Brief Definitive Report

Selective Activation of the Calcium Signaling Pathway by Altered Peptide Ligands

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Summary

We previously demonstrated that altered peptide ligands (APL) can partially activate T cells, resulting in multiple distinct functional phenotypes, including the induction of anergy. Such APL stimulate a unique pattern of T cell receptor (TCR) phospho-ζ species, and lack associated ZAP-70 kinase activity. While these data suggested that selective signaling pathways downstream of the TCR/CD3 molecules are activated upon APL stimulation, they did not directly demonstrate this. Thus, we pursued intracellular signaling events successfully stimulated by APL. Because our previous studies showed that cyclosporin A (CsA) completely inhibited anergy induction, we assessed whether TCR ligation by APL cause a rise in cytosolic calcium (Ca^{++}). Our results show that these ligands can induce Ca^{++} transients, in contrast to data generated using analogue peptides in other antigen systems. These opposing results may reflect differences in the intracellular signaling pathways utilized by different APL, or may be due to the exquisite sensitivity of the assay used here. Importantly, the APL-stimulated Ca^{++} induction is both initiated and sustained at lower levels than that stimulated by a strong agonist signal, but resembles that stimulated by a weaker agonist stimulus. Alone, the less than optimal Ca^{++} induction does not cause anergy, because ionomycin treatment together with the APL does not result in a proliferative signal. Instead, we propose that a combination of this and other signaling pathways induces T cell anergy. Overall, these data support the concept of differential signaling in T cells, as a direct consequence of the phosphotyrosine status of the TCR/CD3 molecules.

The T cell receptor (TCR) is composed of the antigen-specific αβ heterodimer, and the CD3 complex responsible for translating the extracellular binding events into the biochemical pathways involved in T cell activation (1, 2). The CD3 γ, δ, and ε molecules each contain a single signaling module, called an immunoreceptor tyrosine-based activation motif (ITAM), characterized by the consensus sequence $Yxx(L/I)x_{(6-8)}Yxx(L/I)$, whereas the TCR ζ chain expresses three such ITAMs (3). Upon ligand binding the tyrosines of the ITAMs become phosphorylated, allowing the modules to serve as templates for binding downstream SH2-domain containing proteins involved in T cell activation (4, 5).

Studies in which individual TCR/CD3 chains were expressed in isolation of the others formally demonstrated that at least two independent signaling modules exist in the TCR unit (6–8). However, the systems used could not address whether selective signaling events can be triggered upon TCR engagement of ligand in normal T lymphocytes. The recent identification of APL, which have been shown to selectively activate a variety of effector functions, (reviewed in 9–11) provided a convenient tool to begin to test this query. Initial investigations showed that APL stimulated a unique phospho-ζ pattern in T cell clones, which was directly related to binding of only the unphosphorylated form of ZAP-70 and a lack of ZAP-70 kinase activity (12, 13). These results implied that some intracellular signaling events downstream of the TCR/CD3 molecules were being activated by APL, but there was no direct data to prove this.

Here we pursued signaling events actively engaged upon receptor ligation of APL. We show that such ligands can induce an increase in intracellular Ca^{++} ($[Ca^{++}]_i$) in the T cells, indicating successful engagement of the signaling pathway responsible for cytosolic Ca^{++} elevation. The phenotype stimulated by APL differs both quantitatively and qualitatively from that stimulated by a strong immunogenic ligand, and likely contributes to the differing functional outcomes of the cells upon receptor engagement of such ligands. These observations provide the first biochemical evidence of engagement of a signaling pathway downstream of the TCR/CD3 chain phosphorylation by APL.

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Materials and Methods

Mice. Female CBA/J and B10.BR/SgSnJ mice were purchased from the National Cancer Institute (Bethesda, MD) or The Jackson Laboratory (Bar Harbor, ME) and were used at 5–10 wk of age.

