Kinetics and Extent of T Cell Activation as Measured with the Calcium Signal

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Summary

We have characterized the calcium response of a peptide–major histocompatibility complex (MHC)-specific $CD4^+$ T lymphocyte line at the single cell level using a variety of ligands, alone and in combination. We are able to distinguish four general patterns of intracellular calcium elevation, with only the most robust correlating with T cell proliferation. Whereas all three antagonist peptides tested reduce the calcium response to an agonist ligand, two give very different calcium release patterns and the third gives none at all, arguing that (*a*) antagonism does not require calcium release and (*b*) it involves interactions that are more T cell receptor proximal. We have also measured the time between the first T cell–antigen-presenting cell contact and the onset of the calcium signal. The duration of this delay correlates with the strength of the stimulus, with stronger stimuli giving a more rapid response. The dose dependence of this delay suggests that the rate-limiting step in triggering the calcium response is not the clustering of peptide–MHC complexes on the cell surface but more likely involves the accumulation of some intracellular molecule or complex with a half-life of a few minutes.

The binding of T cell receptor to a specific MHC–peptide complex is the critical step in T cell activation as it initiates the intracellular signaling cascade in the T cell (reviewed in reference 1). The first known step in this cascade is the tyrosine phosphorylation of CD3 chains (2, 3). Among other downstream events, these phosphorylations lead to an increase in the level of intracellular calcium (reviewed in reference 4). Initially, calcium is released from intracellular stores, followed by an influx of exogenous calcium through calcium-specific channels in the cell membrane (5, 6). The direct coupling of CD3 phosphorylation to calcium mobilization has been demonstrated by reconstitution of this signaling pathway in COS-1 cells (7).

One major effect of the elevated calcium levels is the activation of calcineurin. This leads to the translocation of the cytoplasmic component of the T cell–specific transcription factor NF-AT[1] to the nucleus and the initiation of transcription (8). The shape and mobility of T cells are also regulated by calcium (9, 10). Changes in intracellular calcium levels can be observed directly at the single cell level using ratiometric fluorescent dyes (9–14). While there is general agreement that the calcium signal is a specific consequence of T cell activation, the form and dose response of the signal remain controversial.

Apart from the calcium signal, other signaling pathways are activated following the engagement of the TCR. These include the pathway leading to the activation of the ERK kinases of the MAP kinase family via ras and raf (reviewed in reference 1). In addition, other T cell surface receptors such as B7 and cytokine receptors have been demonstrated to contribute to T cell activation (15, 16).

In addition to the TCR, the T cell coreceptor CD4 also binds to MHC class II, although its binding is not antigen specific. The cytoplasmic tail of CD4 can associate with the tyrosine kinase lck and recruit lck to the TCR complex (17). But even when the lck kinase domain is inactive (18) or in the absence of the CD4–lck association (19, 20) CD4 can contribute to the activation of T cells.

Altering antigenic peptides without decreasing their ability to bind to the MHC can have dramatic effects on T cell responses (reviewed in references 21–24). Some altered peptides elicit only a subset of the responses induced by the original peptide and thus have been labeled partial agonists (25, 26). Some inhibit an otherwise productive response to the original peptide and can thus be considered antagonists

[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; MCC, moth cytochrome C; NF-AT, nuclear factor of activated T cell.

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(27). Such antagonist peptides alone trigger some biochemical events in T cells, resulting in partial phosphorylation of the CD3-ζ chain (28–32). It has been found that the biological effect of a series of altered peptides correlates, with very few exceptions, with the half-life of the corresponding MHC–peptide–TCR complexes in vitro (28–30). Such a correlation has also been suggested based on work with agonists (33) and on theoretical grounds (34, 35).

In a previous study (36), we correlated different biological responses elicited by a set of altered peptides. This has been done in the presence or absence of blocking antibodies against CD4. We find that the different biological responses can be organized into a hierarchy of signaling, from ones elicited by most altered peptides to ones elicited only by the wild-type peptide and variants of it with minimal alterations. In this study, we characterize the form, frequency, and time course of the calcium response to different peptides at the single cell level. We show that the calcium signal reflects the postulated hierarchy of signaling and is predictive of subsequent T cell proliferation. In addition, we measure a delay between T cell–APC contact and the onset of the calcium signal, which is dependent on the strength of the activating stimulus. We suggest a model to explain this delay in the onset of the calcium signal in which the rate-limiting step in triggering the calcium response involves the accumulation of an intracellular signaling molecule with a half-life of a few minutes.

Materials and Methods

Peptides and Antibodies. Peptides were synthesized with standard FMOC chemistry and purified by reverse phase HPLC. Anti-CD4 antibody GK1.5 was purchased from Becton-Dickinson (Mountain View, CA).

Cell Line. The moth cytochrome *c* (MCC)-specific 5C.C7 T cells were derived from the 5C.C7 TCR transgenic mouse (37, 38). Spleen cells were primed with 10 μM MCC peptide and expanded for 8 d in IL-2 containing medium. The T cells (1×10^6 cells/ml) were restimulated with irradiated (3,000 rads) B10.BR spleen cells and 10 μM MCC peptide. After 10 d of culture and expansion in the presence of IL-2, $CD4^+$ cells were sorted by flow cytometry and restimulated in 10-d intervals.

