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# Signal Thresholds and Modular Synergy During Expression of Costimulatory Molecules in B Lymphocytes

Krishnamurthy Natarajan, Naresh C. Sahoo, and Kanury V. S. Rao<sup>1</sup>

We analyzed intracellular pathways modulating surface densities of CD80 and CD86 in B cells activated through ligation of the Ag receptor, and the adhesion molecule CD54. Whereas B cell Ag receptor (BCR) cross-linking alone stimulated increased expression of CD86, up-regulation of CD80 required dual stimulation with anti-IgM and anti-CD54. The principal downstream component contributed by BCR signaling, toward both CD80 and CD86 induction, was the elevated concentration of free cytoplasmic  $Ca^{2+}$ , recruited by way of capacitative influx. This alone was sufficient to generate an increase in CD86 levels. However, CD80 enhancement required the concerted action of both intracellular  $Ca^{2+}$  concentration and CD54-initiated pathways. The nexus between anti-IgM and anti-CD54 stimulation, in the context of CD80 regulation, was identified to involve a self-propagating process of sequential synergy. The first step involved amplified accumulation of intracellular cAMP, as a result of cross-talk between BCR-mobilized  $Ca^{2+}$  and CD54-derived signals. This then facilitated a second synergistic interaction between  $Ca^{2+}$  and cAMP, culminating in CD80 expression. Our findings of distinct signal transducer requirements, with the added consequences of cross-talk, offers an explanation for variable modulation of costimulatory molecule expression in response to diverse physiological stimuli. Importantly, these results also reveal how concentration threshold barriers for recruitment of individual second messengers can be overcome by constructive convergence of signaling modules. *The Journal of Immunology*, 2001, 167: 114–122.

Extensive studies from a variety of laboratories have detailed both the initiation and propagation of protein phosphorylation cascades that emanate upon ligation of the B cell Ag receptor (BCR)<sup>2</sup> (1–6). This process is initiated by the tyrosine phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) sequences within  $Ig\alpha$  and  $Ig\beta$  by the *src* family of protein tyrosine kinases. As a consequence of this, at least three major pathways are recruited. These include pathways that are dependent upon phospholipase C $\gamma$ , the Rho family of GTPases, or the Ras/phosphatidylinositol-3-kinase (4–6). Although much of the current emphasis of research on signal transduction in lymphocytes has focused on the initial events that follow receptor triggering, the various downstream mechanisms that eventually culminate into modification of lymphocyte function remain unknown.

One important consequence of BCR ligation is the regulated expression of a variety of cell surface molecules such as the MHC class II, costimulatory, and adhesion molecules (7–11). It is the cumulative phenotypic composition of the surface that then defines functional properties of the B cell, either in terms of its Ag presenting or migratory capabilities (10, 12, 13). Although BCR cross-linking constitutes the principal trigger that initiates activation of B cells and their induction into a response, it is now be-

coming clear that this effect can be modulated by signals generated from other molecules on the cell surface (14). For example, whereas coligation of BCR with the CR2/CD19/TAP-1 complex leads to increased B cell proliferation (15, 16), cross-linking of BCR with the Fc $\gamma$ RIIb coreceptor has been found to be inhibitory (17–20). Similarly, reciprocal desensitization of MHC class II and BCR signaling has also been recently demonstrated (21).

Thus, while the regulatory influences of cell surface accessory molecules on BCR signaling is being increasingly recognized, the mechanisms through which independently generated biochemical pathways interface and, thereby, modulate cell function remains poorly understood. To gain further insights into this aspect, we examined the induction of CD80 (B7-1) and CD86 (B7-2) in splenic B cells under a defined set of conditions. As shown earlier by us (22) and others (10), moderate cross-linking of the BCR was sufficient to enhance surface levels of CD86 but not CD80. The latter required provision of an additional stimulus either in the form of a cytokine or ligation of a second molecule on the B cell surface (22–24). In this study, the additional stimulus used was anti-CD54 (ICAM-1) which, in conjunction with anti-IgM treatment, led to a marked enhancement in CD80 levels. Using such a system, we demonstrate how distinctions in both the concentration and spectrum of key second messenger intermediates can account for the distinct induction thresholds of these two molecules. Furthermore, our data also reveal the existence of a novel process of sequential synergy that is involved during CD80 up-regulation. Finally, present findings also provide a preliminary insight into how B cell responsiveness—at least in the context of costimulatory molecule expression—can be variably modulated by diverse physiological stimuli.

## Materials and Methods

### Materials

mAbs against mouse molecules CD80 (clone 1G10), CD86 (clone GL-1), CD54 (clone 3E2), B220 (clone RA3-6B2), and isotype-matched controls were obtained from BD PharMingen (San Diego, CA). Goat anti-mouse IgM was purchased from Sigma (St. Louis, MO). Ab-coated magnetic

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<sup>2</sup> Abbreviations used in this paper: BCR, B cell Ag receptor; PKC, protein kinase C; PKA, protein kinase A; db-cAMP, dibutyryl cAMP; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate; H89, *N*-[2-(*p*-bromocinamyl)aminoethyl]-5-isoquinolinesulfonamide; KN-62, 1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; fluo-3-AM, fluo-3-acetoxymethyl ester;  $Ca_i^{2+}$ , intracellular calcium ion.

beads for the purification of B lymphocytes were obtained from Miltenyi Biotec (Auburn, CA). Inhibitors to various kinases such as genistein, calphostin C, 1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), calmidazolium, cyclosporin A, EGTA, and other reagents such as guanidine isothiocyanate and dibutyl-cAMP (db-cAMP) were obtained from Sigma (St. Louis, MO). Ionomycin, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), *N*-[2-((*p*-bromocinamyl)aminomethyl)-5-isoquinolinesulfonamide (H89), fluo-3-acetoxymethyl ester (fluo-3-AM), Abs to Ser<sup>133</sup>-phosphorylated CREB, and CREB were purchased from Calbiochem (San Diego, CA). Intracellular cAMP levels were measured using the BIOTRAK EIA cAMP detection kit from Amersham (Arlington Heights, IL). Primers specific for the  $\beta$ -actin gene were obtained from Promega (Madison, WI).

In these studies, the anti-IgM and anti-CD54 Abs were used as their F(ab)<sub>2</sub> fragments, which were obtained by digestion with pepsin, followed by purification over a protein G-Sepharose column as described earlier (25).

### Animals

Female BALB/c mice (6–8 wk old), maintained under pathogen-free conditions, were obtained from the small experimental facility of our institute.