Ag, Antibodies, and Reagents. The Hb(64-76) peptide and APL were synthesized, purified, and analyzed as described (14). APL have been defined previously (11) and are referred to using the one letter amino acid code of the substituted amino acid residue and its position. The amino acid sequence for the peptides used in this study are: Hb(64-76) = GKKVITAFNEGLK; S70 = GKKVITSFNEGLK; M70 =GKKVITMFNEGLK, and Q72 = GKKVITAFQEGLK. fura-2/AM was purchased from Molecular Probes (Eugene, OR) and ionomycin from Sigma (St. Louis, MO).

Cell Lines. The generation, characterization and propagation of the Th1 clone PL.17 has been previously described (15). The DCEK Hi7 fibroblasts, expressing I-E^k (16) were used as APC in proliferation, tolerance induction, and Ca^{++} assays.

Proliferation Assays. The proliferative response of PL.17 to the various stimuli was performed as described (17), using T cells and DCEK Hi7 fibroblasts at 3×10^4/well and the peptides at the indicated concentrations. In some experiments ionomycin was included for the duration of the assay at 0.5, 1, or 1.5 μM as indicated.

Tolerance Induction. The tolerance induction assay was performed essentially as previously described, with the inclusion of EGTA (0.5 mM) where indicated (17). After separation from the APC, T cells were rested for 24 hr in RPMI 1640 complete medium (17), before being challenged in a proliferation assay as above, using 2×10^4 T cells/well, irradiated B10.BR splenocytes as APCs (2,000 rads, 5×10^5/well), and Hb(64-76) as antigen (0.01–10 μM). T cells (2×10^4/well) were also incubated with IL-2 alone (20 U/well), and cells from all stimulations proliferated equivalently under these conditions.

Ca^{++} Analysis. T cells were labeled with fura-2/AM (5 μM) for 45 min at 37°C. Glass coverslips containing peptide-pulsed (100 μM, 2 hr at 37°C) DCEK Hi7 APC monolayers were affixed to a Leiden cover slip dish, and mounted on a PDMI-2 open perfusion micro-incubator (Medical Systems Corp., Greenvale, NY) on a Zeiss Axiovert microscope. The labeled T cells were added to the mounted coverslips, maintained at 37°C with 10% CO_2, and recordings were carried out for 30 min using a Dage MTI CCD 72 camera and intensifier. Alternative 340 nm and 380 nm light stimuli provided excitation ratio imaging to be performed with a FL-4000 imaging system (Georgia Instruments, Roswell, GA) with values being recorded at 2-s intervals. Images were processed by a Matrox MVP image processing card mounted in a PC computer, stored on a Panasonic TQ3031F optical memory disk recorder, and printed on a SONY UP-5000 color video printer. The intracellular calcium concentration was estimated from the 340/380 ratios by comparison to a standard curve generated using buffered calcium standards (Molecular Probes).

Results

Role of Ca^{++} Ionophore and Extracellular Ca^{++} in APL-induced T Cell Responses. To examine the involvement of Ca^{++} in partial T cell activation, we utilized the PL.17 Th1 clone specific for the Hb(64-76)/I-E^k determinant. The S70 APL did not stimulate any proliferation of the PL.17 T cells, but it could induce a state of anergy. Because no phosphatidylinositol (PI) hydrolysis was detected upon stimulation with this APL (17), one reasonable conclusion was that no cytosolic Ca^{++} increase occurred in the T cells after stimulation with APL. Alternatively, the assay system used may not have detected low levels of PI hydrolysis stimulated by APL. To explore directly the role of Ca^{++} in APL-induced signaling, the $[Ca^{++}]_i$ was raised pharmacologically using ionomycin. Ionomycin addition was inhibitory to the proliferative response induced by agonist ligand (Fig. 1 *A*), previously shown to be a result of direct inhibition of responsiveness to endogenously produced IL-2 (18). Importantly, addition of ionomycin at any dose tested, together with the S70 APL, did not stimulate proliferation or any IL-2 production by the T cells (Fig. 1 *A* and data not shown). Thus, artificial cytosolic Ca^{++} elevation did not convert the APL to a full agonist. Furthermore, Ca^{++} chelation by EGTA had no effect on the APL-induced T cell anergy (Fig. 1 *B*), showing that anergy could still be induced in the absence of extracellular Ca^{++}. The possible participation of Ca^{++} from intracellular stores, however, still remained.