Calcium Imaging. For calcium imaging, 5C.C7 T cells were loaded for 30 min at room temperature with 2 μM Fura-2 in 10% FCS in PBS with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Chinese hamster ovary (CHO) cells transfected with I-E^k were grown in 8-well coverslips (Nunc, Naperville, IL) and peptide loaded with 25 μM peptide for 4–16 h. Peptide loading efficiency throughout this time range was almost constant. After washing unbound peptide away with 5% FCS in PBS with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, Fura-2–loaded T cells were added onto a patch of confluent CHO cells on the microscope stage to start the experiment. Intracellular calcium in T cells was determined using C-Imaging-1280 System hardware and the Simca Quantitative Fluorescence Analysis software package; both from Compix, Inc., Imaging Systems (Mars, PA). The imaging system was coupled to a Nikon Diaphot 300 inverted microscope, which was equipped with the epifluorescence attachment and a 75W Xenon arc lamp. Alternate excitation of Fura-2–loaded T cells at 340 and 380 nm was achieved using a Ludl high speed dual filter wheel (Ludl Electronic Products, Hawthorne, NY) controlled by the Simca software. Images were collected with a CCD camera (Dage-MTI CCD72) in combination with a SuperGenII intensifier, (Dage-MTI, Michigan City, IN) to amplify fluorescence. Analysis of intracellular calcium and the generation of figures was achieved with the Simca software package. Per run, 30 to 40 cells were picked at random and analyzed. Data of different runs were pooled. For the figures, data of different experiments in which the topic of the figure was studied in direct comparison of the conditions under question was pooled. Only the 102S titration series (part of Figs. 2 and 3) shows a single representative experiment. The fluorescence of the Fura-2–loaded T cell is only visible when the T cell is right on top of the CHO layer. Because the T cells are added to the chamber from the top, their appearance in the fluorescence image is taken as the time of first contact.

Kinetic Model. One signaling component, A, will be converted into another, B, with the rate constant k_1, triggered by the MHC–peptide–TCR contact. Both the concentration of peptide and the type of peptide, as described by the half-life of its MHC–peptide–TCR complex (28–30), influence k_1. Because partial agonist and antagonist peptides give a qualitatively different signal the effect on k_1 of changing the peptide type shall be much more drastic than changing the peptide concentration. Using a first order kinetics, and assuming that the concentration of B before the MHC–peptide–TCR contact is 0, the concentration of A is A_0, B would form over time with $B(t) = A_0 \times (1 - e^{(-k1t)})$. We now suggest that a minimal amount of B has to form to propagate the signal, the trigger concentration B_{tr}. The time t_{tr} that is needed to accumulate B_{tr} is given by: $t_{tr} = -k_1^{-1} \times \ln(1 - B_{tr}/A_0)$. From this formula it can be seen that the smaller k_1 is, the longer it will take to reach the trigger concentration. However, one problem with this simple description is that even very weak signals would trigger activation, given enough time. As we do not observe this we suggest that, either directly or indirectly, B reverts back to A with the rate constant k_{-1} that for reasons of simplicity is assumed to be independent of the MHC–peptide–TCR contact. The rate of the formation of B then becomes $B(t) = A_0 \times (1 + k_{-1} / k_1) \times (1 - e^{(k_1+k_{-1})t})$. Using this model, the maximum amount of B that is formed depends on the ratio of k_{-1} to k_1. The smaller k_1 is relative to k_{-1}, the smaller this maximum level will be. Using weak activation conditions, as very low concentrations of peptide or antagonist peptides by themselves, the trigger level is never reached.

Results

Different Stimuli Elicit Distinct Calcium Signals. A number of TCRs have been characterized that recognize the MCC peptide (88–103) restricted by I-E^k. Different variants of the peptide have been studied in several respects: (*a*) peptide binding to I-E^k (39), (*b*) kinetic rate constants for the binding of the peptide–MHC to the 2B4 and 5C.C7 TCR (30, 33, 40), (*c*) ability to stimulate IL-3 production, proliferation, and acid release in a 5C.C7 line (36, 39). Taking advantage of this extensive data set, we chose one of the best studied of these models, 5C.C7, to characterize the changes in the concentration of intracellular calcium after T cell activation in response to different stimuli. The particular 5C.C7 line we chose to study is derived from the $CD4^+$ cells of the spleen of 5C.C7 TCR transgenic mice (37; Fazekas de St. Groth, B., and M.M. Davis, unpublished

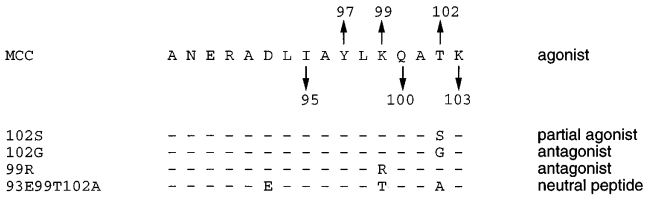

Figure 1. Peptides used. At the top of the figure the sequence of the MCC peptide is shown. The main TCR contact residues are indicated with an upward arrow, the main MHC contact residues with a downward arrow. Numbers indicate amino acid residue numbers of the MCC protein. Underneath, the altered peptides are listed. Only the residues that deviate from the wild-type sequence are shown. The mutation at the position 93 has no effect on either MHC binding or T cell stimulation (39, 44).

results). It is maintained by periodic restimulation with antigen (36). As an APC, we have used CHO cells transfected with I-Ek, which can effectively stimulate a variety of T cells (41, 42).

The calcium response to well-characterized peptides in the MCC family was studied (Fig. 1). For all of them the anchor residues to the MHC are unchanged, and thus binding to I-E^k is very similar (39). Two of the three major TCR contact residues, positions 99 and 102 (39, 43), were changed more or less drastically in different peptides.

The changes in the level of intracellular calcium during T cell stimulation were recorded from single cells for 16–18 min, using the calcium-sensitive fluorescent dye, Fura-2 (45). In a single cell assay differences in the response within a population of responders can be resolved, allowing more extensive analysis. This proved to be crucial in particular for the characterization of different types of calcium signals. The experiments were done at 25°C. Relative differences in type, frequency, and timing of the calcium signal are the same as at 37°C, but the movement of the cells after activation is considerably less (Wülfing, C., and M.M. Davis, data not shown). This reduction in cell movement at 25°C allows a more precise determination of the form of the calcium signal. In our assays, we first recorded the time between APC–T cell contact and the onset of the calcium signal. Second, the form of the calcium signal after the onset, as judged by the emission of the dye Fura-2 when subsequently excited at 340 nm and 380 nm, as well as the ratio of these emissions, was classified. Analyzing not only the ratio, but also the single emission traces, allows us to distinguish between specific elevation of intracellular calcium and ratio changes caused by the movement of the cells before activation.

