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# Protein kinase C activators inhibit the antigen receptor-coupled polyphosphoinositide phosphodiesterase in murine B lymphocytes

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Protein kinase C activators (e.g. PMA) inhibit the inositol phosphate release generated by crosslinking antigen receptors (sIgM and sIgD) on murine B lymphocytes with anti-receptor antibodies. Unlike other $Ca^{2+}$-mobilizing receptor systems, the antigen receptor signal transduction pathway in B cells is not interrupted by PMA at the level of receptor/G-protein or G-protein/polyphosphoinositide phosphodiesterase coupling. In these cells, PMA, presumably by activating protein kinase C, inhibits inositol phosphate release by direct effects on the polyphosphoinositide-specific phosphodiesterase.

Antigen receptor; Phorbol ester; Inositol phosphate; Negative feedback inhibition; Polyphosphoinositide phosphodiesterase inhibition; (Murine B lymphocyte)

## 1. INTRODUCTION

Crosslinking of surface immunoglobulin (sIg) receptors by anti-Ig antibodies activates essentially all quiescent B lymphocytes and causes a substantial proportion of them to synthesize DNA [1,2]. Ligation of these antigen receptors provokes the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to inositol trisphosphate ($IP_3$) and diacylglycerol (DAG) [3,4]. Generation of these second messengers leads to the mobilization of intracellular $Ca^{2+}$ stores [5] and the activation of protein kinase C (PKC) [6].

PKC-activating phorbol esters (e.g. PMA) inhibit anti-Ig-induced B cell activation [7,8]. However, these agents can also stimulate DNA synthesis in lymphocytes, either alone or in synergy with other stimuli [9,10]. The inhibitory effects of phorbol esters on anti-Ig-induced B cell activation correlate with their capacity to suppress receptor-stimulated $PIP_2$ hydrolysis and $Ca^{2+}$ mobilization [8,11–13]. These findings support the concept of PKC-mediated feedback inhibition of receptor-directed inositol phosphate production proposed for other $Ca^{2+}$-mobilizing systems (e.g. [14–17]).

It has recently become evident that sIgM and sIgD receptors on B lymphocytes, in common with many other $Ca^{2+}$-mobilizing receptors, are coupled to the polyphosphoinositide-specific phosphodiesterase (PPI-PDE) via a G-protein termed $G_p$ [18,19]. Such a signalling cascade provides several potential sites of regulation: at the level of ligand-receptor interaction, receptor/G-protein coupling, G-protein/PPI-PDE coupling or at the level of PPI-PDE itself. We here demonstrate that PKC activators, such as PMA, inhibit signalling via the antigen receptors on B cells by direct inhibition of PPI-PDE activity.

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*Abbreviations:* anti-Ig, anti-immunoglobulin antibodies; DAG, 1,2-diacylglycerol; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); $IP_3$, inositol trisphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PPI-PDE, polyphosphoinositide phosphodiesterase

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## 2. MATERIALS AND METHODS

### 2.1. *Reagents*

*myo*-[$^3$H]Inositol (spec. act. 110 Ci/mmol) was from Amersham International (Amersham, England). GTP$\gamma$S was from BCL (Lewes, Sussex, England). Protease inhibitors and phorbol esters were from Sigma (Poole, Dorset, England). Reduced streptolysin-*O* was from Wellcome Diagnostics (Beckenham, Kent, England). Monoclonal rat anti-mouse $\mu$ (anti-$\mu$; b.7.6) and mouse anti-Igh-5$^a$ (anti-$\delta$; AMS28.11) were purified as described in [18].

### 2.2. *Assay of inositol phosphate release*

Briefly, splenic B cells were purified from CBA/Ca (Igh-5$^a$) mice and labelled with [$^3$H]inositol (0.5–2.5 $\mu$Ci/$10^6$ cells) as previously described [18]. The cells were then washed and resuspended at $20 \times 10^6$ cells/ml in the permeabilization buffer (adjusted to 10 nM $Ca^{2+}$ with CaEGTA buffers) described in [18]. After equilibration at 37°C for 10 min, the cells were incubated in the presence and absence of PMA for 10 min. Reduced streptolysin-*O* (0.5 IU/ml: final conc.) was added and after 5 min the permeabilized cells were diluted two-fold (final volume 300 $\mu$l) into tubes containing $Ca^{2+}$ (buffered with 3 mM EGTA), anti-Ig antibodies (50 $\mu$g/ml) and GTP$\gamma$S. After a final 10 min incubation, the reactions were terminated and the inositol phosphates were separated [18]. Stimulation of intact cells with anti-Ig was carried out in supplemented RPMI 1640 medium plus 5% (v/v) fetal calf serum.

