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SYNERGISM BETWEEN DIACYLGLYCEROLS AND CALCIUM IONOPHORE IN THE INDUCTION OF HUMAN B CELL PROLIFERATION MIMICS THE INOSITOL LIPID POLYPHOSPHATE BREAKDOWN SIGNALS INDUCED BY CROSSLINKING SURFACE IMMUNOGLOBULIN

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Resting human tonsillar B cells were stimulated to divide by heat killed Staphylococcus aureus Cowan strain 1 which was shown to induce hydrolysis of phosphatidylinositol 4, 5-bisphosphate known to give rise to diacylglycerol and an increase in cytosolic free calcium. Addition of the diacylglycerols, 1-oleoyl-2 acetyl glycerol or sn-1, 2-dioctanoylglycerol, together with the calcium ionophore ionomycin to B cell cultures induced marked cell proliferation whereas these agents were ineffective when used alone. Both diacylglycerols were shown to compete with [^{3}H] phorbol 12,13 dibutyrate in binding to protein kinase C. These data support the hypothesis that synergism between cytosolic calcium and endogenous diacylglycerol, which activates protein kinase C, is involved in signal transduction in the proliferation of human B cells.

The membrane and signal transduction events elicited by various factors which act sequentially to drive B and T lymphocytes from a resting stage, through activated, proliferating and differentiating steps to functionally mature cells is presently an area of intense interest. Recently Imboden and Stobo have shown that crosslinking of the T3 receptor of T lymphocytes, which is linked to mitogenesis, stimulates breakdown

The abbreviations used are: BCGF, B cell growth factor; DAG, diacylglycerol; DiC8, sn-1, 2-dioctanoylglycerol; EGF, epidermal growth factor; IL1, IL2 and IL3, interleukin 1, 2 and 3; IP1, IP2 and IP3, inositol mono, bis and trisphosphate; OAG, 1-oleoyl-2-acetyl-glycerol; PDBu, phorbol 12, 13 dibutyrate; PDGF, platelet-derived growth factor; PI 4,5-P_2 phosphatidylinositol 4,5 bisphosphate; PKC, protein kinase C; SAC, Staphylococcus aureus Cowan strain 1; sIg, cell surface immunoglobulin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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of PI 4,5-P_2 giving rise to IP3 and DAG (1). IP3 liberates Ca++ from intracellular stores and DAG activates the enzyme protein kinase C (2). These observations suggest that the increase in PI turnover and Ca++ mobilisation which are known to occur during lymphocyte mitogenesis (3-5) are a consequence of breakdown of PI 4,5-P_2 and its subsequent resynthesis. A general role for inositol phospholipid metabolism in the control of cell growth is supported by the observation that binding of the mitogen PDGF to 3T3 cells results in breakdown of PI 4,5-P_2 (6). Nishizuka has proposed that activation of PKC by DAG and mobilised Ca++, which may activate additional kinases, act synergistically to elicit physiological responses (2).

The polyclonal B cell mitogen SAC interacts with and crosslinks surface immunoglobulin molecules and is able to induce B cells to proliferate in the absence of accessory cells or accessory cell factors (7,8). In this study, in view of the above T cell observations, we have investigated whether crosslinking of sIg by SAC leads to breakdown of PI-4,5-P_2. Recently we reported that resting (G_o) human B cells can be stimulated to divide using TPA together with calcium ionophores ionomycin and A23187, at concentrations which have no effect alone (9). A similar synergism between TPA and ionophores has also been reported in studies of human T lymphocytes (10,11). The addition of TPA, which binds to PKC, and ionophore, to effect a rise in cytosolic Ca++ concentration, presumably replace the two signals supplied by PI 4,5-P_2 breakdown. We have investigated this further by looking at whether the synthetic diacylglycerols, OAG and DiC8, which compete with PDBu for binding to protein kinase C, synergise with the calcium ionophore ionomycin in activating the proliferation of human resting B cells.

MATERIALS AND METHODS

SAC was obtained from Calbiochem-Behring, Cambridge, UK, ionomycin was from Calbiochem, La Jolla, CA. and 1,2-diolein and propidium iodide were obtained fom Sigma, Poole, Dorset. DiC8 was prepared by the method of Davis et al (12) from dioctanoylphosphatidylcholine (Avanti Polar Lipids, Inc.). [^{3}H] thymidine, [^{3}H] inositol and [^{3}H]PDBu were obtained from Amersham Int, Amersham, UK. Cell surface marker analyses of B cells was performed as previously described (13). The purification of resting B cells and their growth analysis were as previously described (9). The assay for the inhibition of [^{3}H]PDBu binding by the diacylglycerols was essentially as described by Davis et al (12) except that cells were harvested on glass fibre filter discs. For the analysis of water-soluble inositol phophates, G_o B cells were labelled for 4 hours in Hank's-HEPES medium containing 5μCi [^{3}H] inositol per million cells, washed twice in growth medium then reseeded in costar plate wells (0.6ml) at 3 to 4 million per well. Treatments were added in 30μl of media and reactions were terminated and products analysed on Dowex resin columns (formate form) by the method of Berridge et al (14).

