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Differential responsiveness of human B lymphocytes to phorbol ester and calcium ionophore based on their state of activation

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SUMMARY

For tonsil B cells of a particular high density (below 65% Percoll), both phorbol myristate acetate (PMA) (5 ng/ml) and calcium ionophore A23187 (500 nM) were required to induce RNA synthesis, significant DNA synthesis also occurring in the presence of 12,000 MW B-cell growth factor (BCGF). In contrast, PMA alone, even at 1 ng/ml, was a sufficient stimulus to induce strong DNA synthesis in low-density B cells (45–50% Percoll) and strong proliferative responsiveness to BCGF in intermediate-density B cells (55–65% Percoll). In these latter B-cell populations, A23187 (500 nM), acted synergistically with non-mitogenic PMA doses to induce strong DNA synthesis, the PMA dose required being 5–50 times lower in low-density B cells (0·1–1 ng/ml) than in intermediate-density B cells (5 ng/ml). Preactivation for 30 hr with anti-Ig antibodies plus BCGF, known to drive B cells into late G1, rendered high-density B cells responsive to PMA (1–10 ng/ml) with high, dose-related DNA synthesis. These data indicate that the B-cell mitogenicity of a given nanomolar dose of PMA depends on the more advanced state of activation of B cells. It was also found that the above optimal dose of A23187 (500 nM) paradoxically inhibited the PMA-induced DNA synthesis of low-density B cells and *in vitro* preactivated high-density B cells. Data obtained with low-density B cells suggest that a calcium influx during the PMA-induced proliferative phase of B cells may provide a negative signal for the DNA synthesis.

INTRODUCTION

It has been suggested that, depending of the degree of activation of B lymphocytes, the interaction of the antigen with the membrane immunoglobulins (Ig) may result in an abortive signal or ensure a more rapid response (Thompson *et al.*, 1984; Scott & Klinman, 1987). With regard to this, *in vitro* studies have shown that large (or low-density) and small (or dense) B cells differ in their responsiveness to soluble or immobilized anti-Ig antibodies and T-independent antigens (Muraguchi *et al.*, 1983; Defranco *et al.*, 1982; Thompson *et al.*, 1984; Boyd & Metcalf, 1984; Cambier, Heuser & Julius, 1986; Richard *et al.*, 1986). These data suggest that resting and non-resting B cells may differ in the signalling pathway through the surface Ig (sIg) receptors. If this were the case, it can be expected that resting and non-resting B cells may also differ in their responsiveness to phorbol esters (PMA) and calcium ionophore (A23187) since the PMA-induced activation of protein kinase *C* (PKC) and the A23187-mediated intracellular calcium increase mimic the biochemical events generated by the sIg cross-linking (Bigsterbosch *et al.*, 1985; Chen, Coggeshall & Cambier, 1986; Wilson *et al.*, 1987). These biochemical events are thought to determine the B-cell activation process induced by sIg ligation and, accordingly, PMA and A23187 act in concert to activate murine and human B lymphocytes, mimicking the stimulatory effects of Ig ligands (Monroe & Kass, 1985; Clevers *et al.*, 1985; Guy *et al.*, 1985; Engel *et al.*, 1987; Klaus *et al.*, 1986; Roifman *et al.*, 1987). Data reported here demonstrate that, depending on their state of activation, human B lymphocytes exhibit a differential sensitivity to the stimulatory effects of PMA and A23187.

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MATERIALS AND METHODS

Cell preparations

Tonsils were obtained at tonsillectomy from 5–12-year-old patients with chronic tonsillitis. Tonsil B lymphocytes were purified by two cycles of complement-mediated lysis using a mixture of monoclonal antibodies (mAbs) CD3 (Cris-7), CD4 (EDU-2), CD8 (109-2D4) and CD14 (Cris-6), which were produced in our laboratory by Dr R. Viella and were clustered in the *2nd International Workshop On Human Leukocyte Antigens.* Tonsil T lymphocytes were purified by two cycles of complement-mediated lysis using mAbs EDU-1 (anti-HLA Class II antigens), CD20 (B-C1), CD24 (B-C2) and CD14 (Cris-6). B-C1, B-C2 and EDU-1 have also been produced in our laboratory (Anegón *et al.*, 1986; Engel *et al.*, 1987; Colombany, Lepage & Kalil, 1983). Density fractionation of purified tonsil

B cells was performed using Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) made isotonic with 0·15 M NaCl. Discontinuous Percoll gradients with 5% increment from 40% to 70% were used (see the Results and Discussion).