### Enrichment of B lymphocytes

This was achieved by negative selection, through magnetic sorting, essentially as described previously (22). Briefly, splenocytes from 6- to 8-wk-old BALB/c mice were first depleted of adherent cells by panning over plastic plates. From this, T cells were removed by two rounds of incubation with a mixture of magnetic beads individually coated with anti-CD4, anti-CD8, and anti-CD90 (Thy 1.2) (Miltenyi Biotec). Furthermore, the IgG<sup>+</sup> population was also removed by additional incubation of cells with anti-mouse IgG-coated microbeads. Separation was achieved using MACS columns (Miltenyi Biotec). The purity of the resulting population of B cells obtained in this fashion was 95–98% when measured as B220-PE-stained cells by flow cytometry.

### Stimulation of B cells and analyses of CD80 and CD86 expression by flow cytometry

Enriched B cells were plated in 96-well plates at  $3 \times 10^5$ /well in 0.1 ml of RPMI 1640 containing 10% FCS and antibiotics (RPMI 10). Depending on the requirement, cells were cultured with the F(ab)<sub>2</sub> fragment of anti-IgM (10  $\mu$ g/ml), and/or anti-CD54 (10  $\mu$ g/ml), and/or ionomycin (0.5  $\mu$ M). In initial experiments, stimulation times were varied from 2 to 24 h for CD86 analysis, and from 2 to 60 h when CD80 levels were being monitored. Although a 2-h stimulation time with anti-IgM was sufficient to induce CD86, significant CD80 up-regulation required between 8 and 12 h of treatment with the combination of anti-IgM plus anti-CD54. Consequently, a standard stimulation time of 12 h was used for all of the experiments reported here. For the effects of inhibitors, cells were pre-equilibrated with EGTA (3 mM), genistein (60  $\mu$ g/ml), calphostin C (0.1  $\mu$ M), calmidazolium (20 nM), KN-62 (5  $\mu$ M), cyclosporin A (8  $\mu$ M), or H89 (100 nM) for 1 h before addition of the appropriate stimuli. At the end of incubation, the cells were washed thoroughly in ice-cold HBSS to remove all traces of excess stimuli and inhibitors, and cultured again in complete medium (RPMI 1640 containing 10% FCS) for the requisite time. Based on the kinetics of induction of CD80 and CD86 (see Fig. 1A, c), CD86 levels were measured at 24 h poststimulation with anti-IgM or ionomycin, whereas CD80 was measured at 60 h after addition of the appropriate stimulants. The viability of cells was at least 95%, as determined by trypan blue exclusion, for all the analyses reported here.

At the end of the requisite culture period, the Fc receptors on the cells were blocked with anti-CD16/CD32 (BD PharMingen) before analysis. Following this, cells were stained with biotinylated anti-B220 (1  $\mu$ g/10<sup>6</sup> cells for 1 h at 4°C) and then labeled with streptavidin-PE. After adequate washes (PBS with 0.5% BSA and 0.01% azide), cells were subjected to a second round of staining with FITC-conjugated Abs against either CD80 or CD86 (1  $\mu$ g/10<sup>6</sup> cells for 1 h at 4°C) and then fixed in buffer containing 0.1% paraformaldehyde.

Two-color analysis was conducted on a FACSCalibur flow cytometer (BD Immunocytometry Systems, Mountain View, CA). Logarithmically amplified fluorescence data were collected on  $5 \times 10^4$  viable cells as determined by forward scatter intensity and by exclusion of propidium iodide-stained cells.

### Quantitation of CD80 and CD86 mRNA levels

Total cellular RNA was extracted from cultured cells at the time point indicated in figure legends using guanidine isothiocyanate followed by acid phenol treatment. Total RNA (10  $\mu$ g) was fractionated on a formaldehyde

agarose gel, transferred onto Hybond-N nylon membrane (Amersham), and subsequently probed with <sup>32</sup>P-labeled gene-specific full-length probes for CD80 and CD86. The blots were exposed to x-ray films and developed later. Following this, blots were stripped off the initial probe and then hybridized with <sup>32</sup>P-labeled probes for  $\beta$ -actin as loading controls.

### Monitoring intracellular Ca<sup>2+</sup> levels

Enriched B cells ( $2 \times 10^7$ ) were incubated with 1  $\mu$ M fluo-3-AM in 1 ml of culture medium for 45 min at 37°C. Thereafter, the cells were washed thoroughly in HBSS and finally resuspended at  $1 \times 10^6$  cells/ml of culture medium alone or in medium containing either 3 mM EGTA or 50  $\mu$ M TMB-8. After a 15-min incubation at room temperature, cells were stimulated with the appropriate agents and the resultant variation in intracellular Ca<sup>2+</sup> was monitored by flow cytometry.

### Estimation of intracellular levels of cAMP

Enriched B cells that had been cultured for 12 h were transferred into serum-free medium and equilibrated over an additional 4 h of culture. Following this the cells were washed and resuspended in serum-free medium at  $1 \times 10^6$  cells/ml, and 100- $\mu$ l aliquots were plated into wells of a 96-well tissue culture plate. Quadruplicate sets of wells were stimulated with the appropriate agent (or mock-stimulated with carrier only) for 1 h at 37°C. The resulting intracellular cAMP levels were then measured in cell lysates using the BIOTRAK EIA kit (Amersham) strictly following the protocol recommended by the manufacturer.

To determine the effects of exogenous addition of db-cAMP on intracellular cAMP levels, cells prepared as above were incubated with varying concentrations of db-cAMP for 1 h at 37°C. Following this, the cells were washed extensively in serum-free medium to remove any traces of excess db-cAMP. The remaining procedure was identical with that described above.

### Analysis of CREB phosphorylation

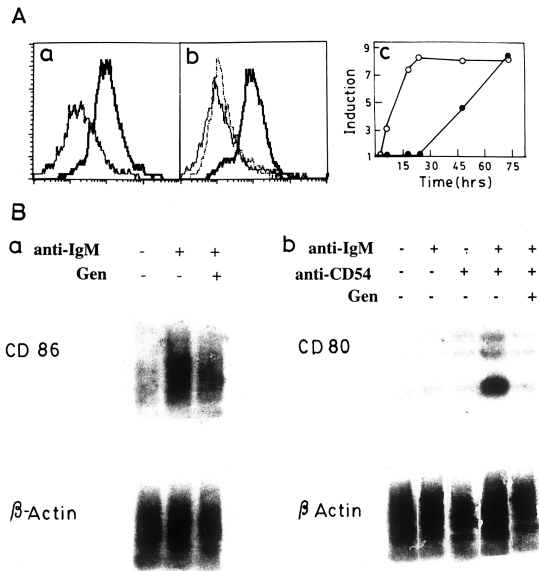
Enriched B cells were equilibrated either in the presence or absence of various inhibitors for 1 h before stimulation with the appropriate agents for 30 min at 37°C. At the end of this the cells were chilled on ice, washed with ice-cold PBS, and lysed in lysis buffer (10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA, 0.5% Nonidet P-40, and 2 mg/ml each of aprotinin, leupeptin, and pepstatin). The resulting nuclear pellet was then extracted in buffer containing 20 mM HEPES (pH 7.9); 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; and 2 mg/ml each of aprotinin, leupeptin, and pepstatin.