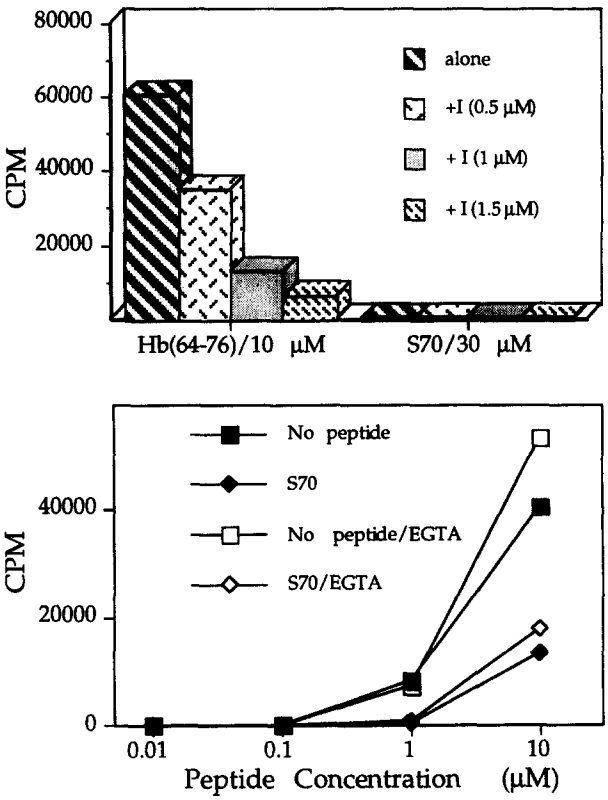

Figure 1. Effects of ionomycin and EGTA on S70 stimulated responses of PL.17. (*A*) PL.17 cells were stimulated with DCEK Hi7 APC and Hb(64-76) (10 μM) or the S70 APL (30 μM), together with varying concentrations of ionomycin. Proliferation was determined as described in *Experimental Procedures*. (*B*) T cells were incubated overnight with APC alone or together with the S70 APL, with or without the addition of EGTA (0.5 mM), and anergy induction assessed as the ability of the T cells to proliferate in response to fresh APC and Hb(64-76). The data are representatives of three (*A*) and two (*B*) independent experiments.

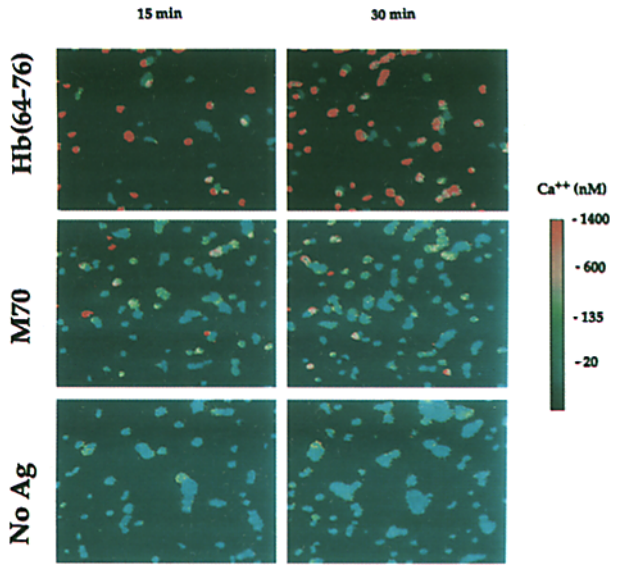

Figure 2. Ca^{++} response of PL.17 cells after stimulation with Hb(64-76) or the M70 APL. This is a pseudocolor representation of $[Ca^{++}]_i$ in individual T cells stimulated with either Hb(64-76) or M70 APL (100 μM), or DCEK Hi7 APC alone, using a fluorescence image processing system. Views of the same fields are shown 15 and 30 min after addition of the T cells to the APC monolayers. The scale relating the color of the T cells to the level of $[Ca^{++}]_i$ is shown. This is a representative of five independent experiments.