Cell surface marker analysis

An indirect immunofluorescence technique was used and fluorescent cells were assessed in a FACS-Analyser (Becton-Dickinson, Mountain View, CA), as described elsewhere (Anegón *et al.*, 1986).

Cell cultures and assessment of DNA and RNA synthesis

Cultures were done in RPMI-1640 (Eurobio, Paris, France) supplemented with 10% FCS (Eurobio) and 1% glutamine (Flow Laboratories, Irvine, Ayrshire, U.K.) and 50 μg/ml of gentamicin. Microcultures were done in 96 well flat-bottomed plates (Nunc, Roskilde, Denmark) in a final volume of 0·2 ml/well. Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemicals Co., Taufkirchen, FRG) and A23187 (Sigma) were stored at −20° diluted (1 mg/ml) in ethanol (Merck, Darmstadt, FRG) until used in the cultures. Affinity-purified goat antibodies ($F(ab')_2$ fragments) specific for human Ig (anti-Ig) (Tago Inc, Burlingame, CA) were used at 50 μg/ml, low-molecular weight (12,000 MW) (BCGF Cellular Products Inc., Buffalo, NY) at 10% (v/v), and phytohaemagglutinin (PHA) (Welcome Diagnostics, Dartford, U.K.) at 0·5% (v/v). RNA and DNA synthesis was evaluated by measuring, respectively, the uptake of [^{3}H]uridine and [^{3}H]thymidine (Amersham Int., Amersham, Bucks, U.K.) (1 μCi/microwell) during the last 16 hr of culture periods. Macrocultures were performed in 25-ml tissue culture flask (Nunc) and viable cultured cells were isolated by centrifugation over a Ficoll–Hypaque gradient (Pharmacia).

RESULTS

Density-fractionated tonsil B cells differ in their proliferative responsiveness to PMA and A23187

Figure 1 shows two representative experiments (out of five) with each density-fractionated B-cell population. Low-density B cells (Percoll 45–50%) showed strong DNA synthesis after a 3-day culture with PMA alone at 1 or 5 ng/ml; this response, even with 5 ng/ml of PMA, was poor in intermediate-density B cells (Percoll 55–65%) and absent in high-density B cells (Percoll 65–70%). When PMA (0·1–5 ng/ml) was used together with A23187 (500 nM), different patterns of responsiveness were observed depending on the density of B cells. In low-density B cells, A23187 acted in synergy with non-mitogenic doses of PMA (0·1 or 1 ng/ml) to induce strong DNA synthesis, while it inhibited the strong DNA synthesis induced by 1 or 5 ng/ml of PMA. In intermediate-density B cells, A23187 (500 nM) acted in synergy with 5 ng/ml of PMA, to induce strong DNA synthesis, although in a few experiments poor responses were obtained (Fig. 1, right). In high-density B cells no synergistic effects were observed with any dose of PMA (0·1–5 ng/ml) plus A23187 (500 nM). A23187 by itself was ineffective and was used at a concentration of 500 nM (or 261·8 ng/ml) because in preliminary experiments using density-non-fractionated tonsil B cells, this dose was found to be more appropriate than 250 nM or 100 nM, confirming the results from other workers (Clevers *et al.*, 1985; Fig. 3).