Nuclear extracts from  $1 \times 10^7$  cells were each resolved on 10% SDS-polyacrylamide gels and subsequently transferred onto a nitrocellulose membrane (Hybond C<sup>+</sup>; Amersham). The resulting blots were probed with rabbit Abs specific for Ser<sup>133</sup>-phosphorylated CREB, followed by HRP-labeled secondary Abs. Blots were developed by chemiluminescence using the ECL kit (Amersham). To verify comparable loading of proteins, bound Abs were stripped off, and the blots were subjected to a second round of screening with anti-CREB Abs.

## Results

### BCR-dependent induction of CD86 and CD80 levels in B cells

Stimulation of splenic B cells with anti-IgM Abs (see *Material and Methods*) induced increased surface expression of CD86 (Fig. 1A), although no concurrent effect on CD80 was noted (data not shown). However, enhanced surface expression of CD80 could readily be obtained upon dual stimulation of cells with a combination of anti-IgM and anti-CD54 (ICAM-1) Abs (Fig. 1A, b). Both of these stimuli were found to be obligatory, because addition of either component alone had no detectable effect on CD80 levels. This contrasted with the effect on CD86, where stimulation with the combination of anti-IgM and anti-CD54 did not yield any significant enhancement in levels over that obtained with anti-IgM stimulation alone (data not shown). In addition to distinct stimulus requirements, the kinetics of induction of these two molecules also differed as shown in Fig. 1A, c. When compared with CD86, CD80 showed delayed induction becoming apparent only several hours after initiation of stimulation (Fig. 1A, c). This observed enhancement in surface densities of CD80 and CD86 in response to the appropriate stimuli also correlated well with a concomitant increase in the respective mRNA levels as shown in Fig. 1B. The



**FIGURE 1.** Induction of CD80 and CD86 in murine B cells. *A*, Cell surface staining for CD86 (*a*) or CD80 (*b*) in B220<sup>+</sup> gated cells that were either left unstimulated (thin line) or stimulated (thick line) with either anti-IgM (*a*) or anti-IgM plus anti-CD54 (*b*). A parallel set of cells were also stimulated in the presence of genistein. Although the results for CD80 are shown by the dashed line (*b*), the corresponding profile for CD86 was superimposable over that obtained for unstimulated cells. *c*, Kinetics of induction of CD86 (○) and CD80 (●) in appropriately stimulated cells. *B*, Northern blot analyses, using cDNA probes, for the corresponding mRNA species is also shown in *B*. For CD86, mRNA extracts were prepared at 24 h after initiation of stimulation, whereas extracts for CD80 mRNA analysis were prepared 48 h later. Data in *A* are representative of at least five separate experiments, whereas those in *B* are from one of three experiments.

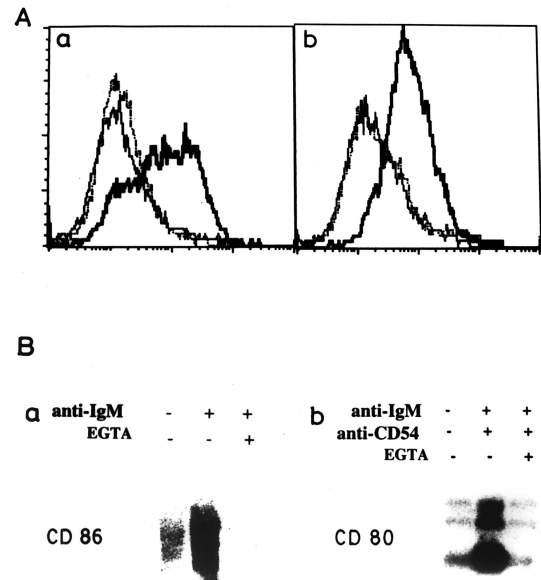
magnitude of increase in cellular mRNA content observed in Fig. 1*B* may likely reflect increased transcriptional activation of genes for both CD80 and CD86. However, the alternate possibility that this increase derives from enhanced message stability cannot presently be ruled out.

Induction of both CD80 and CD86 in response to the appropriate stimuli could be inhibited by the inclusion of genistein, a broad spectrum inhibitor of protein tyrosine kinases (26). This inhibition was exercised at the level of cell surface protein (Fig. 1*A*), as well as at that of the respective mRNAs (Fig. 1*B*). These latter results imply the dependence of both CD80 and CD86 regulatory pathways on the tyrosine phosphorylation events initiated upon receptor ligation.

#### *Intracellular calcium ion ( $Ca_i^{2+}$ ) concentrations modulate expression of both CD80 and CD86*

To dissect biochemical mediators of the above response, we next examined the effects of addition of a variety of pharmacological inhibitors. In the course of these studies, we observed that addition of either TMB-8 or EGTA completely abolished both anti-IgM-dependent CD86 up-regulation and CD80 up-regulation mediated by anti-IgM plus anti-CD54. Whereas TMB-8 is known to inhibit  $Ca^{2+}$  release from intracellular stores (27), EGTA blocks its influx from the extracellular milieu (28). Importantly, the inhibitory effects of these two agents were enforced not only at the level of cell surface protein, but also at that of the corresponding transcripts. The results with EGTA as a representative are shown in Fig. 2.

The inhibitory effects of TMB-8 and EGTA together implicated a role for  $Ca_i^{2+}$  in the mediation of expression of both CD80 and

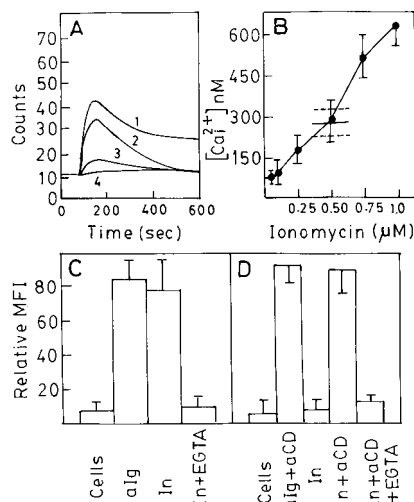


**FIGURE 2.** EGTA inhibits induction of both CD80 and CD86. The effects of EGTA addition (dashed line) on either anti-IgM-dependent CD86 induction (*a*) or anti-IgM plus anti-CD54-mediated CD80 up-regulation (*b*) are shown in *A*. In both cases, unstimulated cells are represented by the thin line, and staining in cells stimulated in the absence of EGTA are depicted by the thick line. The corresponding effects of EGTA on the respective mRNA levels are also shown in *B*. Data presented here are a representative of four separate experiments.