Under different activation conditions, we find four types of calcium signals. Each starts with a rapid rise in intracellular calcium. However, the calcium levels after this initial rise vary. Using the MCC peptide, more than 95% of the cells respond with full signal, as shown in Fig. 2 and described in Table 1. With the altered peptides, we find three qualitatively different variants of the full signal, a reduced, partial, and transient signal (Fig. 2; Table 1). These describe the increasingly weaker calcium responses that we see and have predictive value.

Table 1. *Types of Calcium Signals*

Type	Onset	Form of the signal over time
Full	Singular	Sustained high
Reduced	Singular	Declining or fluctuating above resting calcium level
Partial	Multiple	Fluctuating down to resting calcium level
Transient	Singular	Rapid return to resting calcium level

Four general types of calcium signals are observed according to the number of rapid rises from baseline, *onset*, and the form of the calcium signal after the initial rise, *form of the signal over time*. Corresponding single cell calcium traces are shown in Fig. 2.

Calcium Responses Change with Different Peptides Fig. 3 summarizes the T cell calcium signaling triggered by the peptides used in this report. Using the MCC peptide the response is full, as discussed above. For the partial agonist peptide 102S, the frequency of signaling is the same as for the MCC peptide, but for almost a third of the cells the signal is not complete. For the antagonist 102G peptide, both the frequency of signaling and the signal type are significantly different. The antagonist peptide 99R gives no signaling at all above background. The background level is defined using no peptide or the neutral MCC93E99T102A variant, which shows no biological response in acid release and proliferation assays and no measurable affinity to the 2B4 TCR. In both of these cases, we see no full signals and reduced or partial signals in less than 10% of the T cells (Fig. 3).

To determine the effect of low concentrations of stimulating peptide in a defined manner, the MCC and 102S peptides were each serially diluted into the neutral peptide 93E99T102A, such that the total peptide concentration during loading of the APCs is kept constant at 25 μM. As shown in Fig. 3, diluting the MCC peptide 1:1,000, resulting in a 25 nM MCC concentration, has no effect on the frequency or the type of the calcium signal. At a 1:10,000 dilution of the MCC (2.5 nM), more than 85% of the cells still respond with a full signal (see Fig. 5 *A*). When 102S is diluted into MCC93E99T102A in a dilution series ranging from 25 μM to 25 nM, the signaling frequency throughout the series is still almost 100% (Fig. 3). However, at a 25 nM (1:1,000 dilution), 70% of the cells respond with a reduced signal, as opposed to 30% at 25 μM.

The altered or absent calcium response for the peptides 102S, 102G, and 99R correlates well with the other biological effects of these peptides (Table 2). The loss of extended elevation in the calcium signal in the 102S dilution series correlates with a profound reduction in the ability of 102S to induce proliferation (36). The strongly reduced or absent calcium signal of the 102G and 99R antagonist peptides correlates with their lack of ability to induce any proliferation. As shown by Rabinowitz et al. (36) the acidification response is an early T cell activation event with both