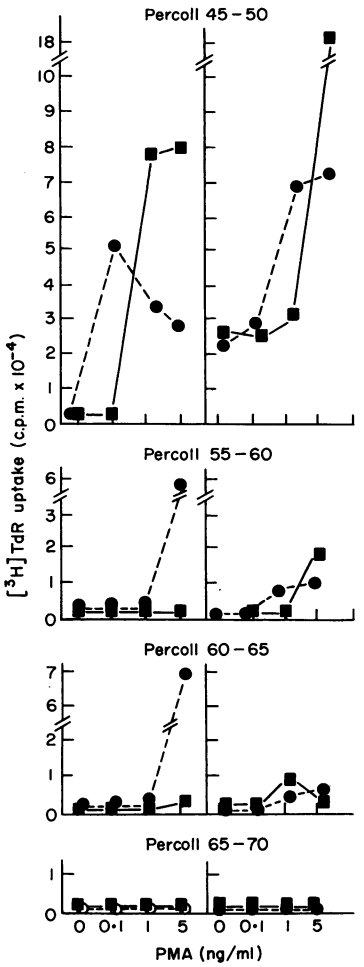

Figure 1. Proliferative responsiveness of density-fractionated tonsil B cells (2×10^5/well) to several doses of PMA in the absence (■) or presence (●) of A23187 (500 nM). Two representative experiments with each density-fractionated B-cell population are shown. The [^{3}H]thymidine uptake was evaluated after 64 hr of culture. Results indicate mean c.p.m. of triplicate cultures with SD below 10% of the mean.

The PMA-induced DNA synthesis of low-density tonsil B cells is not due to residual contaminating T cells

CD3$^+$ and CD14$^+$ cells in purified B-cell fractions were $\leq 1\%$ and undetectable, respectively; and more than 96% of cells were sIg$^+$ and HLA class II$^+$. Moreover, PHA did not induce significant DNA synthesis; and the levels of DNA synthesis induced by PMA plus PHA were not higher than those induced by PMA alone. As shown in Table 1, these latter controls were found to be a very sensitive functional marker of T cells in a set of experiments where a known number of autologous T cells was added to purified high-density B cells. These results indicate that even if contaminating T cells in B-cell populations were 5%, the strong PMA-induced DNA synthesis of low-density B cells would not be due to their influence. Moreover, this number of T cells would be reflected in controls with PHA and PHA plus PMA.

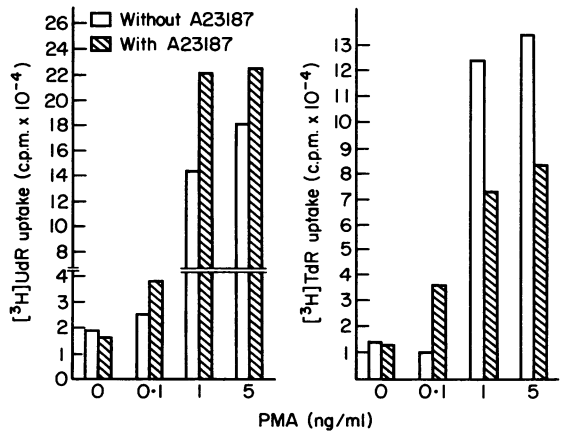

Figure 2. RNA synthesis (a) and DNA synthesis (b) of low-density tonsil B cells (2×10^5/well) cultured with several doses of PMA and A23187 (500 nM). The [^{3}H]uridine and [^{3}H]thymidine uptake was evaluated after 40 and 64 hr of culture, respectively. Bars indicate mean values of triplicate cultures with SD below 10% of the mean.

Time–course of A23187-mediated inhibition on the PMA-induced DNA synthesis of low-density B cells

A23187 did not inhibit the RNA synthesis observed after 40 hr of culture with PMA, whereas it inhibited the DNA synthesis observed 24 hr later (Fig. 2). This suggests that the A23187-mediated inhibition is not due to a cytolytic action and that A23187 acts during the PMA-induced proliferative phase, inhibiting the DNA synthesis itself. The results obtained with the following two-stage cultures support this notion. Low-density B cells were precultured for 30 hr (primary culture) with either PMA (5 ng/ml) or PMA (5 ng/nl) plus A23187 (500 nM); viable cultured cells were isolated, washed three times and recultured for 30 additional hr (secondary culture) without or with graded concentrations of A23187 (100, 250, 500 nM). As shown in Fig. 3, the strong DNA synthesis induced by PMA was not inhibited but was even increased when A23187 was also present during the primary culture. In contrast, the addition of A23187 in the secondary culture caused a dose-related inhibition on the DNA synthesis induced by either PMA or PMA plus A23187, while cell viability was not affected.