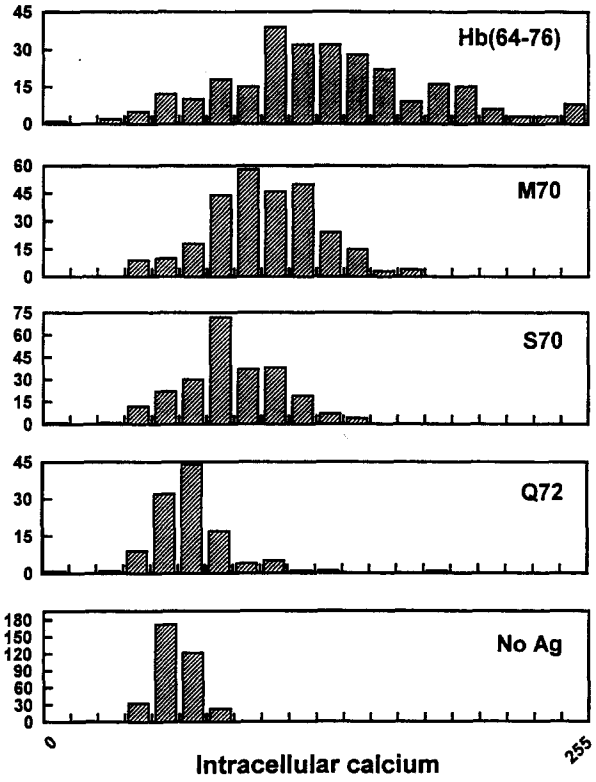

Figure 3. Ca^{++} response of PL.17 after stimulation with various APL. T cells were stimulated as in Fig. 2, using the peptides indicated (100 μM), and the $[Ca^{++}]_i$ of all T cells in multiple fields were quantitated after 30 min. The number of T cells with relative $[Ca^{++}]_i$ at each level is presented on a scale of 0-255 arbitrary units. Total number of cells counted for each group were: 276 for Hb(64-76), 281 for M70, 243 for S70, 116 for Q72, and 357 for no peptide. Mean ± SD were 133 ± 48 for Hb(64-76), 104 ± 26 for M70, 88 ± 23 for S70, 70 ± 20 for Q72, and 56 ± 11 for no peptide. The data are representatives from five independent experiments.

Receptor Ligation by APL Stimulates a Rise in $[Ca^{++}]_i$. A direct examination of the $[Ca^{++}]$ in individual T cells was made by imaging fura-2 fluorescence (19–21). Fura-2/AM loaded PL.17 T cells were added to peptide-pulsed APC monolayers, and the level of Ca^{++} in individual T cells determined using a video imaging system. Shown in Fig. 2 are pseudocolor representations of $[Ca^{++}]_i$ in individual T cells after 15 and 30 min of stimulation with various ligands. Hb(64-76) stimulated a strong Ca^{++} rise in the majority of T cells with near maximal levels detected quickly and sustained over the time traced (Fig. 2, *A* and *B*). In contrast, stimulation of the T cells with APC alone did not lead to significant rise in Ca^{++} levels (*E* and *F*). Presentation with the M70 APL resulted in a measurable increase in $[Ca^{++}]_i$ in most T cells in the field (C, D). The Ca^{++} rise in individual cells was lower than that stimulated by Hb(64-76), but it still represented a significant and specific activation of this biochemical pathway.

Stimulation of Increased $[Ca^{++}]_i$ by APL Occurs in the Majority of T Cells. To ensure that the fields observed in Fig. 2 were representative of the T cell population as a whole, data was collected and examined from several fields after 30 min stimulation with the individual peptides. Hb(64-76) stimulation resulted in T cells exhibiting various $[Ca^{++}]_i$, ranging from very low to the maximal level detectable using fura-2/AM. Thus, at any one time, individual T cells within a population are in different stages of activation, likely a reflection of their individual times of contact with peptide-pulsed APC. Moreover, Hb(64-76) can stimulate maximal detectable levels of $[Ca^{++}]_i$ in a significant proportion of the cells. In contrast, the M70 APL induced significant $[Ca^{++}]_i$ in the majority of cells, but the maximal detectable level was never achieved. This confirms the phenotype shown in Fig. 2, highlighting the inability of this APL to stimulate a maximal rise in cytosolic Ca^{++}.