CD86. Consequently, we next examined the effects of BCR and CD54 ligation on  $Ca_i^{2+}$  mobilization. Consistent with prior findings (for a review, see Ref. 29), stimulation of B cells with anti-IgM resulted in a biphasic recruitment of  $Ca^{2+}$  ions into the cytoplasm. A rapid, but transient, increase was evident immediately upon addition of anti-IgM (Fig. 3*A*). Although these levels declined rapidly, they nevertheless stabilized at concentrations significantly above the baseline (Fig. 3*A*). Although the first, rapid, phase has been attributed to 1,4,5-inositol triphosphate-mediated release from intracellular stores, the second phase of low but sustained elevation has been ascribed to represent capacitative influx mediated by the calcium-release activated channels (CRAC) (29–31). These latter channels are activated upon exhaustion of the internal pool, although the mechanism by which this occurs is not known (31).

In keeping with the above interpretation, addition of TMB-8 markedly inhibited  $Ca_i^{2+}$  mobilization by B cells, in response to anti-IgM stimulation (Fig. 3*A*). As opposed to this, EGTA addition selectively suppressed anti-IgM-mediated capacitative  $Ca^{2+}$  influx, with only a relatively minor effect on recruitment from intracellular stores (Fig. 3*A*).

Distinct from the effects of anti-IgM, stimulation of B cells with anti-CD54 had no detectable influence on  $Ca_i^{2+}$  concentrations (Fig. 3*A*). Furthermore, dual stimulation with both anti-IgM and anti-CD54 also produced no additive effect, yielding a profile identical with that obtained upon stimulation with anti-IgM alone (data not shown). Our findings of the absence of any effect of anti-CD54 on  $Ca_i^{2+}$  concentrations in B cells is in keeping with prior findings. In a recent study, van Horsen et al. (32) also did not detect  $Ca_i^{2+}$  mobilization in B cells upon CD54 ligation although CD54 cross-linking has been shown to enhance  $Ca_i^{2+}$  concentrations in rat endothelial cells (33). Thus, whereas  $Ca_i^{2+}$  was identified as a common mediator of both the CD80 and CD86 regulatory pathways, its intracellular mobilization appeared to exclusively derive



**FIGURE 3.**  $\text{Ca}_i^{2+}$  mobilization and its effect on CD80 and CD86 levels. fluo-3-AM-loaded B cells were stimulated with anti-IgM either in the absence (profile 1) or presence of either EGTA (profile 2) or TMB-8 (profile 3), and the subsequent recruitment of  $\text{Ca}_i^{2+}$  was monitored by flow cytometry (A). The corresponding effect of stimulating cells with anti-CD54 is also shown (A, profile 4). In a separate experiment, cells were treated with graded concentrations of ionomycin, and the peak  $\text{Ca}_i^{2+}$  obtained at each concentration was averaged over a 10-min period. The graph in B depicts the mean ( $\pm$ SD) of four separate determinations. The line intersecting the graph indicates the mean  $\text{Ca}_i^{2+}$  concentration obtained during the EGTA-sensitive phase of anti-IgM-stimulated mobilization (A), where the dashed lines above and below represent the SD from the mean ( $n = 6$ ). C and D, Effects of indicated stimuli on cell surface densities of CD86 (C) and CD80 (D). Cells, Unstimulated; aIg, anti-IgM; In, 0.5  $\mu\text{M}$  ionomycin; aCD, anti-CD54. Values presented are in terms of modal fluorescence intensity (MFI) in B220<sup>+</sup> gated cells for each group, and are the mean ( $\pm$ SD) of four separate determinations.

due to BCR and not CD54 triggering. Furthermore, the inhibitory effect of EGTA on both CD80 and CD86 induction also localizes the capacitative influx phase of BCR-dependent  $\text{Ca}_i^{2+}$  recruitment as the key regulatory component.

The above identification of a role for BCR-mediated capacitative  $\text{Ca}_i^{2+}$  influx also prompted us to next single out the effects due to this second messenger. Influx of  $\text{Ca}_i^{2+}$  from the extracellular milieu can readily be achieved by the use of ionophores such as ionomycin (34, 35). However, to maintain parity with the native situation, we first performed titration experiments to determine that dose of ionomycin that would induce  $\text{Ca}_i^{2+}$  influx to a level that was comparable with that obtained upon anti-IgM (or anti-IgM plus anti-CD54) stimulation. For this, B cells were treated with varying concentrations of ionomycin, and the resultant  $\text{Ca}_i^{2+}$  influx was monitored. The cumulative results from such experiments are shown in Fig. 3B. It is evident from this figure that treatment of cells with an ionomycin concentration of 0.5  $\mu\text{M}$  mimics the capacitative entry phase of anti-IgM-stimulated recruitment of calcium ions.

To ascertain the specific effects of this  $\text{Ca}_i^{2+}$  concentration, we next used 0.5  $\mu\text{M}$  ionomycin as the stimulant. As shown in Fig. 3C, this treatment alone was sufficient to generate an increase in surface CD86 expression. Importantly, the magnitude of this effect was comparable to that obtained upon anti-IgM stimulation and, furthermore, was EGTA sensitive (Fig. 3C). In contrast to its effect on CD86, treatment of cells with 0.5  $\mu\text{M}$  ionomycin alone had no detectable effect on surface CD80 levels (Fig. 3D). However, CD80 up-regulation was readily achieved with a combination of 0.5  $\mu\text{M}$  ionomycin and anti-CD54 (Fig. 3D). The extent of en-

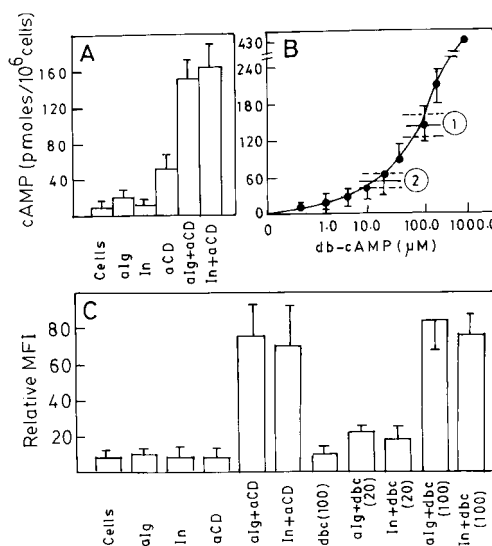
hancement was comparable to that observed with the combination of anti-IgM plus anti-CD54 (Fig. 3D). As expected, this effect was also inhibited upon addition of EGTA (Fig. 3D).