These data contrast with those obtained with intermediate-density B cells, where the poor DNA synthesis induced by the presence of PMA in the primary culture was synergistically increased by A23187 (500 nM) added either to the primary or secondary culture. Moreover, no inhibition occurred when A23187 was present in both the primary and secondary cultures. Note that 500 nM concentration of A23187 was more appropriate than 250 and 100 nM concentrations to achieve a synergistic effect with 5 ng/ml of PMA (Fig. 3).

Earlier activation events induced by PMA and PMA plus A23187 in high-density B cells and intermediate-density B cells

The activation of B cells, i.e. the G0 to the G1 phase transition, can be assessed by measuring RNA synthesis or DNA synthesis to exogenous BCGF (Kehrl, Muraguchi & Fauci, 1984; Monroe & Kass, 1985; Maizel *et al.*, 1982). We next examined whether PMA plus A23187 were capable of inducing these earlier

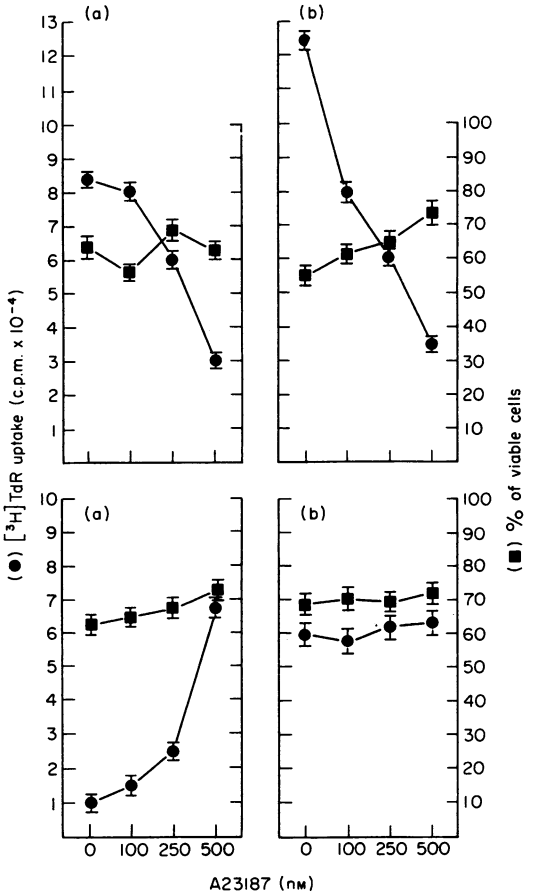

Figure 3. Time–course of A23187-mediated inhibition of PMA-induced DNA synthesis of low-density B cells. Low-density B cells (above) and intermediate density B cells (below) were precultured (10^6/ml) for 30 hr with (a) 5 ng/ml of PMA and (b) 5 ng/ml of PMA plus A23187 (500 nM). Viable cultured cells were isolated, washed three times and cultured (10^5/well) for 30 additional hr with or without A23187 (100, 250 and 500 nM). The [^{3}H]thymidine uptake was evaluated during the last 16 hr. Results indicate mean values (±SD) of cell counts (■) and [^{3}H] thymidine c.p.m. (●) in quadruplicate cultures.

activation events in high-density B cells that failed to show DNA synthesis when stimulated with these compounds. A representative experiment (out of two) is shown in Fig. 4. It was found that A23187 acted in synergy with PMA to induce high levels of RNA synthesis, significant DNA synthesis also occurring when BCGF was also present. A23187 and PMA used individually failed to induce RNA synthesis and DNA synthesis to BCGF (Fig. 4). In these experiments, two-stage microcultures were performed to exclude a possible A23187-mediated inhibitory effect, similar to the one observed in low-density B cells, that could act preventing the proliferative response of high-density B cells to PMA plus A23187.