We next investigated the ability of several other analogues to cause Ca^{++} transients in the T cells to determine if there was a correlation between this and the functional partial activation phenotype. Similar to M70, the S70 APL stimulated a significant rise in $[Ca^{++}]_i$ in most cells examined, with no cells reaching the maximal detectable level. The mean $[Ca^{++}]_i$ reached was slightly lower than that stimulated by M70 (Fig. 3 legend), consistent with the functional performances of these two analogues as partial activators (12). Finally, the control Q72 peptide, which has no detectable functional effect, was unable to stimulate any significant rise in cytosolic Ca^{++} in PL.17.

APL Stimulate a Lower Level of Intracellular Ca^{++} Than Immunogenic Ligand. To ascertain if the difference in $[Ca^{++}]_i$ stimulated by immunogenic ligand and the APL was quantitative or qualitative, we compared the $[Ca^{++}]_i$ induced by different concentrations of Hb(64-76) and M70. As shown

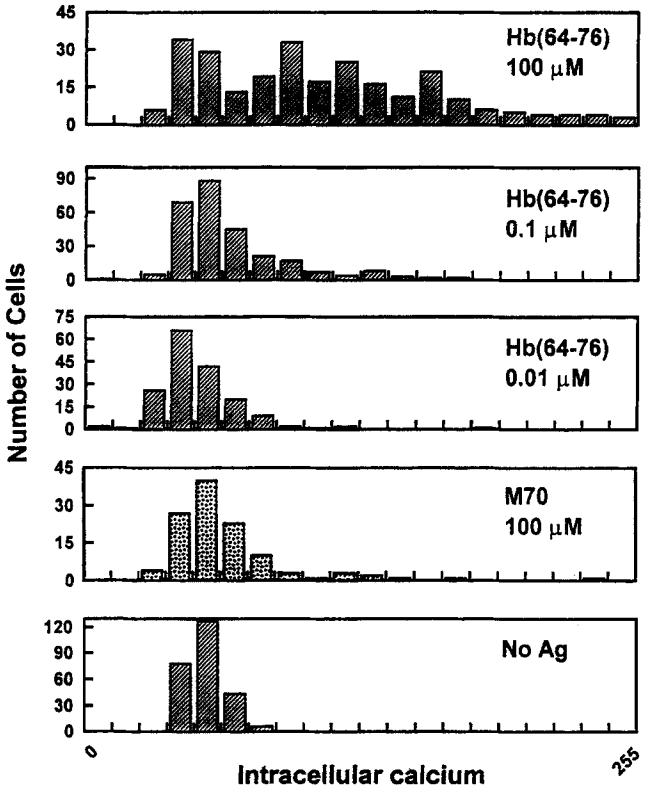

Figure 4. Comparing the Ca^{++} response of PL.17 T cells after stimulation with various concentrations of Hb(64-76) or the M70 APL. T cells were stimulated as in Fig. 3 above, using the indicated concentrations of Hb(64-76) or the M70 APL, and $[Ca^{++}]_i$ determined after 30 min and plotted as in Fig. 3. Total number of cells counted for each group were: Hb(64-76) stimulation: 260 for 100 μM, 272 for 0.1 μM, and 172 for 0.01 μM; 116 for M70, and 255 for no peptide. Mean ± SD were: Hb(64-76) stimulation: 109 ± 52 for 100 μM, 67 ± 26 for 0.1 μM, and 53 ± 20 for 0.01 μM; 67 ± 28 for M70, and 50 ± 9 for no peptide. The data are representatives from three independent experiments.