The collective observations in Fig. 3, C and D, that 0.5  $\mu\text{M}$  ionomycin could duplicate the effects of anti-IgM on both CD80 and CD86 up-regulation, further underscores the relevance of  $\text{Ca}_i^{2+}$  in both pathways. Importantly, these findings support that BCR-mediated capacitative  $\text{Ca}_i^{2+}$  influx is at least sufficient to account for the effect of anti-IgM on induction of both CD80 and CD86. In the context of the present system, the results in Fig. 3 also reveal a dual role for  $\text{Ca}_i^{2+}$ . Whereas on the one hand it ensures CD86 induction, it also appears to act in concert with CD54-dependent pathways to facilitate CD80 up-regulation.

#### *cAMP is the second intracellular messenger that governs CD80 expression*

Our identification of the regulatory intermediate provided upon BCR stimulation encouraged us to explore for a possible counterpart that could be implicated in the CD54-dependent pathways. In this connection, earlier studies have shown that addition of db-cAMP could result in CD80 up-regulation in B lymphoma cells (36). However, the db-cAMP concentration required was high (600  $\mu\text{M}$ ), and the extent of enhancement ( $\sim$ 2-fold) was modest (36). In addition to this, it has also been demonstrated in rat astrocytes that engagement of CD54 by specific Ab induced intracellular cAMP accumulation (37). Taken together, these studies raised the possibility that cAMP could well constitute a mediator of CD54-dependent effects on CD80 levels.

We first examined the effects of either anti-IgM, ionomycin (0.5  $\mu\text{M}$ ), anti-CD54, or relevant combinations of these, on cAMP levels within B cells. The results from such experiments are summarized in Fig. 4A. As expected on the basis of earlier reports (38),



**FIGURE 4.** Regulation of intracellular cAMP and its consequences on costimulatory molecule expression. The data in A depict the effect of the various stimuli used on intracellular cAMP concentrations in purified B cells. In a separate experiment, cells were also stimulated with indicated concentrations of db-cAMP, and the resultant intracellular accumulation was measured (B). The lines intersecting the graph, numbered 1 and 2, represent intracellular cAMP concentrations (dashed lines representing SD) obtained in response to stimulation of cells either with anti-IgM plus anti-CD54, or with anti-CD54 alone, respectively. The data in C depict cell surface CD80 staining in B220<sup>+</sup> gated cells in response to the indicated stimuli. Cells, Unstimulated; aIg, anti-IgM; In, 0.5  $\mu\text{M}$  ionomycin; dbc (20), 20  $\mu\text{M}$  db-cAMP; dbc (100), 100  $\mu\text{M}$  db-cAMP; aCD, anti-CD54. Values are the mean ( $\pm$ SD) of at least four separate experiments.

stimulation of B cells with anti-IgM had no significant effect on cAMP concentrations (Fig. 4A). This was also true when ionomycin was used instead (Fig. 4A). In contrast to this, addition of anti-CD54 was found to produce a near 5-fold increase, implying that CD54 stimulation also leads to accumulation of intracellular cAMP in murine B cells (Fig. 4A). Interestingly, however, a combination of anti-IgM and anti-CD54 as stimulants proved markedly more effective, yielding a near 15-fold elevation in cAMP concentrations (Fig. 4A). In other words, although BCR triggering alone was ineffective, it nevertheless appeared to synergize with CD54 stimulation to generate an amplified cAMP response. Equally significant here was our finding that the synergistic effect of anti-IgM on CD54-dependent cAMP accumulation could readily be duplicated upon substituting it with 0.5  $\mu\text{M}$  ionomycin (Fig. 4A). A comparable extent of enhancement in intracellular cAMP concentrations was obtained regardless of whether anti-IgM or ionomycin was used in conjunction with anti-CD54 (Fig. 4A). Based on these latter results, we infer that anti-IgM-mobilized  $\text{Ca}_i^{2+}$  constitutes at least the principle mediator of the synergistic effects of anti-IgM on CD54-dependent enhancement of intracellular cAMP. Consistent with such an interpretation, the amplifying effects of both anti-IgM and ionomycin on CD54-dependent cAMP accumulation could be abolished by the simultaneous inclusion of EGTA in the cultures. In both instances, the cAMP responses were similar to that obtained upon stimulation with anti-CD54 alone (data not shown).

To directly evaluate the consequences of enhanced cAMP concentrations on CD80 levels, we next used its analog db-cAMP. However, to maintain equivalence with the native situation, we sought to use db-cAMP at a concentration that would mimic the effects of either anti-CD54 alone or that of anti-CD54 plus anti-IgM. For this, titration experiments were first performed wherein purified B cells were treated with varying concentrations of db-cAMP, and the consequent intracellular levels then measured. The results from such experiments are summarized in Fig. 4B. From these results it was estimated that a 20  $\mu\text{M}$  concentration of db-cAMP would mimic the effects of anti-CD54 alone. In contrast, the cAMP response upon dual stimulation with anti-CD54 and anti-IgM (or ionomycin) could be duplicated by using db-cAMP at a concentration of 100  $\mu\text{M}$  (Fig. 4, compare A and B).

Our next objective was to assess the effect of the above titrated doses of db-cAMP, either alone or in conjunction with other stimuli, on CD80 expression. The results from such experiments are depicted in Fig. 4C, and several interesting aspects may be noted here. Although the combined stimulation with anti-IgM and anti-CD54 leads to amplified cAMP accumulation, this alone appeared to be insufficient to explain the effect on CD80 levels. This became evident from the fact that treatment of cells with 100  $\mu\text{M}$  db-cAMP alone had no significant effect on surface CD80 densities (Fig. 4C). Interestingly, though, a combination of 100  $\mu\text{M}$  db-cAMP and anti-IgM proved extremely effective, producing an enhancement in CD80 levels that was comparable to that obtained upon stimulation with anti-IgM plus anti-CD54 (Fig. 4C). In other words, in addition to cAMP, up-regulation of CD80 also required an added contribution from BCR-dependent pathways.

Another important finding in Fig. 4C was the fact that the stimulus of anti-IgM could again be substituted with 0.5  $\mu\text{M}$  ionomycin, without any loss in potency. Thus the combination of 100  $\mu\text{M}$  db-cAMP and 0.5  $\mu\text{M}$  ionomycin was as effective as the parent stimulus of anti-CD54 plus anti-IgM at up-regulating CD80 on B cells (Fig. 4C). Therefore, these data suggest that the BCR-mobilized  $\text{Ca}_i^{2+}$  by way of capacitative influx again constitutes the key entity responsible for cooperating with cAMP-dependent pathways, to increase CD80 expression.