RESULTS AND DISCUSSION

The B cell mitogen SAC was tested for its ability to stimulate hydrolysis of PI 4,5-P_2. Figure 1A shows a timecourse of accumulation of inositol phosphates after the addition of SAC and a lithium block to cultures of purified resting (G_o) B cells. There is a constant accumulation of total inositol phosphates over the tested 120 minute period. In parallel cultures, a subsequent rise in [^{3}H]thymidine uptake was observed 48 to 64 hours later (data not shown). The identity and amounts of the individual inositol derivatives were analysed after 60 seconds of SAC stimulation and the elution profiles for batch elutions from formate resin columns are shown in Figure 1B. The third peak, shown using standards to be IP3 and thus one of the products of PI 4,5-P_2 hydrolysis, shows a rapid SAC induced rise.

As described previously, breakdown of PI 4,5-P_2 gives rise to an IP3 induced Ca++ mobilisation and 1, 2-diacylglycerol. Hence, we investigated whether synthetic diacylglycerols together with the calcium ionophore ionomycin could bypass the normal process of PI 4,5-P_2 hydrolysis and initiate B cell

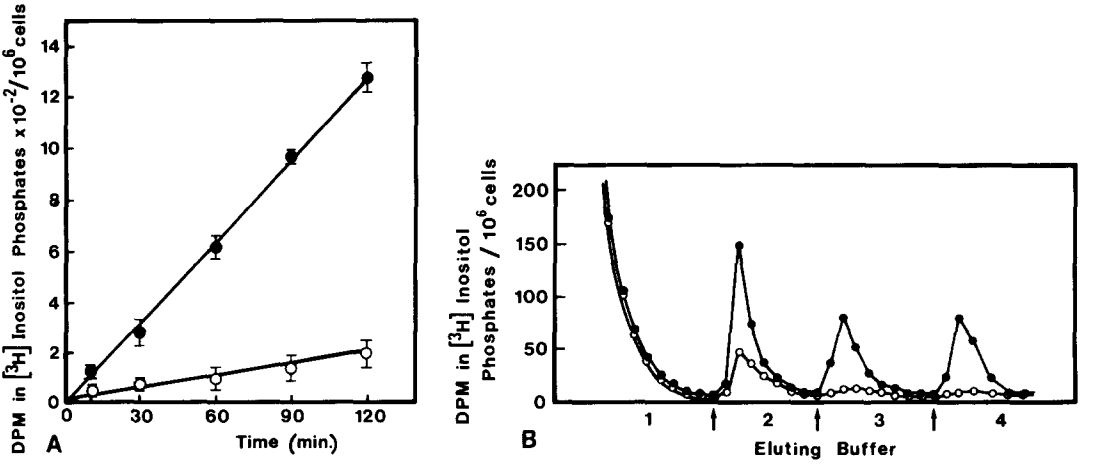

Figure 1. A. Aliquots of [^{3}H]Inositol labelled human B lymphocytes in medium with 7.5mM LiCl received SAC (1 in 20,000) ● or an equal volume of media ○. At the times indicated, the total inositol phosphates (IP + IP2 + IP3) were determined. The results are expressed as amounts in [^{3}H]Inositol phosphates per 10^6 cells and the points ± S.E.M. of three replicates from three separate experiments.

B. The separation of water-soluble inositol phosphates from [^{3}H]Inositol pre-labelled human B cells which are stimulated for 60 seconds with SAC ● or are unstimulated ○. The cell labelling, extraction and column elution are as described in Materials and Methods. Each point is the mean of three separate determinations and the elution profiles were similar in the three experiments. The peaks 1, 2 and 3 represent the amounts of IP1, IP2 and IP3 respectively.

proliferation. Table 1 shows that the diacylglycerols, OAG and DiC8, and ionomycin when added separately to cultures of purified resting (G_o) B cells induced minimal DNA synthesis. In contrast, when cultures were treated with ionomycin together with OAG or DiC8 a marked increase in [^{3}H]thymidine incorporation was observed which is both ionomycin and diacylglycerol dependent. The observed increases in the number of cells in the G2/M phase of the cell cycle (see Table 1) and number of viable cells in culture over several days show that the combined signals stimulate cell proliferation and not just DNA synthesis. Blast cells observed in proliferating cultures were B cells as revealed by staining with the pan-B reactive monoclonal antibody B4. In all cases > 96% of blast cells expressed this antigen.