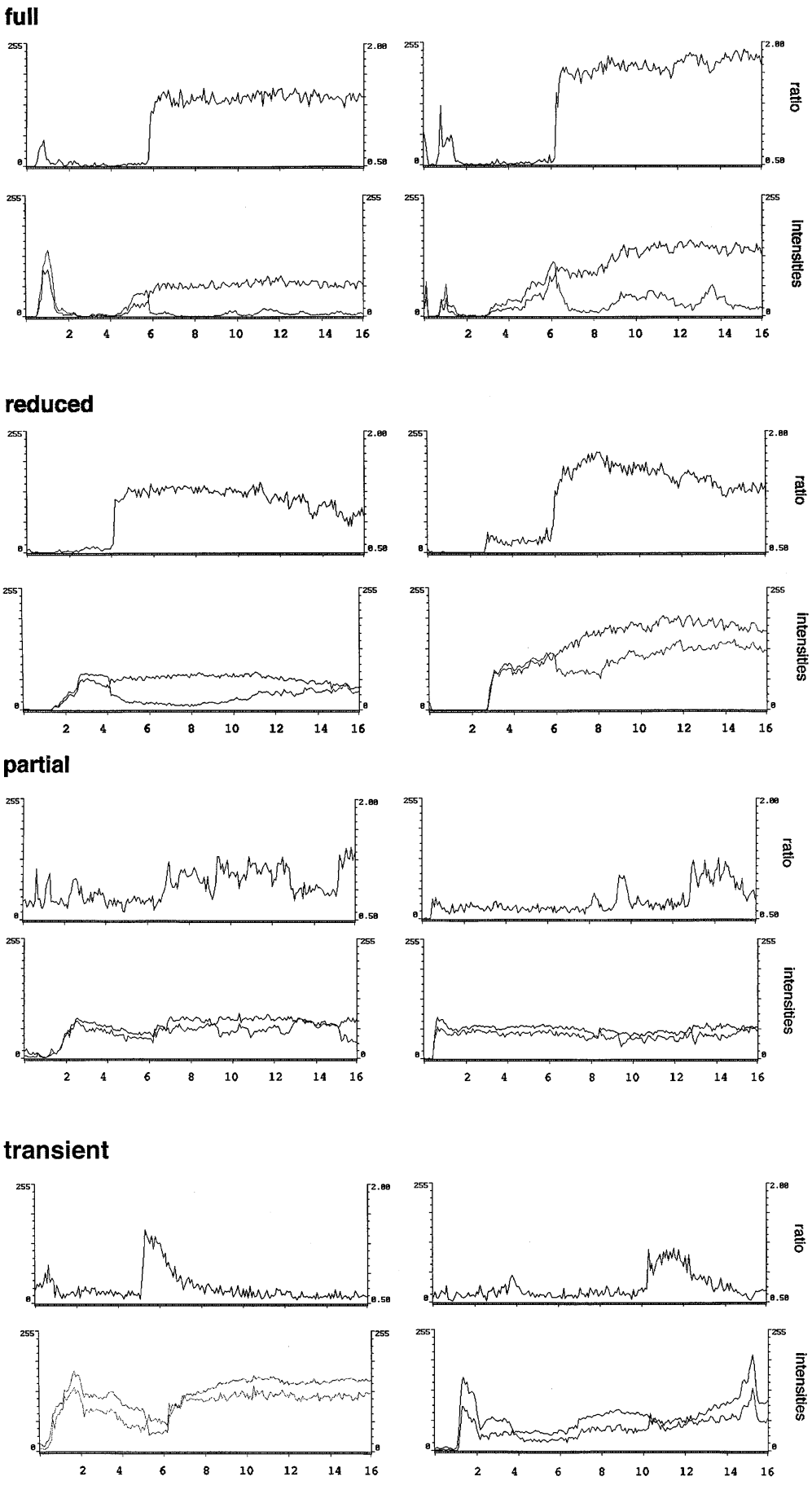

Figure 2. Types of calcium signals. For each signaling type, as indicated by the name at the top left and defined in Table 1, two examples are given that each consist of a pair of panels. In the top panel (single trace image), the ratio of the emission intensities at 340 nm over 380 nm is shown. This is proportional to the intracellular calcium level. In the bottom panel (double trace image), separate emission intensities at 340 nm and 380 nm are shown. The emission intensity at 380 nm is stronger than the emission intensity at 340 nm at the beginning of the recording and can thus be identified. Occasional strong fluctuations at the beginning of the images in both wavelengths and the ratio (e.g., left partial panel) are explained by out of focus fluorescence while the T cell settles into the plane of focus of the objective. It can be identified in the single wavelength images by very low intensities followed by a parallel increase in both wavelength. The individual wavelength traces help in the distinction between full and reduced signals. In a reduced signal the 380 nm trace does not fall close to (*right panel*) or does not stay close to (*left panel*) baseline after activation.

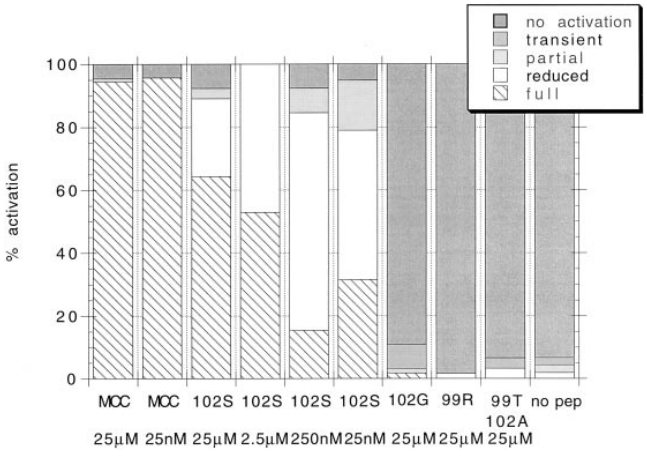

Figure 3. Frequency and type of the calcium response to MCC and altered peptides. The percentage of each signal type for different peptides at different dilutions. Signal types are given in the legend. Peptide names are given in the first row at the bottom of each column. 99T102A stands for the peptide 93E99T102A. When MCC or 102S has been diluted into the neutral peptide 93E99T102A, the concentration of MCC or 102S is given in the bottom row. The total peptide concentration is always 25 μM.

MCC and 102S giving identical dose-response curves. This is reflected in a high calcium signal responder frequency for both. On the other hand, the strongly reduced or absent calcium signal for 102G and 99R correlates with the lack of an acidification response. Because acidification is measured in a bulk assay, the small degree of activation measured in a single cell calcium assay, e.g., for the peptide 102G, might not be detectable above background in the acid release assay. Thus, only a full calcium signal correlates with a proliferation response. In contrast, acid release correlates with a high frequency response, irrespective of signal type.

The Time Between T Cell–APC Contact and Onset of the Calcium Signal Is Determined by the Strength of the Activating Stimulus. The design of our calcium assay allows us to determine the precise time between first APC–T cell contact and the rise in intracellular calcium. We refer to this as the onset delay. This onset delay is composed of two components. Even under the strongest activation conditions, there is a minimal time delay of ~1 min for the activation of the first T cells. Using weaker stimuli, we find that the onset delay increases as the stimulus gets weaker (Fig. 4). This delay is seen both in the time it takes to activate the first cell, as well as in the average onset time of the T cells in a given experiment.