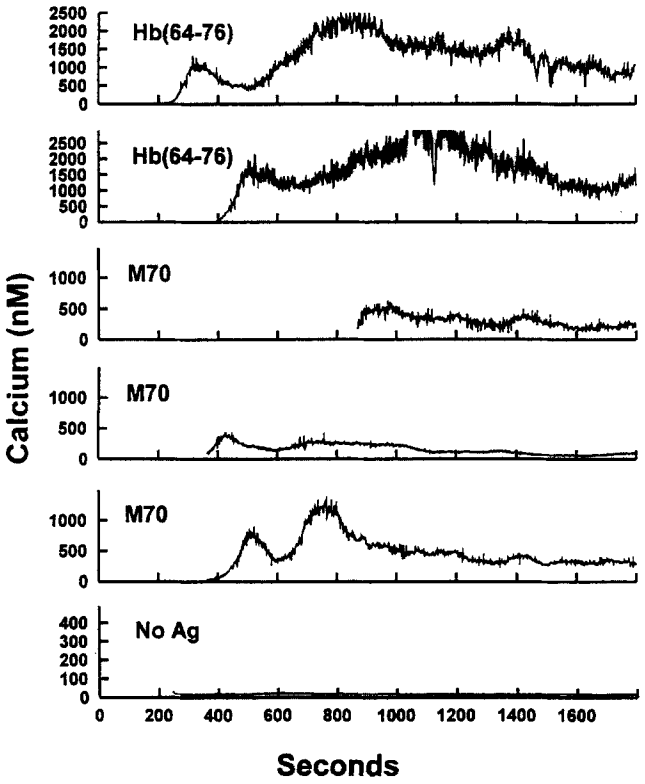

Figure 5. Ca^{++} response of individual T cells over 30 mins stimulation with Hb(64-76) or the M70 APL. PL.17 cells were stimulated as in Fig. 2, with Hb(64-76), M70 APL, or APC alone, and the $[Ca^{++}]_i$ of individual T cells traced and recorded over the 30-min stimulation period. The data are representatives from five independent experiments.

in Fig. 4, the rise in cytosolic Ca^{++} induced by M70 was similar to that stimulated between 0.1 μM and 0.01 μM of Hb(64-76), both in the total number of cells stimulated and the magnitude of the Ca^{++} rise. Thus, the Ca^{++} transients induced by APL or agonist ligand appear to differ quantitatively, with 100 μM of M70 being approximately equal to 0.1 μM of Hb(64-76). Notably, this APL is capable of stimulating much higher $[Ca^{++}]_i$ (for example see Fig. 3), with the variation likely a reflection of the quiescent state of the T cells in individual experiments.

Subtle qualitative differences in Ca^{++} signaling were also noted, as shown by the Ca^{++} tracings of individual cells from Hb(64-76) or M70 stimulated cells (Fig. 5). Hb(64-76) stimulated a high peak response, with $[Ca^{++}]_i$ reaching beyond 2 μM (*top two panels*). Importantly, these high concentrations of Ca^{++} were sustained throughout the 30-min time period assessed. In contrast, although M70 APL also stimulated an initial high level of $[Ca^{++}]_i$, this was much lower than that stimulated by immunogenic ligand, with the highest value detected being ~1 μM (*5th panel*). Intriguingly, this initial concentration was not maintained but quickly dropped to a much reduced but still significant level, which was ultimately sustained throughout the time traced (compare M70 with No Ag). Thus, the APL were able to activate an increase in $[Ca^{++}]_i$ in PL.17, but at a much reduced and less efficiently sustained level than Hb(64-76).

Discussion

While our previous studies showed that several independent signaling pathways are linked to the TCR complex, only components which remained inactive upon APL ligation were defined (12). To understand more thoroughly the complexity of T cell activation, signaling components which are activated successfully by APL had to be identified. Because our previous functional data had implicated a role for calcineurin in the signaling involved in anergy induction, we assumed that the T cells were increasing $[Ca^{++}]_i$ upon APL interaction. Using a direct, quantitative and sensitive approach, we have now been able to show that this is indeed the case. The observation of successful

activation of one intracellular pathway in the absence of ZAP-70 tyrosine kinase activity directly shows that the signaling pathways downstream of the TCR complex can be activated independently of one another. This should allow multiple combinations of biochemical events to occur, culminating in several distinct functional phenotypes.

