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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

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 Ca²⁺, 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 Ca²⁺ 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 Ca²⁺ and CD54-derived signals. This then facilitated a second synergistic interaction between Ca²⁺ 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.

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
beads for the purification of B lymphocytes were obtained from Miltenyi Biotec (Auburn, CA). Inhibitors to various kinases such as genistein, calphos- 

in C, 1-N-[(2-(bist-(5-isouinolinesulfonyl))V-methyl)-1-troxy]-4-phe- 

nopeniprazin (KN-62), calmidazolium, cyclosporin A, EGTA, and other 

reagents such as guanidine isothiocyanate and dibutyryl-cAMP (db-cAMP) 

were obtained from Sigma (St. Louis, MO). Ionomycin, 8-(diethylamino)oc- 

tyl-3,4,5-trimethoxybenzoate (TMB-8), N-(2-((p-bromocinnamyl)amin- 
o)ethyl)-5-isouinolinesulfonamide (H89), fluo-3-acethoxymethyl ester (fluo-

3-AM), Abs to Ser[115]phosphorylated CREB, and CREB were purchased from 

BD Immunocytometry Systems, Mountain View, CA). Logarithmically 

grown lymphoid cells were used for all experiments. B lymphocytes were 

isolated from BALB/c mice (6–8 wk old), maintained under pathogen-free con-

ditions, were obtained from the small experimental facility of our institute. 

Enrichment of B lymphocytes 

This was achieved by negative selection, through magnetic sorting, essen-

ually as described previously (22). Briefly, splenocytes from 6–8 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 IgG2a popu-

lation 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 x 10⁴/well in 0.1 ml of 

RPMI 1640 containing 10% FCS and antibiotics (RPMI 10). Depending on 

the requirement, cells were cultured with the Fab, fragment of anti-IgM 

(10 µg/ml), and/or anti-CD54 (10 µg/ml), and/or ionomycin (0.5 µM). In 

initial experiments, stimulation times were varied from 2 to 24 h for flow 

cytometry 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 CD86 up-regulation required between 8 and 12 h of 

treatment with the combination of anti-IgM plus anti-CD54. Consequently, 

the protocol recommended by the manufacturer.

To determine the effects of exogenous addition of db-cAMP on intra-

cellular 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 washed with ice-cold PBS, 

and lysed in lysis buffer (10 mM HEPES (pH 7.9); 10 mM KCl; 0.1 mM EDTA; 1 mM MgCl₂; 0.5% Nonidet P-40, and 2 mg/ml each of 

aprotinin, leupeptin, and pepstain). The resulting nuclear pellet was then 

extracted in buffer containing 20 µM HEPES (pH 7.9); 0.4 M 

NaCl; 1 mM EDTA; 1 mM EGTA; and 2 mg/ml each of aprotinin, leu-

peptin, and pepstatin.

Nuclear extracts from 1 x 10⁶ cells were resolved on 10% SDS-

polyacrylamide gels and subsequently transferred onto a nitrocellulose 

membrane (Hybond-C; Amersham). The resulting blots were probed with 

rabbit Abs specific for Ser[115]phosphorylated CREB, followed by HRP-

labeled secondary Abs. Blots were developed by chemiluminiscence 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 CD80 and CD86 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 combina-

tion 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 sig-

nificant 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 CD80, CD86 showed delayed induction becoming apparent only several hours 

after initiation of stimulation (Fig. 1A, c). This observed enhance-

ment in surface densities of CD80 and CD86 in response to the ap-

propriate stimuli also correlated well with a concomitant increase in the respective mRNA levels as shown in Fig. 1B. The
Ca2+ by anti-IgM plus anti-CD54. Whereas TMB-8 is known to inhibit dependent CD86 up-regulation and CD80 up-regulation mediated of either TMB-8 or EGTA completely abolished both anti-IgM-examined the effects of addition of a variety of pharmacological To dissect biochemical mediators of the above response, we next analyzed the effects of addition of a variety of pharmacological agents on the expression of both CD80 and CD86. Consequently, we next examined the effects of BCR and CD54 ligation on Ca2+ mobilization. Consistent with prior findings (for a review, see Ref. 29), stimulation of B cells with anti-IgM resulted in a biphasic recruitment of Ca2+ ions into the cytoplasm. A rapid, but transient, increase was evident immediately upon addition of anti-IgM (Fig. 3A). Although these levels declined rapidly, they nevertheless stabilized at concentrations significantly above the baseline (Fig. 3A). 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 Ca2+ mobilization by B cells, in response to anti-IgM stimulation (Fig. 3A). As opposed to this, EGTA addition selectively suppressed anti-IgM-mediated capacitative Ca2+ influx, with only a relatively minor effect on recruitment from intracellular stores (Fig. 3A).