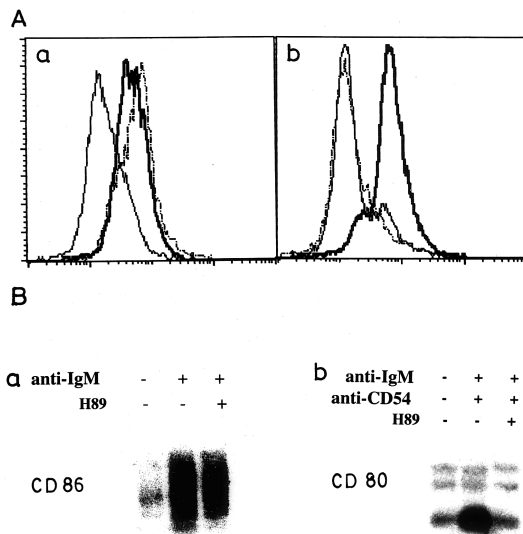
The cumulative results in Fig. 4 also reveal the existence of an iterative interplay between BCR- and CD54-stimulated biochemical pathways. In addition to implicating  $\text{Ca}_i^{2+}$  and cAMP as the two principal second messengers involved, these data also highlight a sequential partitioning of function for the BCR-recruited  $\text{Ca}_i^{2+}$ . At one level this  $\text{Ca}_i^{2+}$  was found to synergize with CD54 stimulation to produce an amplified intracellular cAMP response (Fig. 4A). However, subsequent to this,  $\text{Ca}_i^{2+}$  again appeared to integrate with pathways dependent upon cAMP, to yield an increase in CD80 expression (Fig. 4C). That the synergistic cAMP response was a necessary prerequisite to the latter effect is supported by the data in Fig. 4C. As shown here, reducing the db-cAMP concentration from 100 to 20  $\mu\text{M}$ , a dose that mimics the effects of anti-CD54 alone (Fig. 4B), during stimulation of B cells with either db-cAMP and anti-IgM or with db-cAMP and ionomycin, markedly reduced the efficiency of CD80 induction (Fig. 4C).

#### *BCR- and CD54-dependent pathways also converge to synergize CREB phosphorylation at Ser<sup>133</sup>*

The concerted action of  $\text{Ca}_i^{2+}$  and cAMP signaling pathways on CD80 expression was particularly intriguing, and deserved further scrutiny. However, any information on the transcription factors that directly regulate activation of the CD80 gene is currently lacking. Furthermore, considering the delayed kinetics of induction of CD80 as shown in Fig. 1Ac, it is entirely possible that these two pathways act on early transcriptional events, which then regulate CD80 concentrations either at the level of transcript stabilization or de novo synthesis. Therefore, as an alternative, we explored the effects on phosphorylation of CREB. Activation of CREB has been shown to minimally require its phosphorylation on a Ser residue at position 133 (38–40).

Although it is not known whether CREB in fact influences CD80 expression, several reasons prompted us to choose this particular protein. We have recently identified an octamer sequence (sequence: TGATGTCA) located 713 bp upstream of the transcription start site in the murine CD80 gene, that is capable of binding to CREB in gel-shift assays (K. Natarajan, and K.V.S. Rao, unpublished results). Importantly we found that the induction of CD80 by the combination of anti-IgM plus anti-CD54 could be completely inhibited by the addition of H89, an inhibitor of the cAMP-dependent kinase, protein kinase A (PKA) (41, 42). As shown in Fig. 5, the inhibition was specific for CD80, and was exercised at the level of both cell surface protein and the corresponding mRNA. Because CREB is known to represent the principal substrate for PKA activity (39–41), these findings may implicate, albeit indirectly, a possible role for this transcription factor during CD80 regulation. Finally, it has been shown that Ser<sup>133</sup> of CREB serves as a target for both BCR- and cAMP-dependent pathways in B cells (38, 43). As a result it was anticipated that, at the minimum, CREB could serve as a useful substrate to demonstrate the existence of cooperativity between BCR- and CD54-stimulated pathways.

It has previously been demonstrated that stimulation of murine B lymphoma cells with anti-IgM results in phosphorylation of CREB at Ser<sup>133</sup> (38). The predominant kinase implicated in this appeared to be protein kinase C (PKC) rather than PKA, as identified by the effects of specific inhibitors (38). Consistent with these findings, we observed that stimulation of splenic B cells with anti-IgM also induced Ser<sup>133</sup> phosphorylation of CREB, although the magnitude of this response was significantly lower (~3-fold) (Fig. 6A). Furthermore, this phosphorylation was inhibited to approximately equal extents (between 30 and 40%) by the addition of either the calmodulin-dependent kinase II/IV inhibitor KN-62 (44,

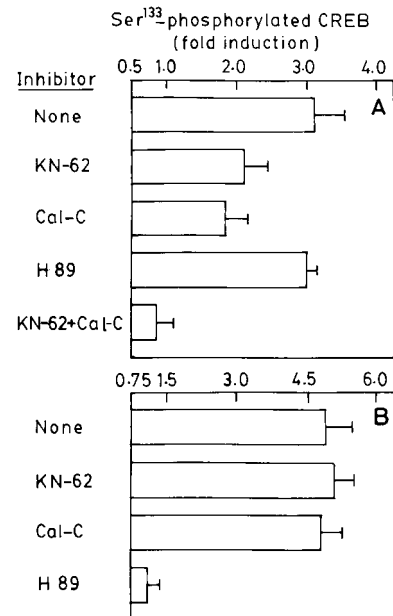


**FIGURE 5.** The PKA inhibitor, H89, inhibits CD80 but not CD86 up-regulation. *A*, Effects of H89 addition on anti-IgM-mediated induction of CD86 (*a*) and anti-IgM plus anti-CD54-dependent up-regulation of CD80 (*b*), respectively. In both cases the thin line represents unstimulated cells, whereas the thick line represents either CD86 (*a*) or CD80 (*b*) in appropriately stimulated cells. In both cases, the dashed line depicts the corresponding levels when the cells were stimulated in the presence of H89 (100 nM,  $2 \times IC_{50}$ ). *B*, Northern blot analyses for either CD86 (*a*) or CD80 (*b*) mRNAs obtained in the individual groups. Parallel analysis for the  $\beta$ -actin message confirmed comparable loading in all cases, although these results are not shown here. Data presented are from one of three separate experiments.

45) or the PKC inhibitor calphostin C (46) (Fig. 6*A*). No significant effect of the PKA inhibitor, H89, could be detected (Fig. 6*A*). Thus, anti-IgM-stimulated phosphorylation of CREB on Ser<sup>133</sup> in B cells appears to be mediated, at least largely, by the combined activities of calmodulin-dependent kinase II/IV and PKC. Consistent with this, addition of a mixture of KN-62 and calphostin C completely abrogated anti-IgM-dependent CREB phosphorylation (Fig. 6*A*).