For the MCC peptide, the onset delay is the shortest with an average of about 3 min and stays unchanged even at 25 nM MCC (a dilution of 1:1,000 into the neutral peptide 93E99T102A) (Fig. 4). 25 μM 102S or 102S diluted only 1:10 into the neutral peptide (2.5 μM) shows an onset delay that is similar to MCC. However, at lower concentrations there is a significant increase in the onset delay with a shift from about 3 min to 6 min for 25 nM 102S (1:1,000 dilution into the neutral peptide) (Fig. 4). Furthermore, the antagonist peptide, 102G, gives an onset delay of 10 min, in combination with a strongly reduced responder frequency. In general, a full calcium signal shows a rapid onset, a slightly reduced calcium signal shows a small onset

Table 2. *Comparison of Calcium Data with Other Biological Assays*

	Acid release		Calcium signal (percentage/quality/onset)		Proliferation	
Peptide	10 μM	10 nM	25 μM	25 nM	10 μM	10 nM
MCC	+	+	100%/full/immediate	100%/full/immediate	+	+
MCC + anti-CD4	+	ND	ND	100%/reduced/immediate	+	+
102S	+	+	100%/reduced/immediate	100%/reduced/delayed	+	±
102S + anti-CD4	+	ND	ND	100%/reduced/delayed	−	−
102G	−	−	15%/reduced/delayed	ND	−	−
99R	−	−	0%	ND	−	−
Neutral peptide	−	−	0%	ND	−	ND
MCC in 102G		ND		80%/reduced/delayed		−
MCC in 99R		±		80%/reduced/delayed		−

The proliferation and acidification data are taken from Rabinowitz et al. (36) and are compared with the calcium data of this study. Peptides are given in the first column. In the bottom part of the table where two peptides are given, the first has been diluted into the second 1:1,000. + *anti CD4* denotes the presence of 100 ng/ml of the anti-CD4 antibody GK1.5. Peptides have been used at the concentration that is indicated at the top of each column. The small peptide concentration in the second calcium data column has been achieved by a 1:1,000 dilution of the peptides into the neutral peptide, resulting in 25 nM concentration. For the acidification and proliferation assays, a plus signifies a strong response, plus/minus signifies weak response, and minus signifies no detectable response. For the calcium assays three readouts are compiled: percentage signifies the percentage of responding cells: 100% signifies virtually all cells respond, 0% signifies no responders. Quality indicates the types of calcium signal: <10% reduced or nil signals are seen or >10% of reduced or nil signals, as indicated by full or reduced. Onset gives the onset time of the calcium signal: >50% of the cells activate within the first 4 min or not, as indicated by immediate versus delayed.

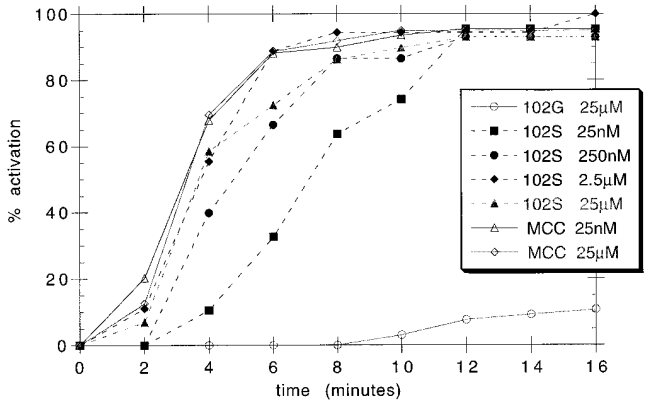

Figure 4. Onset of the calcium response to MCC and altered peptides. Cumulative representation of percent of cells activated (irrespective of the type of signal) during a specific onset time interval. Numbers on the time axis indicate end time point of the interval, i.e., cells activating within 2 min after initial APC contact are shown at time point *2*. Peptide name is given in the legend. When MCC or 102S have been diluted into the neutral peptide 93E99T102A, the concentration of MCC or 102S is also given in the legend. Total peptide concentration is always 25 μM.

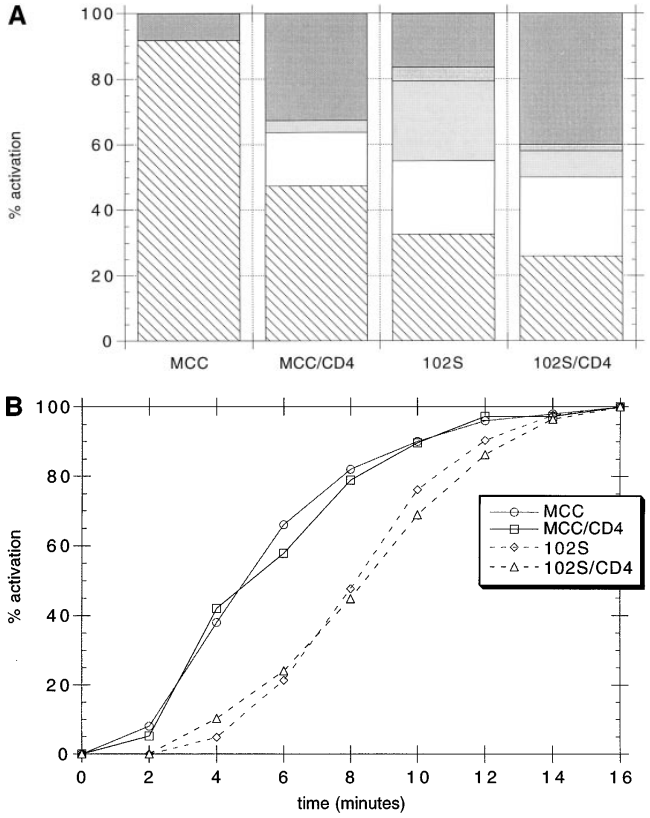

Figure 5. Effect of anti-CD4 antibody GK1.5 on the type and onset of the calcium signals. (*A*) Percentages of each signal type (as explained in Fig. 3). MCC or 102S were diluted 1:1,000 into the neutral peptide MCC93E99T102A, resulting in 25 nM concentration. The type of peptide and the absence or presence of the antibody GK1.5 are indicated under the columns. The antibody concentration is 100 ng/ml. (*B*) Cumulative representation of % cells activated (irrespective of the type of signal) during a specific onset time interval. Time axis as in Fig. 4 *B*. Conditions are as in *A* and are indicated in the header.

delay, and a strongly reduced calcium signal displays a large onset delay. Thus, this delay in T cell activation seems to be an integral part of the response to different ligands.