We also investigated the capacity of PMA alone or PMA plus A23187 to induce proliferative responsiveness to BCGF in those intermediate-density B-cell populations (Fig. 1) which failed to show DNA synthesis when stimulated with PMA plus A23187. In this case, both PMA alone (even at 1 ng/ml) and

Table 1. The PMA-induced DNA synthesis of low-density B cells was not due to residual contaminating T cells.

Tonsil cells	[^{3}H]Tdk uptake* (c.p.m.)			
	Medium	PMA†	PHA†	PMA+PHA†
T cells‡	320±49	10,935±1555	10,935±1090	88,795±2611
Low-density B cells‡	3375±477	103,388±9187	6152±208	106,052±7847
High-density B cells‡	277±10	1762±81	237±10	3315±204
High-density B cells+T cells§	242±17	2846±131	6820±49	17,645±1120

* Evaluated on Day 3. Values are the mean±SD of triplicate cultures.
† PMA, 5 ng/ml; PHA, 0·5% (v/v).
‡ 2×10^5 cells per well.
§ $1{\cdot}9 \times 10^5$ high-density B cells plus $0{\cdot}1 \times 10^5$ autologous T cells per well.

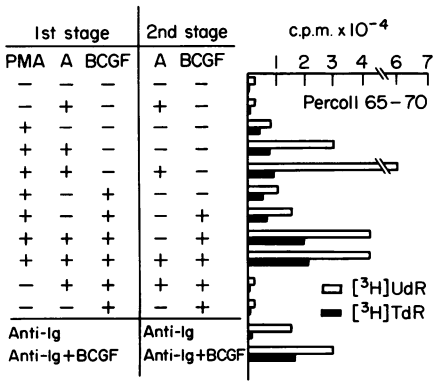

Figure 4. Induction of RNA and DNA synthesis in high-density tonsil B cells by PMA and A23187 in the absence or presence of BCGF using two-stage microcultures. Cells (2×10^5/well) were cultured for 24 hr as indicated (1st stage); microplates were centrifuged, the supernatants discarded, and cells washed once and recultured as indicated (2nd stage). The [^{3}H]uridine and [^{3}H]thymidine uptake was evaluated after 40 and 64 hr, respectively. The results with anti-Ig antibodies plus BCGF are also shown. Bars indicate mean c.p.m. of triplicate cultures with SD below 10% of the mean. A indicates A23187 (500 nM). PMA was used at 5 ng/ml.

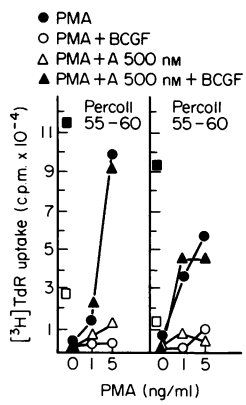

Figure 5. Induction of proliferative response to BCGF by PMA and A23187 in two different intermediate-density B-cell populations (2×10^5 cells/well) failing to show consistent DNA synthesis when stimulated with PMA plus A23187. The results with anti-Ig antibodies in the absence (□) or presence (■) of BCGF are also shown. Results indicate mean c.p.m. of triplicate cultures with SD below 10% of the mean. A indicates A23187.

PMA plus A23187 were similarly effective to induce strong proliferative response to BCGF. A23187 and BCGF, used individually or in combination, had no effects (Fig. 5).

In vitro preactivation renders high-density tonsil B cells responsive to PMA with strong and dose-related DNA synthesis, and the presence of A23187 abolishes this PMA-induced DNA synthesis

We next examined whether *in vitro* preactivation renders high-density B cells responsive to PMA with DNA synthesis and whether A23187 also inhibits this response. As shown in Fig. 6, high-density B cells precultured for 30 hr with anti-Ig plus BCGF, but not those precultured in culture medium alone, were responsive to PMA with high DNA synthesis levels, which showed a linear dependency on PMA dose. This PMA-induced DNA synthesis was abrogated when A23187 (500 nM) was also present. In contrast, and in accordance with the results shown in Fig. 4, A23187 (500 nM) promoted DNA synthesis to BCGF in high-density B cells that were precultured for 30 hr with PMA plus BCGF; the magnitude of this response was similar to that observed with anti-Ig plus BCGF.