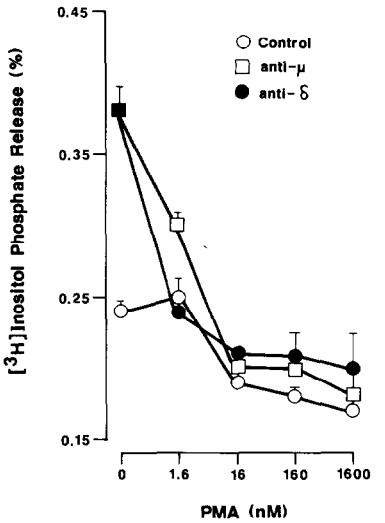



Fig.1. Inhibition of basal and stimulated (sIgM and sIgD-mediated) inositol phosphate release by PMA in intact B cells. [$^3$H]Inositol-labelled B cells were preincubated for 10 min with a range of PMA concentrations before stimulation with medium (○), or with anti-$\mu$ (□) or anti-$\delta$ (●) anti-receptor antibodies. Total inositol phosphate release was measured after 10 min.

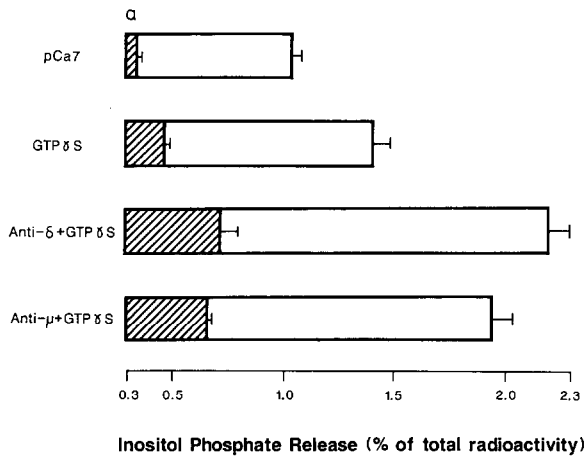
## 3. RESULTS

Pretreatment of intact B cells with PMA effectively blocked the generation of inositol phosphates induced by crosslinking either sIgM or sIgD (maximal inhibition at 16 nM) (fig.1), confirming earlier results [8,11–13]. Moreover, the basal rate of inositol phosphate production was also suppressed by PMA. Using a permeabilized cell system, we have recently reported that both the sIgM and sIgD receptors are coupled to the PPI-PDE via one or more pertussis toxin-insensitive forms of $G_p$ [18]. In the present study we have therefore used this system to determine at which stage in the receptor/G-protein/PPI-PDE signalling cascade PMA exerts its inhibitory effects.

In permeabilized cells, $G_p$ can be directly activated by the nonhydrolysable GTP analogue, GTP$\gamma$S. As expected, in B cells this activation is markedly augmented by co-stimulation of the cells with anti-Ig antibodies (inactive on their own) plus GTP$\gamma$S [18]. This is because optimal activation of the $G_p$-linked PPI-PDE requires its interaction with 'excited' receptors. Thus, if PMA disrupts the G-protein/PPI-PDE coupling, pretreatment of the cells with phorbol ester should inhibit GTP$\gamma$S-induced production of inositol phosphates. On the other hand, if PMA inhibits the coupling between the antigen receptors and $G_p$, the GTP$\gamma$S-induced inositol phosphate production should be unaffected, whilst the augmented release in response to anti-Ig would be abrogated.

These two possibilities were tested in the experiments summarized in fig.2. Pretreatment of B cells with 160 nM PMA led to a comparable (65–70%) inhibition of the basal, GTP$\gamma$S-stimulated and GTP$\gamma$S plus anti-Ig-induced production of inositol phosphates (fig.2a) (16 nM PMA: 60–65% inhibition; 1.6 nM PMA: 5–10% inhibition, results not shown). Thus, although the generation of inositol phosphates was clearly suppressed by PMA, the stimulation indices (calculated as % of the basal rate) of GTP$\gamma$S and GTP$\gamma$S plus anti-Ig-induced activation were unaffected. These conclusions were reinforced by the results shown in fig.2b, which depicts the effects of 1.6, 16 or 160 nM PMA on the direct stimulation of $G_p$ induced by a wide range of GTP$\gamma$S concentrations.

These results suggested that in B cells PMA does



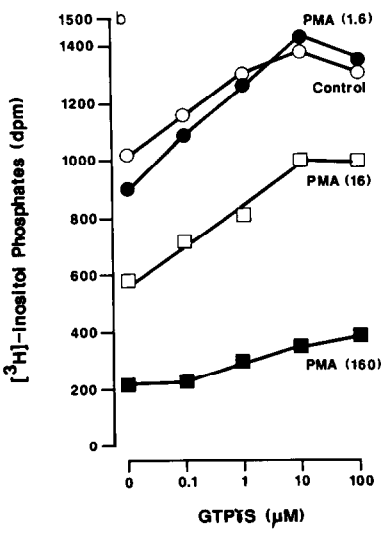




Fig.2. Effects of PMA pretreatment on the GTPγS and GTPγS plus anti-Ig-mediated release of inositol phosphates in permeabilized B cells. [$^3$H]Inositol-labelled, permeabilized B cells were stimulated with (a) GTPγS (100 μM) with or without anti-Ig antibodies. Control cells, open bars; cells pretreated with 160 nM PMA, shaded bars. (b) Cells were pretreated with 1.6, 16, or 160 nM PMA and were then stimulated with the indicated concentrations of GTPγS. Total [$^3$H]inositol phosphate release was measured after 10 min.