In similarity to anti-IgM, stimulation with anti-CD54 also led to a near 5-fold enhancement in Ser<sup>133</sup>-phosphorylated CREB levels (Fig. 6*B*). However, in this case the effect appeared to be principally mediated by PKA because addition of H89 yielded near complete inhibition, whereas neither KN-62 nor calphostin C had any detectable effect (Fig. 6*B*). In other words, although both anti-IgM and anti-CD54 individually drive CREB phosphorylation at Ser<sup>133</sup>, they apparently recruit distinct protein kinases to achieve this.

In comparison with the relatively weak individual potencies of anti-IgM and anti-CD54, simultaneous activation with both of these stimuli produced a markedly pronounced effect, yielding a near 20-fold enhancement in levels of the Ser<sup>133</sup>-phosphorylated form of CREB (Fig. 7*A*). Interestingly, partial inhibition of this effect was obtained with KN-62 (25%), calphostin C (20%), or H89, although the effects of H89 were the most pronounced, where inhibition was >75% (Fig. 7*A*). Simultaneous addition of all three of these inhibitors resulted in a complete inhibition of phosphorylation (Fig. 7*A*). Thus, the synergistic effects of anti-IgM and anti-CD54 on the phosphorylation status of CREB appear to reflect the cumulative effects of the protein kinases individually recruited by these stimuli. However, it was notable that a substantial proportion of the enhanced phosphorylation of CREB at Ser<sup>133</sup> was dependent upon PKA activity (Fig. 7*A*).



**FIGURE 6.** Effects of anti-IgM and anti-CD54 on CREB phosphorylation in B cells. Purified B cells were stimulated with anti-IgM (*A*) or with anti-CD54 (*B*) either in the presence or absence of the indicated protein kinase inhibitors as described in *Materials and Methods*. Subsequently prepared nuclear extracts were then resolved by gel electrophoresis, and the extent of CREB phosphorylation was determined by Western blot analysis using Abs specific for Ser<sup>133</sup>-phosphorylated CREB (*Materials and Methods*). Following this, bound Ab was eluted, and the blots were reprobed with Abs against the CREB protein. The extent of enhancement in Ser<sup>133</sup>-phosphorylation was determined by densitometric scanning of the resulting autoradiograms, and is presented as fold induction over that in unstimulated cells after normalizing for CREB protein levels in each lane. Values are the mean ( $\pm$ SD) of three independent determinations. Cal-C, Calphostin C.

The synergistic influence of anti-IgM and anti-CD54 seen in Fig. 7*A* could also be duplicated by substituting these stimuli with 0.5  $\mu$ M ionomycin and 100  $\mu$ M db-cAMP, respectively (Fig. 7*B*). Notably, the effects of the various inhibitors tested also yielded results comparable with that obtained for anti-IgM plus anti-CD54 stimulation (Fig. 7, compare *A* and *B*). Thus, although KN-62 and calphostin C inhibited CREB phosphorylation at Ser<sup>133</sup> by 25 and 20%, respectively, inhibition by H89 was by as much as 80%. At one level, these data confirm that, in similarity with CD80 induction, Ca<sub>i</sub><sup>2+</sup> and cAMP again constitute the principal intracellular mediators that direct the downstream effects of BCR and CD54 ligation on CREB phosphorylation. This is particularly highlighted by the fact that the kinase intermediates recruited by ionomycin plus db-cAMP in this process are the same as those mobilized by anti-IgM plus anti-CD54. In either case a biased requirement for PKA activity could also be noted. Therefore, the results in Fig. 7 clearly identify the existence of a productive cross-talk between BCR-stimulated Ca<sub>i</sub><sup>2+</sup> and intracellular cAMP signaling pathways. This synergy, in turn, is capable of potentiating biochemical responses, at least in terms of CREB phosphorylation.

#### *Cross-talk between second messengers overcomes individual concentration threshold barriers*

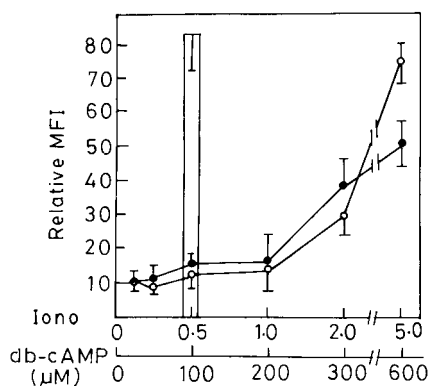
Although our present data demonstrate the requirement for synergistic interactions between Ca<sub>i</sub><sup>2+</sup> and cAMP to facilitate CD80 up-regulation we took note of the fact that these second messengers have also been shown to be capable of independently inducing



**FIGURE 7.** Phosphorylation of CREB on Ser<sup>133</sup> in stimulated B cells. *A*, Results of a Western blot analysis for CREB in either unstimulated cells (*lane 1*) or cells that were stimulated with anti-IgM (*lane 2*), anti-CD54 (*lane 3*), or with a combination of anti-IgM plus anti-CD54 (*lane 4*). Additional sets also include cells stimulated with anti-IgM plus anti-CD54 in the presence of the inhibitors KN-62 (*lane 5*), Calphostin C (*lane 6*), H89 (*lane 7*), or a combination of all three inhibitors (*lane 8*) (*Materials and Methods*). *Top*, Results obtained using Abs specific for the Ser<sup>133</sup>-phosphorylated form of CREB (P-CREB). Subsequent to this, bound Abs were eluted, and the blots were probed once again with Abs against the CREB protein (*bottom*, CREB). *B*, Results of a similar experiment except that cells were stimulated with 0.5  $\mu$ M ionomycin (*lane 2*), 100  $\mu$ M db-cAMP (*lane 3*), or a combination of both (*lane 4*). Results for parallel sets of cells stimulated with 0.5  $\mu$ M ionomycin plus 100  $\mu$ M db-cAMP in the presence of KN-62 (*lane 5*), Calphostin C (*lane 6*), H89 (*lane 7*), or a combination of all three inhibitors (*lane 8*). Unstimulated cells are represented in *lane 1*. Data are a representative of five separate experiments.

CD80 expression. For example, stimulation of B lymphoma cells with db-cAMP alone has been reported to induce surface CD80 expression (36). In a similar vein, stimulation of chronic myelogenous leukemia myeloid progenitor cells with a calcium ionophore has also been found to result in CD80 up-regulation (47). However, in both cases the concentrations of stimulators used were significantly higher than those used in this study (36, 47). Therefore, it occurred to us that the cross-talk between Ca<sub>i</sub><sup>2+</sup> and cAMP may well serve to minimize individual concentrations required for the induction of CD80 expression.