Blocking CD4 and Antagonism. We also studied the effect of blocking CD4 coreceptor engagement on the 102S and MCC calcium response. In the presence of 100 ng/ml of the anti-CD4 antibody GK1.5, the calcium signal induced by 25 nM MCC or 102S is reduced with respect to the frequency and type of signal (Fig. 5 *A*). The onset is not significantly delayed (Fig. 5 *B*). This suggests that CD4 plays a role predominantly after the onset of the calcium signal. The inhibition of the calcium signal by CD4 correlates with the data of Rabinowitz et al. (36), who have shown that GK1.5 blocks proliferation in response to MCC and 102S.

The peptides 99R and 102G have been shown to act as antagonists in cytokine secretion and proliferation assays (Reay et al., in preparation; 36). Furthermore, the partial agonist 102S can be changed into an antagonist in the presence of 100 ng/ml of the anti-CD4 antibody (36). To assess the effect of antagonists on the calcium response, the MCC peptide was serially diluted into the three different antagonist conditions. For a 1:10,000 dilution of MCC into 102S/GK1.5, resulting in 2.5 nM MCC concentration, 10% of the cells do not respond at all, and 30% respond with a reduced calcium signal (Fig. 6 *A*). This is essentially the same signal seen with the 102S peptide and GK1.5 alone without any MCC (see Fig. 5). A 1:10,000 dilution of the MCC into 99R and 102G leads to a reduction in the frequency of responding cells to less than 40%, and almost all calcium signals are partial (Fig. 6 *B*). Thus, the normal MCC calcium response can be efficiently antagonized under limiting activation conditions. In all three cases, the antagonist effect depends strongly on the concentration of the agonist, as MCC diluted to 1:1,000 shows only a small antagonistic effect, as judged by the slight reduction in type and frequency of the calcium signal (Fig. 6 *A*). Interestingly, however, all three antagonist conditions delay the onset of the calcium signal, ranging from slightly (about 1 min larger) to more than twice as long (Fig. 6 *B*).

Discussion

Intracellular Calcium Elevation and T Cell Responsiveness. We show here that the form of the intracellular calcium traces can vary widely depending on the activation conditions. Under fully activating conditions, the calcium trace shows a rapid rise followed by a smooth plateau. Under suboptimal activation conditions, the intracellular calcium concentration fluctuates considerably more after the initial rise (Fig. 2). Other investigators have described single cell calcium traces for peptide-dependent T cell activation at high concentrations of agonist peptide or with cross-linking anti-CD3 antibody. They found either oscillations (9, 11, 14, 46) or smooth calcium traces (47), or a combination of both forms (12). While these different results may be due to dif-

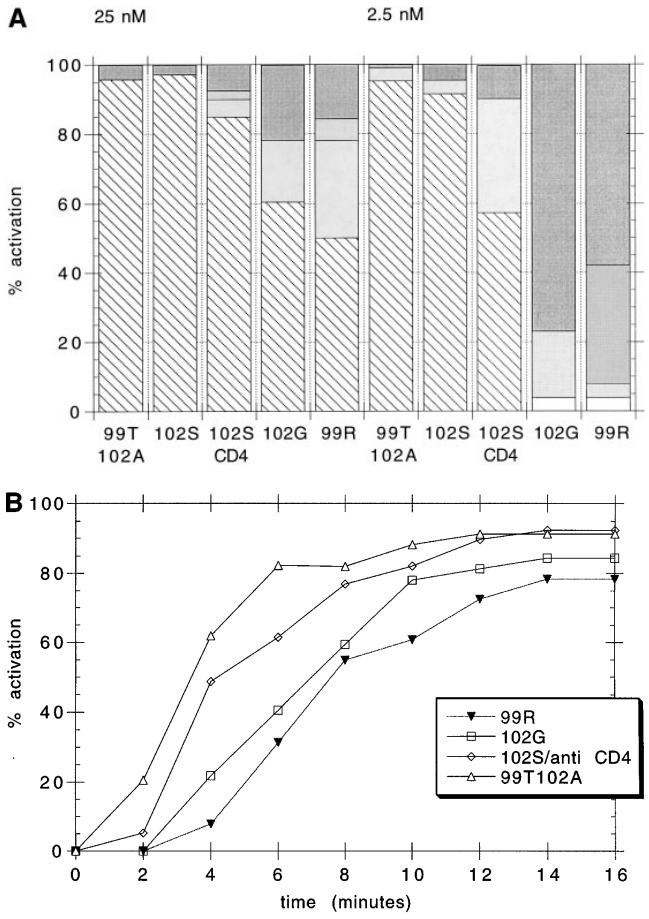

Figure 6. Antagonism. (*A*) Percentages of each signal type (as explained in Fig. 3) for dilution of MCC into different peptides as given below the bars. 99T102A indicates the peptide 93E99T102A. 102S/CD4 indicates 102S in the presence of 100 ng/ml anti-CD4 antibody GK1.5. The left five bars show, as indicated on the top, the data for the 1:1,000 dilutions of MCC, resulting in 25 nM MCC concentration. The right five bars show the data for the 1:10,000 dilutions, resulting in 2.5nM MCC concentration. (*B*) Cumulative representation of percent of cells activated (irrespective of the type of signal) during a specific onset time interval for the 1:1,000 dilutions of MCC into antagonist peptides, as indicated in the header. Time axis as in Fig. 4 *B*.

ferent ligands used, an alternative explanation relates to the degree of transformation of the T cells. When we compare calcium traces from 5C.C7 naive cells, T cells primed once, multiply primed T cells, i.e., the line used in this publication, and an restimulation-independent 5C.C7 T cell line, we find that the incidence of oscillations increases with the degree of transformation of the T cells (Wülfing, C., and M.M. Davis, in preparation). Consistent with this, the studies that show the most oscillatory T cell calcium traces (14, 46) both used hybridomas.