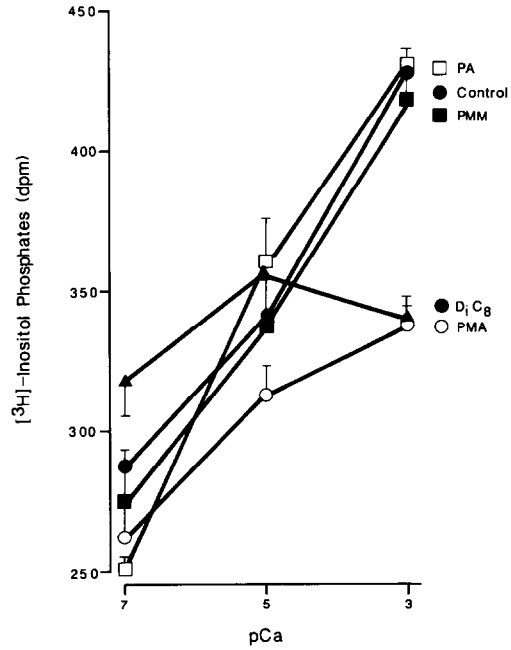



Fig.3. Effects of phorbol esters and diacylglycerol on the $Ca^{2+}$-activated PPI-PDE activity. [$^3$H]Inositol-labelled, permeabilized B cells were preincubated with PMA, phorbol monomyristate (PMM), phorbol monoacetate (PA) (all at 160 nM) or 1,2-dicapryloyl-*rac*-glycerol ($DiC_8$) (160 μM) for 10 min before stimulation with the indicated $Ca^{2+}$ buffers. Total inositol phosphate release was measured after 10 min.

not affect receptor/G-protein coupling, or G-protein/PPI-PDE coupling, but rather may cause direct modification of PPI-PDE itself. In order to measure the basal rate of PPI-PDE activity, the above experiments were carried out at p*Ca* 7 (100 nM), i.e. at the levels of intracellular $Ca^{2+}$ pertaining in unstimulated cells. However, PPI-PDE can be directly activated by high, non-physiological (millimolar) levels of $Ca^{2+}$. As shown in fig.3 the direct activation of PPI-PDE at p*Ca* 3 was effectively blocked by 160 nM PMA. Furthermore, it is likely that this effect is due to activation of PKC, since the inhibition can be mimicked by the metabolizable diacylglycerol, 1,2-dicapryloyl-*rac*-glycerol but not by the nonactive phorbol esters, phorbol monomyristate or phorbol monoacetate (fig.3).

## 4. DISCUSSION

The results of this study indicate that activation of PKC by agents such as PMA directly inhibits the activity of PPI-PDE in B cells. Previous workers [8,11–13] had shown that pretreatment of B cells and B cell lymphomas with PMA inhibits both inositol phosphate generation and intracellular $Ca^{2+}$ mobilization induced by crosslink-

ing sIg receptors with anti-Ig antibodies. Although the precise mechanism had not been defined, it seemed likely that these effects were mediated via PKC [12]. It was also clear that the inhibition by PMA was not due to (i) down-regulation of sIg receptor expression [8,11], (ii) a decrease in receptor-binding affinity or in the rate of capping [8], nor (iii) a decrease in $PIP_2$ substrate available for hydrolysis by PPI-PDE [12,20].

Recently, the use of permeabilized cell systems has allowed the detection of $G_p$ in the sIg receptor/PPI-PDE signal transduction pathway [18,19]. Coupling of cell surface receptors to their effector enzymes (in this case PPI-PDE) by a G-protein provides several potential regulatory sites. Our results show that with sIg receptors on B cells, unlike other $Ca^{2+}$-mobilizing receptor systems, PMA does not interrupt either the receptor/G-protein [21–24] or the G-protein/PPI-PDE coupling [25–27]. Rather, in these cells, PMA and synthetic DAG inhibit signalling through both sIgM and sIgD receptors by affecting the activity of PPI-PDE, presumably via activation of PKC. Similar results have recently been reported for the f-Met-Leu-Phe receptor signalling pathway in HL60 cells (Cockcroft, S. et al., personal communication and [28]). These results are in line with recent findings that both membrane-bound and cytosolic PPI-PDE are substrates for PKC [29]. In conclusion, it is becoming apparent that activation of PKC can exert feedback control on signalling via $Ca^{2+}$-mobilizing receptors at multiple sites.

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