DISCUSSION

Low-density (or large) B cells (banding between 45% and 50% Percoll gradients) correspond to activated B cells in advanced positions along the G1 phase of the cell cycle (Monroe & Cambier, 1982; Defranco *et al.*, 1982; Thompson *et al.*, 1984). Human B cells isolated below 55% Percoll gradients have usually been considered as 'high-density' B cells and considered to represent resting B cells. However, recent data clearly indicate that tonsillar B cells isolated between 55% and 62·5% Percoll gradients mostly correspond to B cells in early activation states (i.e. initial positions of the G0 to the G1 phase transition), which are not detectable by cell cycle analysis (Walker *et al.*, 1986; Clark & Shu 1987). Consequently, in the current study, tonsil B cells banding between 55% and 65% Percoll gradients are designated intermediate-density B cells and assumed to largely represent B cells in early activation states. Only tonsil B cells of a

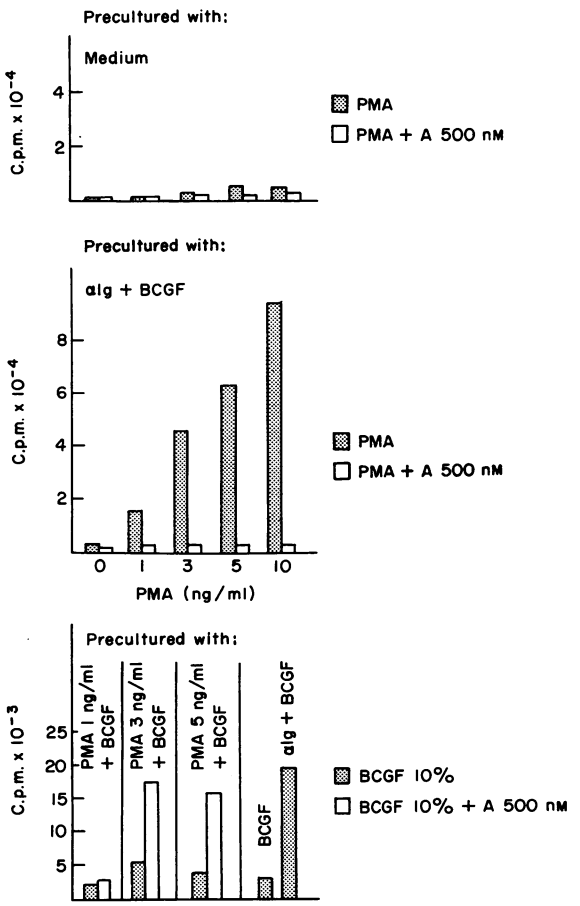

Figure 6. Preactivation of high-density B cells with anti-Ig plus BCGF rendered them responsive to PMA with dose-related DNA synthesis, and A23187 abrogated this response. High-density B cells (2×10^5/well) were precultured for 30 hr as indicated (within the plots); plates were then centrifuged, supernatants discarded and cells washed once with medium and recultured for 64 additional hr in the conditions indicated (on the right). The [^{3}H]thymidine uptake was evaluated during the last 16 hr. The results with anti-Ig plus BCGF are also shown. Bars indicate mean c.p.m. of triplicate cultures with SD below 10%. A indicates A23187.

particular high density (isolated below 65% Percoll gradients) are designated here as high-density B cells and judged as likely candidates to represent resting B cells; of note, the proportion of these tonsillar high-density B cells is low (less than 5% of total B cells).