Based on the current T cell activation pathway model, one would predict that the absence of PI hydrolysis would result in the lack of an elevated $[Ca^{++}]_i$ (1, 2). Contrary to this, although the APL do not stimulate any detectable PI turnover in the T cells (17), they do successfully stimulate a significant rise in cytosolic Ca^{++}. Possible explanations for these apparent disparate findings are i) the insensitive PI turnover assay was unable to detect low levels of PI hydrolysis induced by the APL, or ii) several biochemical routes exist to achieve a release of Ca^{++} from intracellular stores, and one which is independent of IP_3 is used by the APL.

The results shown here not only demonstrate that APL do indeed stimulate a significant Ca^{++} response, but reveal several interesting characteristics of it as compared to that stimulated by agonist ligand. First, the levels achieved never reached the much higher levels stimulated by Hb(64-76), indicating a quantitative difference in the activity of this pathway. Second, the initial Ca^{++} concentrations reached after APL ligation were not sustained, but quickly dropped to much reduced but still significant levels, indicating a qualitative difference in this signal stimulated by the two ligands also. Our initial studies demonstrated that the calmodulin regulated phosphatase calcineurin, and therefore Ca^{++}, was involved in the anergy induction, since CsA treatment completely prevented the phenotype (17). Thus, the data shown here indicate that the Ca^{++} response induced by the APL is adequate to stimulate partial T cell activation, but not sufficient to reach a sustained threshold level required for full activation.

A question remaining unanswered is what properties peculiar to the APL enable them to stimulate this altered Ca^{++} response in the T cells as compared to agonist ligand? Since all of the peptides share similar affinities for the I-E^k molecule, they should all present equivalent ligand complex numbers to the T cells (22). However, ligand affinities for the TCR may differ, affecting the outcome of the interaction. While such measurements for the peptides and TCR in our system are currently unknown, two recent studies have shown that APL have a faster dissociation rate than the agonist ligand (23, 24). Moreover, another report has indicated that sustained signaling leading to T cell activation requires prolonged TCR occupancy, and this is due to an involvement of the actin cytoskeleton (21). By extrapolation, if a critical number of complexes are required to be engaged at any one moment for an individual T cell to reach a high level of $[Ca^{++}]_i$, a faster dissociation rate may prevent this from ever being achieved by the APL. As a result, only intermediate $[Ca^{++}]$ are reached, which are insufficient for full activation of the cells.

While we show that distinct biochemical pathways can be stimulated upon T cell activation by APL, the question remains as to the physiological relevance of such findings. Because selective pathway activation correlates directly with the phosphotyrosine status of the ITAMs, perhaps similar phosphorylation patterns are generated in vivo during various stages of T cell development and peripheral activation, resulting in selective activation of the appropriate signaling pathways for the required functional response, such as positive selection (25, 26). The physiological regulation of the src kinases during thymocyte development (27, 28) may mimic the activation events occurring after TCR engagement of APL, by inducing different ITAM phosphotyrosine states. In support of this, a T cell line expressing a mutant $p56^{lck}$ kinase can initiate a Ca^{++} signal but no IL-2 production (29). Moreover, the APL in our system stimulate less $p59^{fyn}$ activity than immunogenic ligand (J. Sloan-Lancaster, and P.M. Allen, unpublished observations).

Much remains unknown regarding the contributions of individual biochemical events to any one T cell functional phenotype. Studies using APL stimulation have proven extremely useful in isolating individual signal transduction pathways, thus allowing the assessment of their relative importance in isolation as opposed to being encompassed in the normal intracellular biochemical homeostasis created upon full T cell activation. This dynamic and rapidly evolving area of investigation will undoubtedly contribute greatly to the uncovering of the molecular events occurring in many areas of T cell activation.

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