In the system used here, we find that the elevation of intracellular calcium varies from the full response to various reduced or oscillatory patterns and that this correlates with other bioassays as summarized in Table 2. A positive result in the acidification assay is always accompanied by some calcium signaling, whereas a proliferative response correlates with a full calcium signal in most of the cells. Even a small disturbance of the full signal, such as a delayed onset or a response from less than 100% of the T cells, correlates with an inability of the stimulus to induce proliferation of the whole population. This suggests that the need for paracrine help may require the proliferation of the large majority of the individual cells in a population. Furthermore, other intracellular signaling pathways that are necessary for full T cell activation may be activated independent of the calcium signal to some extent. Nevertheless, the importance of a sustained calcium signal has been shown by Timmerman et al. (48), who have demonstrated that only a sustained calcium elevation signal is able to keep NF-ATc in the nucleus. Similarly, Negulescu et al. (46) have shown that a sustained elevation of the intracellular calcium concentration is necessary for the activation of an IL-2 promoter reporter construct. From the data of Karttunen and Shastri (49) and Valitutti et al. (50), it also appears that a full calcium stimulus must be maintained for at least 30–60 min to induce an irreversible commitment to T cell activation, particularly at lower peptide concentrations. Given the importance of the sustained phase of the calcium signal, a small reduction might contribute significantly to a severe reduction in proliferation.

During activation, the T cell receives a wide variety of signals through a large number of individual receptors that have to be integrated within the signaling pathway to allow the cell to decide whether to proliferate or not. Our data allows us to estimate to what extent the calcium signal is already integrated. The pattern of elevation of the intracellular calcium elicited by different peptides is not only quantitatively different, but qualitatively as well. Antagonism is more effective at a higher dilution of the agonist peptides into the antagonist. A blocking anti-CD4 antibody reduces the calcium signal. These three observations suggest that a T cell has counted and qualified the different signals through TCR and CD4 before the elevation of the intracellular calcium concentration. In the process of signal integration, the calcium signal thus seems to be much closer to the final proliferation decision than to the events elicited directly by the single engaged receptors.

Altered Ligands. With respect to TCR antagonist ligands, recently Sloan-Lancaster et al. (47) have shown that several antagonist ligands can induce weak calcium fluxes and suggest that this might be part of an antagonist signal. Our own data shows that whereas two different antagonist stimuli (102G alone or 102S + αCD4 antibody) generate detectable, but reduced calcium responses, one (99R) does not. This indicates that the mobilization of intracellular calcium is not necessary for antagonist peptide–MHC ligands to block a response. Thus, their primary effect may lie upstream in the T cell signaling process. Similarly, Windhagen et al. (26) have described an antagonistic peptide that does not induce a calcium signal. Also consistent with a more TCR proximal effect of antagonism are the data of Sloan-Lancaster et al. (32) and Madrenas et al. (31) showing alterations in CD3-γ phosphorylation and subsequent ZAP-70 association as a consequence of antagonist ligand engagement.