Assuming these criteria, current data indicate that the B-cell mitogenicity of a given nanomolar dose of PMA is determined by the more advanced state of activation of B cells. Data suggest that for B cells in advanced positions along the G1 phase as represented by low-density B cells, PMA alone, even at 1 ng/ml, constitutes a potent proliferative stimulus. In accordance with this notion, *in vitro* preactivation rendered high-density B cells responsive to PMA with high and dose-related DNA synthesis. Preactivation was done with anti-Ig plus BCGF for 30 hr because, in the presence of conventional BCGF, the number of anti-Ig-stimulated B cells which achieve late G1 at this time of culture is higher than in the absence of BCGF (Kehrl *et al.*, 1984). Conventional BCGF, i.e. derived from culture supernatants of PHA-stimulated blood mononuclear cells, correspond to the low molecular weight (12,000 MW) BCGF, one of the major human T cell-derived lymphokines showing BCGF activity for human B cells (Maizel *et al.*, 1982); such 12,000 MW BCGF has been purified to homogeneity, its gene cloned and its low- and high-affinity receptors on activated B cells characterized (Mehta *et al.*, 1985; Mehta, Grant & Maizel, 1986; Sharma *et al.*, 1987).

In low-density and intermediate-density B cells, non-mitogenic PMA doses acted in synergy with A23187 (500 nM) to induce strong DNA synthesis. The magnitude of the non-mitogenic PMA dose required for this synergy was also determined by the more advanced state of activation of B cells, and was 5–50 times lower in low-density (0·1–1 ng/ml) B cells than in intermediate-density B cells (5 ng/ml). In these latter B-cell populations, but not in high-density B cells, PMA alone, even at 1 ng/ml, induced strong proliferative responsiveness to BCGF. The synergistic effects of PMA and calcium ionophore for DNA synthesis in intermediate-density B cells (55–65% Percoll) are consistent with data reported by other authors using tonsil B cells banding below 57·5% (Guy *et al.*, 1985) or below 62·5% Percoll gradients (Walker *et al.*, 1986).

In high-density B cells (banding below 65% Percoll), both PMA (5 ng/ml) and A23187 (500 nM) were required to induce RNA synthesis, significant DNA synthesis also occurring in the presence of BCGF. Perhaps in quiescent B cells the induction of DNA synthesis by PMA plus A23187 has a delayed kinetics or requires higher doses of PMA and A23187 than used here. For murine resting B cells, PMA and A23187 also act in synergy to induce RNA synthesis (Monroe & Kass, 1985). Moreover, B cells from mice maintained in germ-free conditions, probably representing true quiescent and 'virgin' B cells, do not show DNA synthesis to anti-Ig antibodies (Boyd & Metcalf, 1984). Whether the DNA synthesis induced by PMA or PMA plus A23187 in highly purified B cells is growth factor-independent or mediated by autocrine growth factors is, at present, unknown.

According to the current model for the synergism between calcium increase and phorbol esters, the calcium increase acts by 'priming' the PKC for activation by phorbol esters (Wolf *et al.*, 1985; May *et al.*, 1985). Exposure of B cells to ligands of Ig for a few min induces intracellular calcium elevation that persists for hours (Bijsterbosch *et al.*, 1985; Wilson *et al.*, 1987). Moreover, it has been shown recently that the 12,000 MW BCGF also induces intracellular calcium elevation (Ledbetter *et al.*, 1988). Thus, the intracellular calcium increase caused by both anti-Ig and BCGF may account for the increased responsiveness to PMA of *in vitro* preactivated high-density B cells. It is conceivable that low-density B cells have been similarly preactivated *in vivo* via the interaction with the antigen and growth factors. On the other hand, a possible calcium-dependent activation of calpain might also contribute to affect the PKC system in activated B cells (Melloni *et al.*, 1985).

A23187 paradoxically inhibited the PMA-induced DNA synthesis of activated B cells. Data obtained with PKC inhibitors and calcium channel blockers indicated that the G0 to the G1 transition of B cells is dependent on both PKC activity and calcium entry, while proliferation is dependent on PKC activity but independent of calcium entry (Monroe & Kass, 1985; Dugas *et al.*, 1986). The present data suggest that a calcium influx during the PMA-induced proliferative phase of B cells may provide a negative signal for the DNA synthesis. One possible

mechanism for this effect might be the activation of a calcium-dependent endonuclease activity similar to the one associated with thymocyte apoptosis (Wyllie, 1980). Apoptosis is also a physiological event within the proliferating B cells of the germinal centre (reviewed by Duvall & Wyllie, 1986). Whether such endonuclease exists in proliferating B cells and can be activated by a calcium influx remains to be determined.

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