To examine for such a possibility, purified B cells were independently stimulated with varying concentrations of either ionomycin or db-cAMP. In addition, for comparative purposes, a parallel set of cells was also stimulated with the combination of 0.5  $\mu$ M ionomycin and 100  $\mu$ M db-cAMP. The extent of surface CD80 induction in each of these cases was then assessed, and the cumulative results are presented in Fig. 8. However, although both db-cAMP and ionomycin were independently capable of producing enhanced CD80 expression, their efficiency was poor (Fig. 8). Relatively higher concentrations of either of these two agents were required to produce a significant effect (Fig. 8). In contrast to this, the combination of ionomycin and db-cAMP proved extremely effective, yielding optimal CD80 enhancement with only suboptimal concentrations of the individual stimuli (Fig. 8). Therefore, these results reveal that the concerted action of second messenger-dependent signaling modules can subvert concentration thresholds



**FIGURE 8.** Thresholds for Ca<sub>i</sub><sup>2+</sup> and cAMP for induction of CD80. Purified B cells were stimulated with indicated concentrations of either ionomycin (Iono, ○) or db-cAMP (●). Following this, the subsequent expression of surface CD80 was monitored by flow cytometry. In addition, a parallel set of cells was also treated with a combination of 0.5  $\mu$ M ionomycin and 100  $\mu$ M db-cAMP for comparative purposes. The resultant enhancement in surface CD80 expression is shown by the open column. Values are the mean ( $\pm$ SD) of five separate experiments.

required for independent action, at least in the context of CD80 regulation.

## Discussion

Although studies continue to detail the early biochemical events initiated upon BCR cross-linking, the importance of modulation of these pathways by accessory signals is also being increasingly realized. One way of viewing the signaling cascades, initiated upon stimulation of a given cell surface molecule, can be as analogous to the wave fronts generated upon perturbation at a single source. In such a case, the interference between ‘wave-fronts’ of independent origin may then be expected to lead to either a ‘constructive’ or ‘destructive’ outcome. A good example of the former is the coligation of BCR with the CR2/CD19/TAP-1 complex, where B cell responsiveness is heightened (15, 16). In contrast, the cross-linking of BCR with the Fc $\gamma$ RIIb coreceptor, where B cell responsiveness is attenuated (17–20), may be considered as a case resulting from destructive interference.

Although the existence of signal interference is now well established, our understanding of the various modes by which they can occur is sparse. It was with the intent of gaining additional insights into this aspect within B lymphocytes that this study was undertaken. It was anticipated that our present experimental system, using anti-IgM and anti-CD54 as the stimuli, would shed light on how BCR-dependent pathways service CD86 expression on the one hand, and concomitantly integrate with CD54-dependent pathways to modulate CD80 levels on the other.

The identification of Ca<sub>i</sub><sup>2+</sup> as an obligatory mediator for the induction of both CD80 and CD86 provided us with a reference point for further study. This was particularly true given that this intermediate was solely a product of BCR-triggering, where the second phase of recruitment—by capacitative influx—proved to be at least sufficient to account for the effects of anti-IgM. It was at this stage of our investigations that at least two distinct effector modes for BCR-mobilized Ca<sub>i</sub><sup>2+</sup> had become apparent. On the one hand, this second messenger was independently capable of driving CD86 up-regulation. Direct evidence in support of such a conclusion could be afforded by experiments wherein stimulation of B cells with a pretitrated dose of ionomycin proved to be as potent as anti-IgM in this effect. However, in addition to this, BCR-dependent Ca<sub>i</sub><sup>2+</sup> was also shown to act in concert with CD54-driven pathways to provide for an amplified cAMP response. It was this increased accumulation of intracellular cAMP that then set the stage for a second round of cooperativity between Ca<sub>i</sub><sup>2+</sup> and cAMP, resulting in enhanced surface expression of CD80. The direct involvement of both Ca<sub>i</sub><sup>2+</sup> and cAMP in the latter process could be substantiated by the fact that a combination of ionomycin and db-cAMP at their predefined concentrations was as effective as



the parent stimulus of anti-IgM plus anti-CD54 at up-regulating CD80 levels.

The interactions between BCR-mobilized  $\text{Ca}_i^{2+}$ - and CD54-dependent signaling pathways could also be evidenced at the level of phosphorylation of CREB on Ser<sup>133</sup>. Although a role for CREB in CD80 regulation is yet to be demonstrated, these results at the very least provide biochemical confirmation for the productive nature of this interaction. Although the increase in Ser<sup>133</sup> phosphorylation of CREB, due to activation of cells with anti-IgM plus anti-CD54, was found to involve kinase intermediates independently recruited by the individual stimuli, the observed predominant involvement of PKA activity is intriguing. It would be interesting to see whether the potentiatory effects of anti-IgM plus anti-CD54 stimulation are also exercised at the level of PKA activation. Importantly though, this synergistic effect could be demonstrated to be again mediated by the two second messengers,  $\text{Ca}_i^{2+}$  and cAMP, providing additional evidence for a constructive interference between these two signaling modules.

It has been previously reported in murine B cells that enhancement of intracellular cAMP levels before BCR ligation has inhibitory effects on both 1,4,5-inositol triphosphate generation and  $\text{Ca}_i^{2+}$  mobilization (48). From this the existence of a cross-talk between cAMP- and BCR-dependent phosphoinositol signaling pathways was inferred (48). Although present data confirm the existence of such a cross-talk, they also suggest that the outcome of this process is strictly dependent upon the timing at which these two pathways are initiated. Thus, in contrast to the attenuating effects of sequential triggering, simultaneous initiation leads to potentiation of at least some of the subsequent responses. However, the biochemical interactions mediating this cross-talk remain unknown, and would clearly be of interest to delineate.

In addition to the above, our present characterization of the interactions between BCR- and CD54-dependent pathways also provides a novel insight into the networking of independently generated signaling modules. As the data suggests, this constitutes a sequential process that is initiated by a cross-talk between the two pathways. The consequence of this interaction then, is to directly promote a second round of synergy and thereby facilitate CD80 up-regulation. In other words, these findings reveal the existence of "dialectical" modes of signal interference, which can contribute toward gain of function for the target cell.

Finally, our delineation of differences in both the concentration and spectrum of the regulatory intermediates involved during expression of CD80 and CD86 provides an empirical explanation for their distinct induction thresholds. Although CD86 expression could be achieved through a relatively moderate augmentation in  $\text{Ca}_i^{2+}$ , concomitant induction of CD80 required the added support from cAMP recruitment. Our observation that cross-talk between Ca- and cAMP-signaling pathways modulates individual concentrations required for CD80 up-regulation is also particularly relevant. Thus, in principle, either independent mobilization of any of these mediators at optimal concentrations, or simultaneous recruitment of both at suboptimal concentrations, should render CD80 expression permissive at least in B cells. It is likely, then, that the versatility offered by such multiple options may well constitute the wherewithal for "intelligent decision-making" when responding to external stimuli.

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