilton, F. H., O'Flaherty, J. T., Ellis, J. M., Swendsen, C. L. & Wykle, R. L. (1983) *J. Biol. Chem.* **258**, 7268–7271
33. Murray, J. J., Dinh, T. T., Truett, A. P., III & Kennerly, D. A. (1990) *Biochem. J.* **270**, 63–68
34. Dougherty, R. W., Dubay, G. R. & Niedel, J. E. (1989) *J. Biol. Chem.* **264**, 11263–11269
35. Mullmann, T. J., Siegel, M. I., Egan, R. W. & Billah, M. M. (1990) *J. Immunol.* **144**, 1901–1908
36. Dawson, R. M. C. (1967) *Biochem. J.* **102**, 205–210
37. Heller, M. (1978) *Adv. Lipid Res.* **16**, 267–326
38. Pappu, A. S. & Hauser, G. (1983) *Neurochem. Res.* **8**, 1565–1575
39. Bocckino, S. B., Blackmore, P. F., Wilson, P. B. & Exton, J. H. (1987) *J. Biol. Chem.* **262**, 15309–15315
40. Martin, T. W. & Michaelis, K. C. (1988) *Biochem. Biophys. Res. Commun.* **157**, 1271–1279
41. Cabot, M. C., Welsh, C. J., Cao, H. & Chabbott, H. (1988) *FEBS Lett.* **233**, 153–157

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