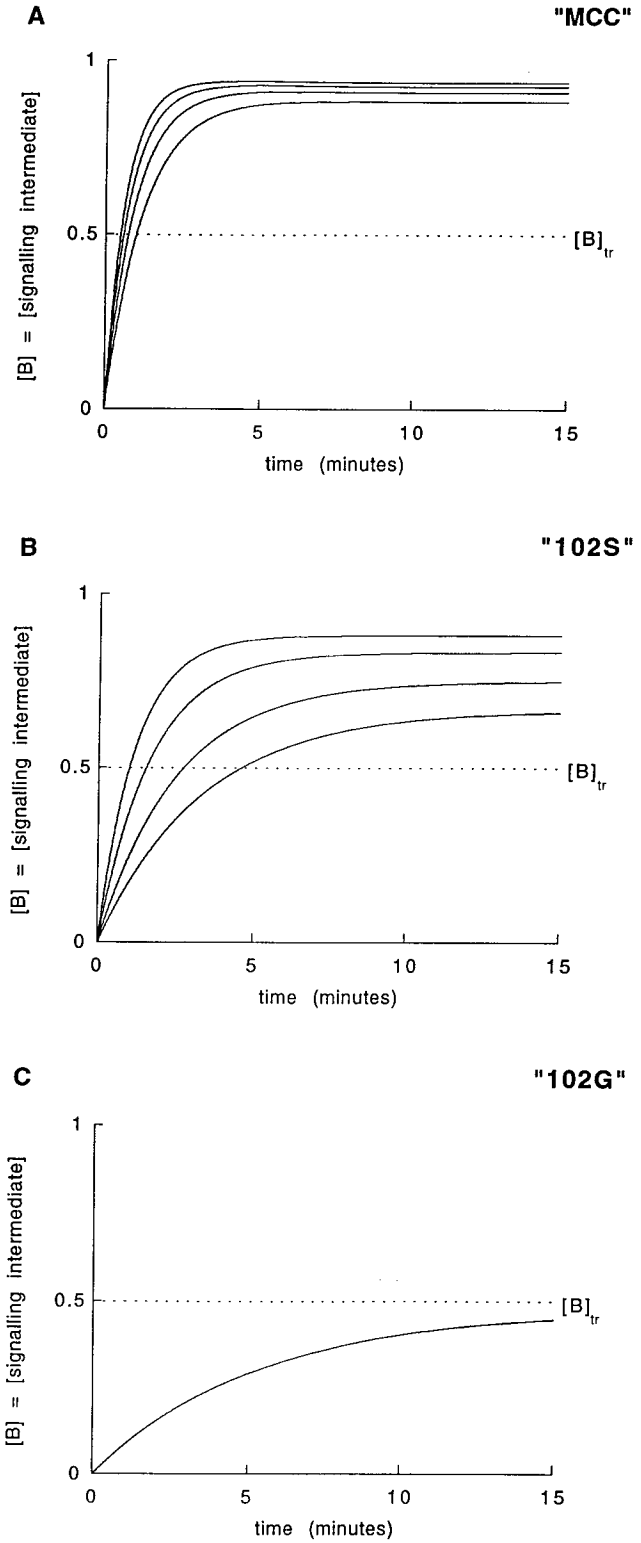

Figure 7. Model of onset effect. The figure represents the accumulation of the critical signaling component B that has to reach the trigger concentration B_{tr} to induce the calcium response. The mathematics of the model is given in the Materials and Methods section. Graphs for different values of the rate constants k_1 and k_{-1} are shown. k_{-1} is set to 1 in all graphs to represent a constant removal of B. The postulated trigger to be reached for the induction of the calcium signal is the production of the half-maximal amount of B. The rate of formation of B, k_1, is varied in the three panels. It varies between 7.5 and 15 for *A*, as to represent the fast formation of the signaling intermediate under different concentrations of the agonist peptide, MCC. No onset delay is seen. k_1 is set to values between 2 and 7.5 in *B* to represent the postulated formation of the signaling intermediate by different concentrations of the partial agonist, 102S. The formation of B is not much faster than its removal. This results in a strong onset effect. In *C*, the rate formation of B is 0.9. Because the rate of formation is smaller than the rate of removal, the trigger concentration is never reached on average, as to represent the antagonist peptide, 102G.

Kinetics of the Onset Delay: Evidence for a Rate-limiting Step in Triggering a T Cell Calcium Signal with a Half-life of a Few Minutes. In this report, we determined the time between initial T cell–APC contact and onset of the calcium response. Comparing all activation conditions, we find that a minimal delay of 45 s is seen for the MCC peptide at high as well as low concentrations. Therefore, this initial delay must reflect events that are independent of the stimulus strength. Likely factors in this time frame are the time required for the establishment of cell–cell adhesion and for the transmission of intracellular signals from the membrane to the calcium stores.

Weak stimuli, such as partial agonist or antagonist peptides, result in an additional onset delay of up to 10 min. Recent studies have demonstrated that, in most cases, partial agonists and antagonists dissociate more rapidly from a given TCR than agonist ligands (28–30). Therefore, most altered peptide ligands should reach steady-state with respect to TCR binding more rapidly than agonist ligands. Furthermore, one characteristic of the onset delay is that large changes in the peptide dose result in only small changes in the activation time (Fig. 4). For example, going from 250 nM to 25 nM of the peptide 102S causes only a 40% increase in the onset time. If the formation of individual TCR–peptide–MHC pairs was the rate-limiting step in the induction of the calcium signal there would be, for concentrations of free MHC–peptide smaller than the concentration of free TCR, a linear increase in the onset time as the peptide concentration decreases. If the formation of TCR–peptide–MHC oligomers was critical there would be an even more dramatic increase in the onset time as a function of peptide concentration. Even more sophisticated models in which TCR–peptide–MHC mono- or oligomers need to interact for some minimal time in order for signaling to occur do not fit the observed peptide dependency of the onset delay (Kantor, R., C. Wülfing, and M.M. Davis, unpublished results). Thus, the increased onset time observed for weak stimuli does not appear to depend on some threshold level of TCR ligation or oligomerization. This is not to say that oligomerization does not occur or that the half-life of the TCR–peptide–MHC complex is not important, as other studies have shown that it is (30, 33), but that these factors may not be rate-limiting at the cellular level.

Instead, we suggest that the rate-limiting factor in this delay reflects intracellular reactions leading to the accumulation of signaling molecules. These reactions could involve a continuous competition between phosphorylation and

dephosphorylation, or between production or breakdown of other parts of the signaling machinery. We hypothesize that T cell activation shifts the balance of this equilibrium towards the accumulation of signaling molecules. A simple kinetic model based on a reaction scheme of this type agrees well with the onset delay that we observe (Fig. 7). The crucial feature of this scheme is that the turnover of the rate-limiting signaling intermediate is determined by an activation strength-dependent first-order forward and an activation strength independent first-order reverse reaction:

$$A \underset{k-1}{\overset{k1}{\rightleftharpoons}} B$$

The concentration over time of this signaling intermediate B is given by $B(t) = A_0 \times (1 + k_{-1} / k_1)^{-1} \times (1 - e^{(k_1 + k_{-1})t})$ (see Materials and Methods for details).

The half-life of these reactions can be derived from our kinetic data. Under the very weak activation conditions of the antagonist 102G most calcium responses occur between 8–12 min. Because only a small percentage of the cells respond, the reaction leading to signaling must be nearly complete (i.e., at the new steady-state) before any responses occur. This is depicted in Fig. 7 *C*. Because the time to reach 95% of a new steady-state level of signaling intermediates is four times the half-time, 2 min is a good approximation for the half-time of the reaction towards the steady-state. At the same time, this is the lower limit for the half-times of the individual forward and reverse reactions of this steady-state. The half-times of this stimulus-independent reverse reaction and this forward reaction for 102G are a few minutes under the additional assumption that to trigger the calcium response the accumulation of the signaling intermediate has to be substantial but not complete (Fig. 7 *C*). The half-times of the forward reaction for stronger activation conditions would be shorter. As these measurements were done at 25°C, one must also allow for a two to four times acceleration in this value at 37°C. In a broader biological picture, we thus suggest that the accumulation of a signaling intermediate triggers the calcium signal. This intermediate is continuously degraded with a half-time of a few minutes. Therefore, every T cell stimulus that results in calcium signaling has to produce this intermediate with a half-time of less than a few minutes.

Other early processes in T cell activation occur within a similar time frame. One example is ζ chain phosphorylation (31, 32), which reaches steady-state within a few minutes for agonist signaling and within 2–10 min for antagonist signaling (32). Such a reaction could produce the postulated critical signaling intermediate.

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