Distinct from the effects of anti-IgM, stimulation of B cells with anti-CD54 had no detectable influence on Ca2+ concentrations (Fig. 3A). 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 Ca2+ concentrations in B cells is in keeping with prior findings. In a recent study, van Horssen et al. (32) also did not detect Ca2+ mobilization in B cells upon CD54 ligation although CD54 cross-linking has been shown to enhance Ca2+ concentrations in rat endothelial cells (33). Thus, whereas Ca2+ was identified as a common mediator of both the CD80 and CD86 regulatory pathways, its intracellular mobilization appeared to exclusively derive magnitude of increase in cellular mRNA content observed in Fig. 1B 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. 1A), as well as at that of the respective mRNAs (Fig. 1B). 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 (Ca2+) 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 Ca2+ 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.
and CD80 (D). Cells, Unstimulated; aIg, anti-IgM; In, 0.5 mM and cells with an ionomycin concentration of 0.5 M. It is evident from this figure that treatment of the EGTA-sensitive phase of anti-IgM-stimulated mobilization (D) was monitored. The cumulative results from such experiments are depicted by the dashed lines above and below represent the SD from the mean (A, profile 4). In a separate experiment, cells were treated with graded concentrations of ionomycin, and the peak Ca\textsuperscript{2+} obtained at each concentration was averaged over a 10-min period. The graph in B depicts the mean (±SD) of four separate determinations. The line intersecting the graph indicates the mean Ca\textsuperscript{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 CD80 (C) and CD86 (D). Cells, Unstimulated; aIg, anti-IgM; In, 0.5 mM ionomycin; aCD, anti-CD54. Values presented are in terms of modal fluorescence intensity (MF1) in B220\textsuperscript{+} gated cells for each group, and are the mean (±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 Ca\textsuperscript{2+} recruitment as the key regulatory component.

The above identification of a role for BCR-mediated capacitative Ca\textsuperscript{2+} influx also prompted us to next single out the effects due to this second messenger. Influx of Ca\textsuperscript{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 Ca\textsuperscript{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 Ca\textsuperscript{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 mM mimics the capacitative entry phase of anti-IgM-stimulated recruitment of calcium ions.

To ascertain the specific effects of this Ca\textsuperscript{2+} concentration, we next used 0.5 mM 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 mM 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 mM ionomycin and anti-CD54 (Fig. 3D). The extent of enhancement 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 mM ionomycin could duplicate the effects of anti-IgM on both CD80 and CD86 up-regulation, further underscores the relevance of Ca\textsuperscript{2+} in both pathways. Importantly, these findings support that BCR-mediated capacitative Ca\textsuperscript{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 Ca\textsuperscript{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 mM), and the extent of enhancement (~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 mM), 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 3](http://www.jimmunol.org/Downloaded.png)

**FIGURE 3.** Ca\textsuperscript{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 Ca\textsuperscript{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 Ca\textsuperscript{2+} obtained at each concentration was averaged over a 10-min period. The graph in B depicts the mean (±SD) of four separate determinations. The lines intersecting the graph indicates the mean Ca\textsuperscript{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 CD80 (C) and CD86 (D). Cells, Unstimulated; aIg, anti-IgM; In, 0.5 mM ionomycin; aCD, anti-CD54. Values presented are in terms of modal fluorescence intensity (MF1) in B220\textsuperscript{+} gated cells for each group, and are the mean (±SD) of four separate determinations.

![Figure 4](http://www.jimmunol.org/Downloaded.png)

**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 intercepting 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\textsuperscript{+} gated cells in response to the indicated stimuli. Cells, Unstimulated; aIg, anti-IgM; In, 0.5 mM ionomycin; dbc (20), 20 mM db-cAMP; dbc (100), 100 mM db-cAMP; aCD, anti-CD54. Values are the mean (±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 μM 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 Ca$^{2+}$ 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 μM 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 μM (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 μM db-cAMP alone had no significant effect on surface CD80 densities (Fig. 4C). Interestingly, though, a combination of 100 μM 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 μM ionomycin, without any loss in potency. Thus the combination of 100 μM db-cAMP and 0.5 μM 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 Ca$^{2+}$ 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 Ca$^{2+}$ and cAMP as the two principal second messengers involved, these data also highlight a sequential partitioning of function for the BCR-recruited Ca$^{2+}$. At one level this Ca$^{2+}$ was found to synergize with CD54 stimulation to produce an amplified intracellular cAMP response (Fig. 4A). However, subsequent to this, Ca$^{2+}$ 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 μM, 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 Ser133**

The concerted action of Ca$^{2+}$ 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: TGATGTCAT) 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 Ser133 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 Ser133 (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 Ser133 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, 

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*Note: The text continues beyond the visible excerpt, but is not included here.*
dependent upon PKA activity (Fig. 7A), although the effects of H89 were the most pronounced, where formation of CREB (Fig. 7A). Interestingly, partial inhibition of this form of CREB (Fig. 7A). In other words, although both anti-IgM and anti-CD54 individually drive CREB phosphorylation at Ser\(^{133}\), 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\(^{133}\)-phosphorylated form of CREB (Fig. 7A). 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. 7A). Simultaneous addition of all three of these inhibitors resulted in a complete inhibition of phosphorylation (Fig. 7A). 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\(^{133}\) was dependent upon PKA activity (Fig. 7A).

The synergistic influence of anti-IgM and anti-CD54 seen in Fig. 7A could also be duplicated by substituting these stimuli with 0.5 \(\mu\)M ionomycin and 100 \(\mu\)M db-cAMP, respectively (Fig. 7B). 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\(^{133}\) 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, \(Ca^{2+}\) 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 \(Ca^{2+}\) 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 \(Ca^{2+}\) 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...
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,++ 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 μM ionomycin and 100 μM 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 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 FcγRIIb 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,++ 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,++ had become apparent. On the one hand, this second messenger was independently capable of driving CD80 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,++ 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 cooperation between Ca,++ and cAMP, resulting in enhanced surface expression of CD80. The direct involvement of both Ca,++ 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 Ca\(^{2+}\) and CD54-dependent signaling pathways could also be evidenced at the level of phosphorylation of CREB on Ser\(^{133}\). 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\(^{133}\) 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 CREB, due to activation of cells with anti-IgM plus anti-CD54, could be achieved through a relatively moderate augmentation in their distinct induction thresholds. Although CD86 expression provides a novel insight into the networking of independently generated signaling pathways. The consequence of this interaction then, is to directly potentiate 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 Ca\(^{2+}\), 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.

References