Table 1. (3H) Thymidine uptake into B cells as D.P.M. per microtitre plate well + (% cells in G2/M of cell cycle)

		Concentration of Ionomycin		
	nM	0	0.8μg/ml	1.6μg/ml
OAG	0	454 (0)	327 (0)	285 (0)
	10	478 (0)	685 (0)	723 (0)
	20	452 (0)	1250 (1.1)	14,217 (6.5)
	40	410 (0)	25,634 (8.4)	39,631 (11.8)
	60	485 (0)	29,831 (9.6)	54,917 (18.9)
	80	609 (0)	32,814 (10.8)	53,112 (17.5)
DiC8	0	515 (0)	374 (0)	258 (0)
	10	545 (0)	10,481 (5.8)	36,021 (13.1)
	20	748 (0)	23,637 (7.2)	55,787 (21.4)
	40	1052 (1.0)	38,851 (11.5)	97,488 (27.8)
	60	1841 (1.8)	55,681 (20.2)	124,110 (36.2)
	80	2341 (2.5)	84,332 (24.4)	191,486 (45.8)

Shown above is the proliferative response, assessed by measurement of (3H) thymidine incorporation, of B cells cultured with a range of concentrations of the diacylglycerols OAG and DiC8 alone and each combined with two concentrations of the calcium ionophore ionomycin. Further progression of the cells through the cell cycle was determined by running propidium iodide stained nuclei through a FACS flow cytometer and is expressed as the percentage of cells in G2-M (in brackets). Values are means of quadruplicates and are representative of two separate experiments.

To ascertain whether the two diacylglycerols act by activating PKC, their ability to competitively inhibit binding of a known PKC ligand, [^{3}H]PDBu, to resting B cells was assessed. The inhibition curves (see Figure 2) show that the [^{3}H]PDBu displacing ability of the diacylglycerols correlates with their ability to synergise with ionomycin in inducing B cell proliferation. The curves may reflect the ability of the diacylglycerols to cross or intercalate with the intact lipid

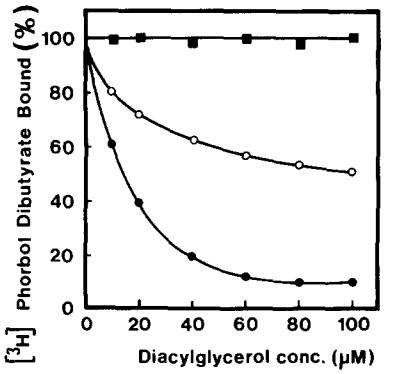

Figure 2. Inhibition of [^{3}H] Phorbol dibutyrate binding to human B cells by; sn-1, 2-dioctanoylglycerol ● , 1-oleoyl 2-acetylglycerol O or 1, 2-diolein ■. The binding inhibitions were carried out using 5nM of [^{3}H]PDBu with non-specific binding determined in parallel using 30uM PDBu, as described in Materials and Methods. Each point is the mean of four determinations. Similar results were obtained in two separate experiments.

bilayer for the following reason. 1, 2-diolein is a good stimulator of PKC in in-vitro assays but was unable to displace [^{3}H]PDBu binding to intracellular PKC and was non-mitogenic for B cells when added together with ionomycin (data not shown).

The synergistic mitogenicity of ionomycin and diacylglycerols and PI 4,5-P_2 hydrolysis observed following sIg crosslinking, show that both Ca++ mobilisation and activation of PKC are required to elicit B cell DNA synthesis and proliferation. However, this does not necessarily provide evidence for a dual system of signalling from the cell membrane to the nucleus which directly initiates cell proliferation as proposed by Berridge (15). Evidence from physiological studies of lymphocyte activation suggests that the stimulated hydrolysis of PI 4,5-P_2 is an early signal in a domino receptor cascade. Antigen, which is the primary stimulus, binding to its receptor gives rise to an increased cell surface presentation of a secondary receptor for IL2 or BCGF. Binding of the appropriate ligand then gives rise to increased presentation of transferrin receptors, which is the commitment event common to all cell types

that occurs late in the G1 phase of the cell cycle. In this respect, it has been shown that TPA binding to PKC induces the expression of IL2 receptors in both T and B cells (16,17). Truneh and colleagues have shown that in T cells synergism between ionophore and TPA can lead to the expression of receptors for IL2 and IL2 factor secretion (11). A parallel situation probably exists in B cells with respect to BCGF receptors and growth factor. Nishizuka et al (18) postulate that synergy between TPA and ionophore replaces the IL1 signal required to initiate T cell proliferation and which is normally provided by monocytes.

In various studies there is considerable evidence that membrane events pertinent to proliferation involve PKC. This enzyme has been shown to phosphorylate the EGF (19), insulin (20) and transferrin (21) receptors and is redistributed in lymphocytes from the cytosol to the membrane when the growth factors IL2 and IL3 bind to their receptors (22, 23). Furthermore, the membrane bound activity of PKC has been shown to be related to the proliferative ability of transformed cells in low calcium medium (24).

Evidence is thus presented that the B cell mitogen SAC, which is known to crosslink sIg, stimulates hydrolysis of PI 4,5-P_2 thus giving rise to increases in cytosolic free calcium and membrane diacylglycerols leading to DNA synthesis. These signals are mimicked by the concurrent addition of diacylglycerols and a calcium ionophore at concentrations which are non-mitogenic for B cells when added alone. The focus of this synergy and also involvement of infrequent subsidiary cell types, which may provide secondary factors, are at present unclear. However, as both diacylglycerol and mobilised Ca++ are required to elicit B cell proliferation, and may both activate

PKC, its involvement in various stages of lymphocyte proliferation is of particular interest.

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